doi:10.1111/evo.13632

You cannot have it all: Heritability and constraints of predator-induced developmental plasticity in a Neotropical treefrog

Justin Charles Touchon[1,2,3] and Jeanne Marie Robertson[4,5]

[1]*Biology Department, Boston University, Boston, Massachusetts 02215*

[2]*Current Address: Biology Department, Vassar College, Poughkeepsie, New York 12604*

[3]*E-mail: jutouchon@vassar.edu*

[4]*Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853*

[5]*Current Address: Department of Biology, California State University, Northridge, California 91330*

Received January 12, 2018
Accepted October 10, 2018

Many organisms have evolved phenotypic plasticity but examples of a heritable genetic basis or genetic constraints for plasticity across environments remain scarce. Tadpoles of the Neotropical treefrog *Dendropsophus ebraccatus* alter tail coloration and shape differently in response to fish or aquatic insect predators. To assess the genetic basis of plasticity we raised 1020 tadpoles from 17 maternal half-sib pairs (34 unique families) individually with chemical cues of fish or aquatic insects, or with cue-free control water. We used Bayesian animal models to estimate narrow sense heritability of morphology and cross-trait genetic correlations in all three treatments, heritability of plasticity in response to each predator, and genetic correlations between responses to fish and insects. Families showed remarkably different responses to predators and heritability was often high (0.45–0.75), as was heritability of plasticity itself (0.42–0.62). We detected strong negative genetic correlations for responses to each predator (–0.45 and –0.59), providing clear evidence of a limit to plasticity. Most importantly, we show that prey genotypes are constrained in their capacity to respond to different types of predators, which likely maintains genetic variation for plasticity in a temporally and spatially dynamic landscape where there is no single adaptive peak.

KEY WORDS: **Anura, Bayesian, phenotypic plasticity, quantitative genetics, wild animal model.**

Phenotypic plasticity, the ability of a single genotype to give rise to multiple potential phenotypes in response to environmental variation, is a widespread phenomenon (Schlichting and Pigliucci 1998; Pigliucci 2001; West-Eberhard 2003). The seeming ubiquity of plasticity in morphology, behavior, coloration, and life history, combined with the prevailing thought that induced phenotypes are not heritable, led many developmental and evolutionary biologists to minimize plasticity in laboratory studies, and ignore it in evolutionary theory until 1980s (West-Eberhard 1989, 2003; Gilbert and Epel 2009; Pfennig et al. 2010). It has since been demonstrated that, at a minimum, plasticity (1) facilitates survival and persistence in novel environments where taxa might otherwise experience poor or reduced fitness, (2) alters predator-prey eco-evolutionary dynamics, (3) reveals cryptic genetic variation, and (4) can accelerate the rate of adaptation in new habitats (Price et al. 2003; Badyaev 2005; Ghalambor et al. 2007; Ledon-Rettig et al. 2010; Fischer et al. 2014; Lind et al. 2015). Populations exposed to novel invasive predators can evolve inducible defensive morphologies in as few as 10–15 generations (Nunes et al. 2014). Further, comparative studies both within and across species demonstrate that inducible phenotypes triggered by environmental variation can become fixed, leading to macroevolutionary changes (Badyaev and Foresman 2000; Gomez-Mestre and Buchholz 2006; Suzuki and Nijhout 2006; Scoville and Pfrender 2010). The potential importance of plasticity to shape evolutionary trajectories has thus gained recognition, leading

some to call for a "new modern synthesis" that includes plasticity as a centerpiece (Price et al. 2003; Schlichting 2003, 2004; West-Eberhard 2005; Ghalambor et al. 2007; Pfennig et al. 2010; Laland et al. 2014, 2015).

Central to the idea that plasticity has a role in phenotypic evolution is that plastic phenotypes can themselves evolve. Thus plasticity, like any other evolutionary trait, must have a heritable and variable genetic basis in a population. That plastic traits can be variable across genotypes has been known for some time (Scheiner and Goodnight 1984; Newman 1988, 1994; Semlitsch 1993; Robinson and Wilson 1996; Reques and Tejedo 1997). However, empirical examples of heritable variation of phenotypic plasticity itself (i.e., the *ability to produce* different phenotypes in response to environmental variation, not merely the phenotypes themselves) remain somewhat scarce (Agrawal et al. 2002; Laurila et al. 2002a; Nussey et al. 2005; Relyea 2005; Kraft et al. 2006a; Ledon-Rettig et al. 2010; Gomez-Mestre and Warkentin 2013; McGhee and Travis 2013). Plasticity evolves when environments are heterogeneous and when indicators of that heterogeneity are reliable (West-Eberhard 2003). That said, the evolution of adaptive plasticity is hypothesized to be constrained by costs of plasticity (i.e., a decrease in fitness incurred by an induced phenotype compared to a fixed phenotype), costs of phenotypes (i.e., a trade-off in fitness that results from producing one phenotype instead of another), and limits to plasticity (i.e., the inability of a genotype with plasticity to produce as optimal a phenotype as that of a specialized, fixed genotype) (DeWitt et al. 1998; van Kleunen and Fischer 2005; Van Buskirk and Steiner 2009; Murren et al. 2015).

Documenting costs of plasticity has proven difficult, but costs of phenotypes and limits to plasticity have been more readily detected (DeWitt 1998; Relyea 2002a; Van Buskirk and Steiner 2009; Murren et al. 2015). Phenotype costs may manifest when organisms face, and potentially attempt to respond to, multiple conflicting cues. For example, although many prey organisms can respond with morphological or behavioral changes to specific predators (Tollrian and Harvell 1999; Van Buskirk 2001; Hoverman et al. 2005; Touchon and Warkentin 2008a), that singular response might not suffice when these prey face multiple predation risks (Turner et al. 1999; Relyea 2004; Lakowitz et al. 2008; Beckerman et al. 2010). In other words, an appropriate (plastic) phenotypic response to one predator may increase risk to a different predator (McIntosh and Peckarsky 1999; Turner et al. 2000), creating an adaptive landscape with multiple adaptive peaks (Wright 1932; Gavrilets 2004). For example, *Planorbella* freshwater snails raised with water bugs (Belostomatidae) develop wider shells, which increases survival with water bugs but decreases survival if snails encounter crayfish predators (Hoverman and Relyea 2009). Because predators and prey are often spatially and temporally heterogeneous within an environment (Gascon 1991, 1992; Touchon and Vonesh 2016), it is therefore easy to imagine how variable genotypes for plasticity could evolve and be maintained with no single optimum phenotype emerging.

Anuran larvae are model organisms for studying the development and consequences of plasticity in response to predation and competition (Benard 2004; Hossie et al. 2017). Tadpoles respond to physical and chemical cues in the environment by altering their morphology, development, coloration, and behavior (McCollum and Leimberger 1997; Lardner 2000; Benard 2006; Touchon and Warkentin 2008a). Many studies of tadpole phenotypic plasticity, including some that examine genetic variation in plasticity, have ignored individual- or family-level variation and focused only on mean responses of populations or species (Van Buskirk et al. 1997; Relyea 2002b; Benard 2006; Touchon and Warkentin 2008a). However, studies that examined family-level variation in tadpole plasticity have generally documented substantial genetic variation for inducible traits, providing an important basis for further exploration (e.g., Laurila et al. 2002a; Relyea 2005; Kraft et al. 2006b; Ledon-Rettig et al. 2010). Understanding the variation that occurs within and between families (i.e., genotypes) sheds light on the genetic variation that underlies the variation in phenotypes.

Tadpoles of the Neotropical treefrog *Dendropsophus ebraccatus* demonstrate opposing phenotypic plasticity in response to at least two different types of predators: fish and aquatic insects. On average, *D. ebraccatus* tadpoles raised with chemical cues of fish predators (but with no physical interaction) develop a shallow clear tail, whereas tadpoles raised with cues of dragonfly larvae or giant water bugs develop a large tail with a conspicuous red and black spot at the posterior end (Fig. 1; Touchon and Warkentin 2008a). The magnitude of this response is further mediated by the physical environment, being strongest when growth rates are constrained by low temperatures (Touchon and Warkentin 2011). These different responses are putatively related to predator avoidance strategies. Fish in this system are generally active hunting predators and the shallow tail shape and translucent color are thought to reduce detection and increase overall swimming speed (Blair and Wassersug 2000; Hoff and Wassersug 2000). Conversely, most dragonfly larvae are sit-and-wait predators (Corbet 1999), and a large tail fin increases turning speed whereas the conspicuous tail spot acts as a lure to direct attacks away from the body (Blair and Wassersug 2000; Hoff and Wassersug 2000; Van Buskirk et al. 2003). Although much is known about the ecological causes and consequences of such inducible phenotypes, we know relatively little about genetic variation for plasticity in the face of multiple opposing predators. Furthermore, there appears to be no knowledge of the genetic correlations of induced phenotypes that are produced in response to different predators.

To address this gap in knowledge, we raised 34 maternal half-sib families of *D. ebraccatus* tadpoles, exposing individuals

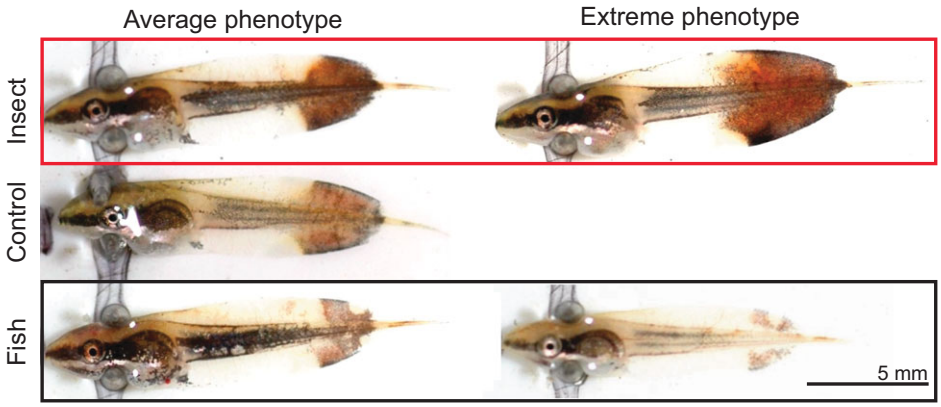

Figure 1. Examples of *Dendropsophus ebraccatus* tadpoles after 10 days or rearing with chemical cues of aquatic insect (top) or fish (bottom) predation, or cue-free controls (middle). Tadpoles on the left provide examples of the average responses observed across all families in this study, whereas tadpoles on the right are examples of the extreme phenotypes induced by insects or fish predation cues.

from each family to fish or aquatic insect cues or predator-free control cues. Here, we are concerned with understanding the genetic nature of limits to plasticity, as opposed to costs of plasticity or phenotypes. Using this system, we asked the following questions: (1) How variable and heritable are the responses to predator cues across families? (2) Is predator-induced plasticity heritable and what is the coefficient of genetic variance in this population? (3) Do phenotypic responses genetically co-vary across traits and inducing environments? We predicted families (i.e., genotypes) would vary in magnitude and direction in response to each of the two predators, and that this variation would have a heritable basis. Although relatively little is known about the heritability of phenotypic plasticity, and particularly of morphological plasticity, morphological traits generally have higher heritabilities than do life-history or behavioral traits, and this pattern holds even for traits under selection (Mousseau and Roff 1987). We therefore predicted that heritability of traits related to the tail (spot size and color) and swimming musculature would be highest, as these are putatively under the strongest selection from the two predators (e.g., Benard 2006). Similarly, given the heterogeneous nature of selection pressure from various predators, we expected the capacity to be plastic should be under strong selection and therefore expected plasticity itself to be highly heritable. Finally, we predicted that trade-offs would exist—families that respond strongest to one predator would have a weaker response to the other and that these would be detected as negative genetic correlations across inducing environments.

Methods

TADPOLE PLASTICITY EXPERIMENT

This study was conducted at the Smithsonian Tropical Research Institute field station in Gamboa, Panama, between 10 August 2006 and 11 September 2006. Between 10 August and 25 August we collected 17 mating pairs of *D. ebraccatus* (two to four pairs on each of six nights) from three adjacent (≤2 km apart) ponds (Ocelot Pond, Bridge Pond, and Quarry Pond) around Gamboa (see Touchon and Vonesh 2016). Pairs were in amplexus when collected but had not begun laying eggs, or females were collected singly and paired with a nearby calling male.

For each pair of frogs, we collected an extra male from the same pond to act as a second mate for the female. Pairs were placed in a 4-L inflated Ziploc bag and returned to a dark room of an open-air laboratory where they were left to mate overnight. *Dendropsophus ebraccatus* usually lays semiterrestrial eggs (Touchon and Warkentin 2008b; Touchon and Worley 2015), and in these experiments they attached eggs to the inside walls of the inflated bags. Females can lay approximately 200–300 eggs in a night (Touchon and Worley 2015). We checked on mating pairs periodically and once a female had laid approximately 100 eggs we removed the pair from the bag, separated the amplectant male, and placed the female into a new bag with the second male. Because eggs are fertilized externally, this ensured that each of the two sets of eggs had a different sire. Frogs were released at the site of capture the following morning. In total, we paired 17 *D. ebraccatus* females to two males each, leading to 34 unique families or 17 maternal half-sib pairs.

Eggs attached to the wall of the plastic bag were mounted vertically on rigid plastic cards that overhung 240-mL plastic cups. A small amount of water (~5 mm depth) was placed in the bottom of the cup to catch hatchlings and eggs were misted multiple times per day with aged tap water to ensure hydration. Eggs hatched after approximately 3 days at which point more water was gently added to the cup and hatchlings were allowed 2 days to develop undisturbed.

On the third day post-hatching, 30 tadpoles were removed from each group of full-sibs and haphazardly assigned to one of three treatments: (1) predator-free control, (2) fish cues, or (3) aquatic insect cues. Hatchling *D. ebraccatus* are approximately 6 mm in total length at hatching (Touchon and Warkentin 2008a). Tadpoles were placed individually into 240-mL transparent plastic cups filled with 125 mL of aged tap water. In total, we raised 1020 *D. ebraccatus* tadpoles in individual cups. All cups were arranged across three vertically stratified shelves in an open-air laboratory with ambient air temperature, protected from the elements and augmented with fluorescent lighting. Cups were allocated to ensure an even representation of treatments across the three shelves and therefore not confound shelf with treatment. However, given the number of cups needing to have water removed and predator cue added each day, we clustered each family (a unique dam × sire combination) and treatment combination together. Thus, for example, one family might have had insect cue cups on the top shelf, control cups on the middle shelf, and fish cue cups on the bottom, whereas the family next to it might have had control cups on the top shelf, dragonfly cue cups in the middle, and fish cue cups on the bottom. This helped to ensure that appropriate cues were always added to their cups.

To prevent fouling of the water (given the small volume of water for each tadpole), food (rabbit chow) was added and feces and uneaten food were removed daily. A total of 25 mL of water was removed each day and replaced with fresh or chemically cued water as appropriate. Fish treatment water was generated by maintaining 30 *Astyanax ruberrimus* fish in 30 L of aged tap water (1 L per fish). Fish were collected with dip nets from a ditch that feeds into Bridge Pond. Insect treatment water was generated by maintaining nine containers of 5 L of aged tap water, each containing three large aeshnid dragonfly nymphs (Aeshnidae: *Anax amazili*), two libellulid dragonfly nymphs (Libellulidae: *Pantala flavescens*), one small unidentified belostomatid, and 10 backswimmers (Notonectidae: *Buenoa antigone antigone*). Although having a mixed "insect" cue may seem unorthodox, separate studies of *D. ebraccatus* tadpoles used *Pantala* dragonfly nymphs and belostomatids and yielded similar phenotypic responses (Touchon and Warkentin 2008a, 2011). *Anax* dragonfly nymphs are known to induce similar changes in tail morphology and color in multiple treefrog tadpoles (Relyea 2001; LaFiandra and Babbitt 2004; Richardson 2006) and backswimmers have similar behavioral effects as water bugs and dragonfly nymphs, for at least some species of tadpoles (Jara and Perotti 2010). We therefore expected all insect predators to induce responses in a similar direction. All predators are common throughout ponds in the study area and feed on *D. ebraccatus* tadpoles (Touchon and Vonesh 2016). Insect predators were collected from Bridge, Ocelot, and Experimental Ponds in Gamboa. Fish were fed a total of 60 tadpoles per day, and each container of insects was fed 10 tadpoles per day; thus, in both treatments two tadpoles were consumed per liter of water per day. Water was removed from all insect containers and mixed together before addition to individual tadpole containers. Predators were replaced if they died or metamorphosed.

Tadpoles were exposed to cues for 10 days, at the end of which they were lightly anesthetized in a bath of tricaine methanesulfonate (MS-222) and photographed dorsally and laterally (see Touchon and Warkentin 2008a for details of photography methods). All tadpoles recovered from the anesthesia and were released at their natal pond. Tadpoles were photographed in groups of five, all from the same family and treatment. However, all photos were given a randomly assigned code and subsequent measurements of tadpoles were done blindly to eliminate measurer bias. We measured tadpole morphology and characterized tail color using ImageJ version 1.34s (Rasband 2012). We measured each tadpole's 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), maximum tail fin depth (TFD), and the area of the conspicuous tail spot (TSA) (see Touchon and Warkentin 2008a for a visual depiction of measurements). These measurements are standard measurements of larval amphibians as they relate to swimming performance, antipredator defenses, and feeding capability (e.g., Van Buskirk et al. 1997; Van Buskirk and Schmidt 2000; Relyea 2001; McIntyre et al. 2004; Dayton et al. 2005; Touchon and Warkentin 2008a). TFD was defined by eye. Because the size of the tail spot is an area, TSA was square root transformed to achieve normality prior to statistical analyses. Similarly, TFD and hue were log-transformed to achieve normality.

Photographs contained a white-and-black standard and images were calibrated to these standards using the "Colour Correct" plugin in ImageJ. Color of the entire tail spot was measured in terms of hue and saturation using the HSB Stack and Measure functions. ImageJ measures hue and saturation values on a scale of 0–255. For hue, zero represents red and increasing values represent the colors of shorter wavelengths; increasing values indicate yellow, then green, and lastly blue. Saturation is the purity of a color relative to its brightness; small values generally indicate achromatic colors (shades of white, gray, and black) that arise from multiple colors (e.g., mix of blue and red) and larger values indicate values that derive from a single color (e.g., only red).

ANALYSES OF PHENOTYPIC RESPONSES TO PREDATORS

Data analyses were conducted in R version 3.3.1 (R Development Core Team 2013). To assess whether tadpoles exposed to predator cues altered their morphology and color, we used linear

mixed effects models in the package lme4 with predator treatment included as a fixed effect (Bates et al. 2013). The fit of all models was checked visually by inspecting qqplots and in all cases where data were transformed, model fit was improved. Because larger tadpoles are likely to also have larger phenotypic measures, we first used the prcomp function to conduct a principal components analysis of all linear morphological measurements to obtain an overall measure of tadpole "size" (e.g., Van Buskirk and McCollum 2000). The first principal component, PC1, accounted for 86% of variation in tadpole size and was included as a covariate fixed effect in models of morphological measures (McCoy et al. 2006). Analyses of color (i.e., hue and saturation) included the square root of TSA as a covariate as prior research has implied that larger tail spots are often more colorful as well (Touchon and Warkentin 2008a). Mother (dam) and Father (sire) nested within Mother (dam) were included as random effects in all models. Pond of origin, shelf in the laboratory, and start date were initially included as random effects but Akaike's information criterion (AIC) revealed that these never increased the fit of models, and in fact generally decreased the fit, and so they were not included in final analyses. Significance of fixed effects was assessed using nested likelihood ratio tests.

ESTIMATING HERITABILITY OF PLASTIC PHENOTYPES

To estimate narrow sense heritability (h^2) and components of genetic variance (additive genetic variance: V_A, paternal effects: V_{Sire}, maternal effects: V_{Dam}, residual genetic variance: V_{Resid}), we used "animal models" (Wilson et al. 2010) fit as linear mixed effects models in the package MCMCglmm (Hadfield 2010). This modeling technique uses Bayesian statistics to estimate variance components based on a pedigree that describes the relatedness of all individuals in the study. Our breeding design was constrained by the need to conduct the experiment at a relatively remote field station in Panama (i.e., we did not have a way to maintain adult frogs in captivity for extended periods of time), but unfortunately does not allow as accurate estimates of genetic and nongenetic maternal effects as a design in which males are mated to multiple females (e.g., North Carolina I).

Markov Chain Monte Carlo (MCMC) models were run for 500,000 iterations with a burn-in of 10,000 iterations, and were thinned every 50 iterations. We defined priors by dividing the observed amount of phenotypic variance for each trait being analyzed by the number of terms in the model (Wilson et al. 2010). All models included PC1 as a covariate and were run both including treatment as a fixed effect as well as within each predator treatment. Autocorrelation and effective sample size were verified manually, and convergence was verified visually by inspecting the trace plots of the posterior distribution. For each combination of phenotype measurement and rearing treatment, we used the deviance information criterion (DIC) to compare among models with four random effects structures ($V_A + V_{Resid}$, $V_A + V_{Sire} + V_{Resid}$, $V_A + V_{Dam} + V_{Resid}$, or $V_A + V_{Sire} + V_{Dam} + V_{Resid}$) and selected the best fitting model to use for estimating h^2 and variance components (note that DIC scores, h^2 estimates, and coefficients of genetic variance for all models are reported in Supporting Information). DIC is similar to the more well-known AIC method of quantifying model fit, but extended for use in Bayesian models (Burnham and Anderson 2002). Using the DIC score, we also calculated the model weights using the MuMIn package in R (Bartoń 2018) as well as the evidence ratio for each model (both reported in Supporting Information). We estimated h^2 and variance components as described in Wilson et al. (2010), and did so separately within each predator treatment (McGhee and Travis 2013). h^2 was calculated as V_A divided by the sum of all variance components included in the model (e.g., $V_A + V_{Sire} + V_{Dam} + V_{Resid}$, or $V_A + V_{Dam} + V_{Resid}$). Instead of reporting variance components themselves, we calculated and report here coefficients of genetic variance (CV_A, CV_{Sire}, CV_{Dam}, and CV_{Resid}). Coefficients of genetic variance provide a scaled and more comparable measure of evolvability than direct variance components themselves (Houle 1992) and were calculated by dividing the square root of each variance component by the size corrected trait mean $\times$ 100. Because all models included a covariate (either PC1 or square root transformed TSA), size-corrected trait means were obtained using the emmeans package in R (Lenth 2018).

Lastly, in an effort to quantify the effects of maternal relatedness, we randomly generated 1000 comparisons of the average tadpole phenotypes between a given family of tadpoles (unique mother and father combination) and their half-sibs (same mother but different father), and an unrelated family of tadpoles (different mother and different father). Each set of three randomly selected groups of tadpoles were always from the same rearing treatment. If phenotypes are highly heritable, we would expect half-sib families to be more similar to one another than to unrelated groups of tadpoles. We compared the distributions of differences in morphology and color measurements with Kolmogorov-Smirnov tests.

ESTIMATING GENETIC CORRELATION OF PLASTIC PHENOTYPIC RESPONSES

We used bivariate models to estimate the genetic covariance and heritability of all pairwise combinations of different aspects of the plastic phenotype within each of the three predator environments. Each model included PC1 as a covariate; models could only include one covariate and thus it was not possible to include TSA as a covariate in models that included tail spot saturation or hue. For each pair of traits, we ran a bivariate MCMC model for 250,000 iterations with a burn-in of 50,000 iterations and thinned every 50 iterations. Priors were defined as above. Random effects were V_A

$+ V_{Resid}$, as this structure most consistently led to the best fitting model in univariate models (see Results).

ESTIMATING GENETIC CORRELATIONS OF PLASTICITY ACROSS PREDATOR TREATMENTS

We estimated the genetic covariance of phenotypic plasticity in opposing predator treatments by fitting bivariate models of resampled datasets. Because each tadpole was only kept in a single environment, we first created bootstrapped datasets of sets of three randomly selected tadpoles (one from each predator treatment) for each family and then separately calculated the magnitude of plasticity (i.e., difference from the control) for tadpoles in the fish and insect treatment. Thus, (1) each family resulted in two measures of plasticity, one for the response to fish and one for the response to insects, and (2) the response variable in resampled datasets was the degree of plasticity and not the raw phenotypic measures themselves. We created 10 randomly sampled sets of tadpoles for each of the 34 families (with replacement allowed). For each trait of interest, we ran 500 bivariate MCMC models, each run for 250,000 iterations with a burn-in of 50,000 iterations and thinned every 100 iterations. This allowed us to estimate the distributions of heritability with each predator and genetic correlations across environments. Priors and random effects (V_A + V_{Resid}) were defined as above.

Results

VARIATION IN RESPONSES TO PREDATOR CUES ACROSS FAMILIES

The morphology of *D. ebraccatus* tadpoles raised with chemical cues from fish or insect predators, or without predator cues at all, differed substantially after 10 days. Tadpoles from the three rearing treatments differed in nearly all morphological and coloration variables measured (Fig. 2; Table S1). In particular, tadpoles raised with insects had relatively shorter tails and bodies, but much larger relative tail fins with larger and more colorful tail spots (Fig. 2). Furthermore, the interaction between size and predator treatment was significant for most aspects of the phenotype, indicating that the allometric scaling relationship between overall size and each aspect of the phenotype was altered (Table S1).

With specific consideration of color, the saturation of the tadpole tail spot differed across predator treatments, even after controlling for variation in TSA, whereas the hue differed across treatments and with tail spot area but not after controlling for variation in tail spot size (Fig. 2I, J; Table S1). Tadpoles raised with insect cues had tail spots with more purely red color (higher saturation), whereas tadpoles raised with fish cues had the most achromatic, least red tails (lower saturation). The interaction between tail spot size and saturation appears to be because the slope of the increase in saturation with increased tail spot size was shallower among tadpoles raised with insects.

Examination of the reaction norms of all 34 families revealed that different genetic backgrounds show marked differences in how they responded to chemical cues of fish or insect predation (Fig. 2). For example, although we observed a general trend for tadpoles raised with insects to have the smallest bodies relative to overall size (Fig. 2B), 10 families were larger when raised with insects than as controls. Similar patterns of the variable directional effects of predator cues among families were evident for all aspects of the phenotype. Variation in TFD was among the strongest and most consistent pattern observed, with tadpoles raised with insects having the deepest tail fins; that said, seven families had shallower tail fins with insects than as controls (Fig. 2D). We even found family-level differences in tail color saturation, a trait that we expected to have relatively consistent responses to predators. Although nearly all families increased saturation with insect cues and decreased saturation with fish cues, variation in the baseline phenotype of different families resulted in some families having greater tail color saturation when raised with fish cues than other families had even when raised with insect cues (Fig. 2I). In other words, differences in baseline tail color saturation among families meant that tadpoles from "low saturation" families—even after an induced increase in tail spot saturation—might still exhibit lower saturation than tadpoles from "high saturation" families that had an induced reduction in tail saturation.

HERITABILITY AND ADDITIVE GENETIC VARIATION FOR PREDATOR-INDUCED PHENOTYPES

In 21 of 30 models, the model fit was worsened by including V_{Sire} or V_{Dam}, or both (Table S2). Thus, we focus on models that include only V_A and V_{Resid} (Table 1; for results of all models see Table S2). Estimates of narrow sense heritability (h^2) were relatively low for TTL, but were considerably higher for most other phenotypic traits such as TFD, TMD, TSA, and tail spot saturation (Table 1). On average, h^2 was somewhat higher for tadpoles raised with insect cues (mean: 0.58) than for those raised with fish cues or as controls (means: both 0.52). In only one instance (tail spot saturation), h^2 was higher for control tadpoles than for either of the induced phenotypes. h^2 was highest (>0.70) for BL, TMW, and HW of tadpoles raised with insect cues and TSA of control and fish-raised tadpoles.

Estimates of CV_A were low for traits related to size (e.g., TL, TFD, HW, etc.), moderately high for tail spot hue and TMW, and very high for TMD, TSA, and tail spot saturation (Table 1). Including sire and dam effects in models reduced estimates of CV_A by an average of 18% (Table S3). When included, maternal effects (CV_{Dam}) were consistently estimated to be larger than paternal effects (CV_{Sire}). Maternal effects were generally of a

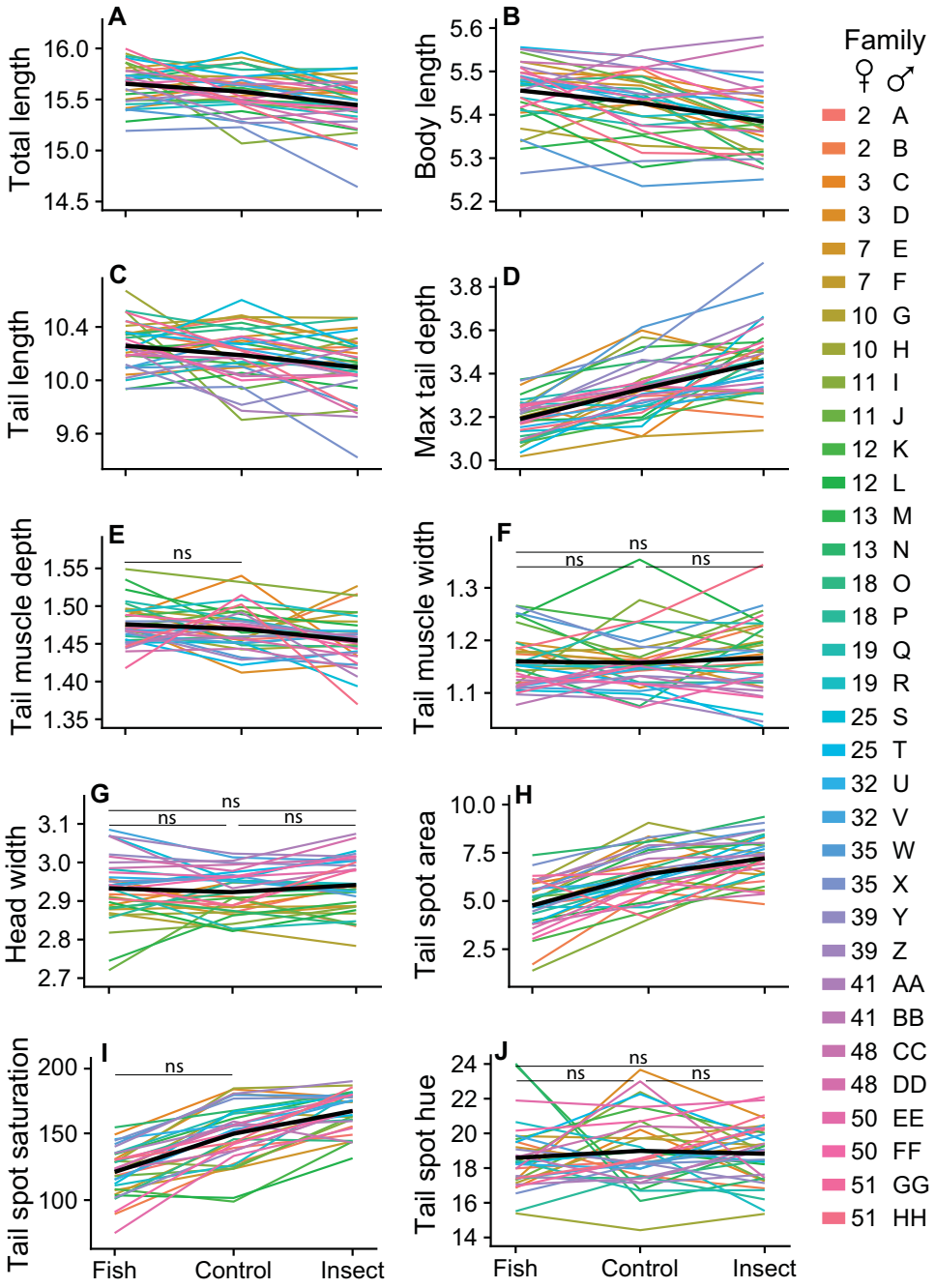

Figure 2. Developmental responses of *Dendropsophus ebraccatus* tadpoles in response to chemical cues of aquatic insect or fish predation, or cue-free controls. Shown are 10 different measures of morphology or coloration. The highly variable responses of each of 34 different families (colored lines) are shown, as well as the mean effects of rearing treatment (thick black lines). Values shown in A–H are estimated marginal means accounting for variation in overall size (PC1). Females were each mated to two different males and no males were used twice. Units for panels A–G are mm and for H is $mm.^2$ Units for I and J scale between 1 and 255; in I larger values indicate pure colors and in J smaller values indicate more red hues (see text for details). Mean differences in rearing treatments were significantly different from one another (all $P < 0.05$), as determined by post-hoc Tukey tests, unless otherwise noted.

similar magnitude to additive genetic effects, with the exception of tail color saturation, where CV_A was substantially larger than CV_{Dam} (Table S3).

A comparison of the differences in the phenotypes between related families and unrelated families revealed that tadpoles that shared a mother were much more similar to one another than

Table 1. Within-environment additive and residual coefficients of genetic variance (CV_A and CV_{Resid}, respectively) and narrow sense heritability (h^2) estimates from Bayesian animal models of *Dendropsophus ebraccatus* tadpoles raised with fish or insect predation cues or in predator-free controls.

Trait	Predator treatment	Covariate	CV_A (95% HPDI)	CV_{Resid} (95% HPDI)	Mean h^2 (95% HPDI)
Total length	Control	PC1	1.42 (1.07, 1.72)	1.31 (1.07, 1.50)	0.22 (0.12, 0.33)
	Fish	PC1	1.36 (1.01, 1.63)	1.24 (1.04, 1.43)	0.21 (0.11, 0.32)
	Insect	PC1	1.61 (1.25, 1.91)	1.38 (1.13, 1.59)	0.31 (0.16, 0.45)
Body length	Control	PC1	1.54 (1.16, 1.84)	1.36 (1.11, 1.57)	0.31 (0.17, 0.46)
	Fish	PC1	1.52 (1.21, 1.78)	1.40 (1.18, 1.59)	0.54 (0.39, 0.68)
	Insect	PC1	7.18 (5.27, 8.72)	4.45 (2.54, 5.77)	0.71 (0.49, 0.93)
Tail length	Control	PC1	2.33 (1.85, 2.75)	2.03 (1.68, 2.33)	0.57 (0.41, 0.71)
	Fish	PC1	2.11 (1.66, 2.50)	1.97 (1.65, 2.25)	0.53 (0.38, 0.68)
	Insect	PC1	2.68 (2.21, 3.12)	1.97 (1.58, 2.31)	0.65 (0.50, 0.79)
Max tail depth	Control	PC1	1.35 (1.01, 1.64)	1.12 (0.87, 1.34)	0.59 (0.40, 0.78)
	Fish	PC1	1.07 (0.81, 1.30)	0.96 (0.77, 1.12)	0.55 (0.36, 0.73)
	Insect	PC1	1.56 (1.20, 1.87)	1.14 (0.85, 1.39)	0.65 (0.46, 0.84)
Tail muscle depth	Control	PC1	9.28 (6.88, 11.51)	9.65 (7.96, 11.09)	0.48 (0.30, 0.67)
	Fish	PC1	8.45 (5.78, 10.85)	8.89 (7.15, 10.34)	0.33 (0.17, 0.51)
	Insect	PC1	10.84 (8.03, 13.23)	9.49 (7.38, 11.12)	0.56 (0.37, 0.75)
Tail muscle width	Control	PC1	5.82 (3.95, 7.55)	4.79 (3.09, 5.95)	0.59 (0.33, 0.86)
	Fish	PC1	5.39 (4.02, 6.59)	4.50 (3.36, 5.34)	0.58 (0.39, 0.79)
	Insect	PC1	7.57 (5.64, 9.11)	4.20 (1.99, 5.61)	0.76 (0.54, 0.96)
Head width	Control	PC1	2.48 (1.61, 3.24)	2.22 (1.47, 2.70)	0.55 (0.29, 0.82)
	Fish	PC1	2.60 (2.03, 3.07)	1.87 (1.41, 2.26)	0.65 (0.48, 0.83)
	Insect	PC1	3.00 (2.16, 3.69)	1.77 (0.72, 2.34)	0.73 (0.50, 0.97)
Tail spot area	Control	PC1	11.09 (9.01, 13.11)	6.78 (4.55, 8.37)	0.72 (0.55, 0.88)
	Fish	PC1	14.85 (11.84, 17.45)	8.43 (5.20, 10.70)	0.75 (0.58, 0.92)
	Insect	PC1	8.57 (6.52, 10.43)	6.27 (4.38, 7.63)	0.65 (0.44, 0.84)
Saturation	Control	TSA	13.33 (9.36, 16.78)	10.80 (7.42, 13.27)	0.60 (0.37, 0.85)
	Fish	TSA	13.79 (8.44, 17.99)	14.47 (10.92, 17.17)	0.47 (0.23, 0.73)
	Insect	TSA	9.58 (6.15, 12.56)	10.51 (8.22, 12.31)	0.45 (0.23, 0.69)
Hue	Control	TSA	4.58 (2.97, 6.01)	4.22 (2.97, 5.13)	0.53 (0.28, 0.81)
	Fish	TSA	4.17 (2.82, 5.34)	3.67 (2.57, 4.42)	0.56 (0.32, 0.81)
	Insect	TSA	3.46 (1.93, 4.70)	5.38 (4.56, 6.06)	0.29 (0.11, 0.50)

Shown are the estimates of CV_A and CV_{Resid} from the best fitting model for each trait in each treatment, which contained just these two parameters in 70% of models. Coefficients of genetic variance for sire and dam effects and results of all models run are included in Supporting Information.
Parentheses contain 95% confidence intervals calculated from the highest posterior density interval (HPDI) of the model.

tadpoles that were unrelated (Kolmogorov-Smirnov tests for all variables $P < 0.00001$; Fig. S1).

CROSS-ENVIRONMENT AND CROSS-TRAIT GENETIC CORRELATIONS FOR PLASTICITY

Bivariate models of plasticity in response to each type of predator revealed strong negative genetic correlations between fish-induced and insect-induced phenotypic responses (Table 2). All genetic correlations were significantly different from zero and ranged from –0.42 to –0.59. Except for tail color saturation, h^2 was higher when tadpoles were raised with chemical cues of fish (0.58) than with cues of insects (0.45; Table 2).

We also investigated cross-trait genetic correlations of different aspects of the phenotype within each predator treatment. In all three predator environments, bivariate models of pairs of phenotypic traits demonstrated both positive and negative genetic correlations between different aspects of the phenotype (Fig. 3). Of note is that TFD, TSA, and tail spot saturation were generally strongly positively correlated among tadpoles in all three rearing environments, whereas most other pairwise comparisons of the phenotype were either uncorrelated or had significant negative genetic correlations (Fig. 3). Genetic correlations, either positive or negative, were least pronounced in tadpoles raised with fish cues.

Table 2. Narrow sense heritability (h^2) of plasticity (change from control) and cross-environment genetic correlations for *Dendropsophus ebraccatus* tadpoles raised with fish or insect predation cues.

Trait	h^2 of plasticity with insect cue	h^2 of plasticity with fish cue	Genetic correlation
Total length	0.452 (0.384, 0.519)	0.596 (0.538, 0.653)	−0.533 (−0.213, −0.812)
Body length	0.417 (0.347, 0.487)	0.599 (0.543, 0.655)	−0.503 (−0.164, −0.800)
Tail length	0.469 (0.398, 0.539)	0.598 (0.541, 0.655)	−0.557 (−0.251, −0.823)
Max tail depth	0.423 (0.345, 0.503)	0.622 (0.566, 0.679)	−0.423 (−0.057, −0.751)
Tail muscle depth	0.489 (0.359, 0.620)	0.604 (0.500, 0.707)	−0.586 (−0.299, −0.832)
Tail muscle width	0.501 (0.422, 0.581)	0.602 (0.545, 0.658)	−0.417 (−0.063, −0.737)
Head width	0.404 (0.332, 0.476)	0.579 (0.516, 0.642)	−0.467 (−0.106, −0.786)
Tail spot area	0.454 (0.380, 0.529)	0.589 (0.528, 0.650)	−0.453 (−0.100, −0.772)
Saturation	0.442 (0.299, 0.585)	0.421 (0.277, 0.564)	−0.423 (−0.034, −0.776)
Hue	0.418 (0.342, 0.494)	0.616 (0.556, 0.677)	−0.477 (−0.135, −0.777)

Parentheses contain either 95% confidence intervals calculated from the distribution of resampled values (plasticity with insect or fish cues) or are the mean values of the upper and lower limits of the HPDI in each resampled model (genetic correlation).

Discussion

Countless plant and animal species have evolved the ability to respond to varying abiotic and biotic environmental conditions—changes in day length or temperature, resource availability, or the presence of predators or competitors—by altering some aspect of their phenotype (Schlichting and Pigliucci 1998; Pigliucci 2001; West-Eberhard 2003). These organisms can alter aspects of their chemical composition, morphology, behavior, coloration, or life history. For such flexible traits to evolve through selection, there must be heritable genetic variation for the flexibility of the trait itself. Here, we demonstrate that predator-induced plasticity (morphology and color) observed in larvae of the Neotropical treefrog *D. ebraccatus* is remarkably heritable and highly variable across different families (genotypes). Importantly, we document strong negative genetic correlations for plastic responses to two different types of predators that induce different phenotypic responses, indicating the presence of constraints on how these plastic traits evolve. In other words, the ability to flexibly respond to one predator is constrained by a genotype's ability to respond to another predator, and there are clear tradeoffs in how animals invest in different aspects of the defensive phenotype.

HOW VARIABLE ARE RESPONSES TO PREDATOR CUES ACROSS FAMILIES?

Like the larvae of several other anurans (Van Buskirk and McCollum 1999; Relyea 2001; Laurila et al. 2002b; LaFiandra and Babbitt 2004; Benard 2006), *D. ebraccatus* tadpoles in this study altered tail morphology and color in response to chemical cues of predators. They developed relatively large tails with colorful tail spots in response to cues of aquatic insects (e.g., dragonfly nymph, diving beetle larvae, or water bug predators), and relatively shallow, colorless tails in response to cues of fish predators. This is the same response documented previously for *D. ebraccatus* to these types of predators (Touchon and Warkentin 2008a, 2011). More importantly, the patterns we observe here only held when looking at the average responses of tadpoles. By considering different genotypes, we were able to demonstrate remarkably variable responses to the same cues of predation (Fig. 2). Although plasticity was often induced by predators, the directionality of responses could not always reliably be predicted. For example, although one of the most pronounced responses of tadpoles to cues of insect predators is the increase in tail fin depth (Fig. 2D), 20% of families had shallower tails with insect cues than when raised as controls. Similarly, although there was little average effect of predators on tadpole TMW (Fig. 2F), 20% of families decreased TMW in response to each predator, and 32% of families increased TMW in response to each predator.

IS PREDATOR-INDUCED PLASTICITY HERITABLE IN *D. EBRACCATUS*?

The plastic phenotypic responses of tadpoles to predation cues were highly heritable with relatively high measures of CV_A (Table 1). Furthermore, plasticity itself was highly heritable, and was more so when tadpoles were responding to chemical cues of fish than insects. This study is one of only a very few to demonstrate narrow sense heritability of predator-induced morphological plasticity (see Relyea 2005; Dingemanse et al. 2009). High broad sense heritabilities of phenotypic traits were found when tadpoles were fed a novel diet (Ledon-Rettig et al. 2010). Although predator-induced morphological changes may be relatively understudied in tadpoles, other flexible traits have been studied, and have reported lower estimates of h^2 and CV_A than we find here. Induced hatching time (Gomez-Mestre and

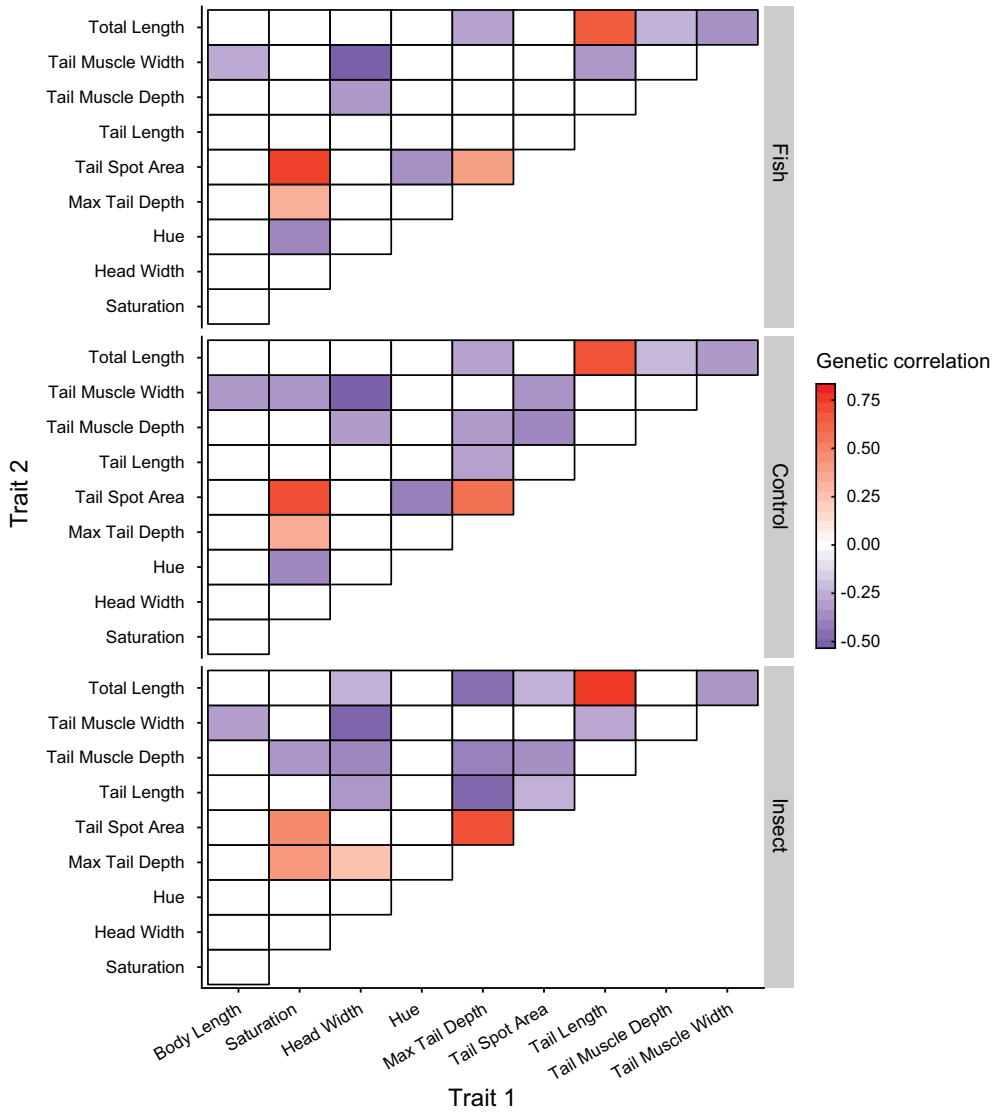

Figure 3. **Cross-trait genetic correlations between each of 10 measures of morphology and coloration for *Dendropsophus ebraccatus* tadpoles raised with chemical cues of aquatic insect or fish predation, or cue-free controls. Cells in blue represent pairs of traits with negative genetic correlations, whereas cells in red represent positive genetic correlations. Cells in white are not significantly different from zero (i.e., traits are not correlated).**

Warkentin 2013), mating behaviors (McGhee and Travis 2013), or exploratory personality (Dingemanse et al. 2009) all show lower heritability and additive genetic variance than we find for morphological traits in *D. ebraccatus*. This was perhaps to be expected, as life-history and behavioral traits are often regarded as having lower h^2 than behavioral or morphological traits (Price and Schluter 1991; Mousseau and Roff 1987). The fact that plastic traits, and the ability to be plastic itself, are heritable and variable demonstrates the mediating role of natural selection in induced phenotypes for *D. ebraccatus* specifically, and could be true, generally, for taxa occurring in highly heterogeneous and variable environments.

DO PHENOTYPIC RESPONSES GENETICALLY CO-VARY ACROSS TRAITS AND INDUCING ENVIRONMENTS AND ARE THERE TRADE-OFFS FOR THE ABILITY TO RESPOND TO EACH PREDATOR?

The most consistent phenotypic responses to predators were in tail spot area and saturation (Fig. 2H, I). Most families either increased tail spot size and saturation when raised with insect cues or decreased them with exposure to fish cues; only a subset of families, and certainly not all, could do both. This stands in contrast to green frog tadpoles, which have shown genetically correlated responses in activity level to both fish and dragonfly nymph predators (Watkins and McPeek 2006). Of particular

interest in our results is that the range of possible phenotypes displayed across all families was much larger than the range of phenotypes produced by any single family. This may seem obvious and intuitive on the surface, but the interpretation of this finding is important for understanding genetic constraints on induced phenotypes. The strong negative genetic correlations for (1) responding to each predator (Table 2) and (2) for producing different aspects of the phenotype (Fig. 3) suggest that genotypes are limited in their ability to respond to multiple predators. For example, a family that produces a high saturation red tail with insect cues is likely unable to also make a very clear tail with fish cues, and vice versa. No genotype demonstrated "perfect plasticity" and therefore most families were predisposed in their response to one predator or the other. If this were not the case, we would have expected to find that the range of phenotypes expressed at the family level would be more similar to the range of variation in phenotypes among families.

The variation in these flexible morphological responses is remarkable as it runs counter to the notion that prey animals should respond in a particular, presumably adaptive, manner with a predator. For example, although the magnitude of plastic responses by wood frog (*Rana [= Lithobates] sylvatica*) tadpoles to dragonfly nymph predators varied among families (Relyea 2005), they generally responded in the same direction (as opposed to our study, which revealed responses in different directions among genotypes). This form of fundamentally different plastic responses has been observed in behavior or life-history traits between populations under different selective regimes (Husby et al. 2010) or between individuals with and without plasticity (Duffy 2010). Recognizing that individuals within a population vary in their phenotypes is paramount to understanding the evolution and maintenance of phenotypic plasticity in a population (Bolnick et al. 2003). Furthermore, such high degrees of individual-level variation in plastic responses are important for understanding many community ecological processes (Hughes et al. 2008; Bolnick et al. 2011; Fischer et al. 2014).

WHAT MAINTAINS VARIATION AND CONSTRAINTS IN PHENOTYPIC PLASTICITY?

Theory has long presumed that the evolution of phenotypic plasticity must be constrained by some forms of costs (e.g., DeWitt et al. 1998). Recent work has delineated costs of plasticity per se from costs of producing mismatched phenotypes or limits to producing different phenotypes by plastic genotypes (Relyea 2002a; Van Buskirk and Steiner 2009; Murren et al. 2015). Our goal was not to investigate costs of plasticity, and our data are not suited for documenting costs as we did not measure fitness, but our finding of strong negative genetic correlations for plastic responses to different predators strongly supports the view that variable selection promotes a diverse set of plastic phenotypic responses (Murren et al. 2015).

Indeed, ours is the first study that we are aware of to investigate heritability of plasticity in response to multiple predators, or multiple forms of any environmental variable for that matter, and as such makes a valuable contribution to our thinking about the maintenance of plasticity. Although relaxed selection (Snell-Rood et al. 2010; Hunt et al. 2011; Leichty et al. 2012) and cryptic genetic variation (Ledon-Rettig et al. 2010; Paaby and Rockman 2014) have recently been proposed as important drivers of the origin of plasticity, few have focused on the processes that serve to maintain plasticity. In our system, and likely many others in nature, prey are under constant but varying selection from multiple predators (including many more than were tested here; Touchon and Vonesh 2016), each exerting selection for a different phenotypic optima. Negative genetic correlations between traits likely stem from negative correlational selection on the responses to two environments (Roff and Fairbairn 2012). In other words, natural selection caused by predators such as fish and dragonflies reduces the ability to respond to both types of predators. This leads to a highly dynamic adaptive landscape wherein different predator communities create different sets of peaks that are occupied by different prey genotypes (Calsbeek et al. 2012). However, instead of plasticity facilitating the jump from one peak to another in the landscape, as has previously been suggested (West-Eberhard 2003; Calsbeek et al. 2012), negative genetic correlations may result in genotypes occupying a high-fitness peak in one environment that is a low-fitness valley in another. That is, in such spatially and temporally variable selective environments, no single plastic genotype is best, and genetic variation for plasticity is maintained.

What else might contribute to such a variable adaptive landscape? The responses we observed by different families may be explained by several nonmutually exclusive hypotheses. (1) There may be other aspects of the phenotype that change in concert with morphology that renders different body and tail shapes equally effective at improving survival. Morphology and coloration are only two parts of the phenotype (Forsman 2015), and changes in behavior (for example) may complement other aspects of the induced phenotype in ways that maintain an adaptive response to predators. (2) Different morphologies may be similarly adaptive under different environmental conditions. The ponds in this study are very close together and contain generally similar predator communities, yet differ in physical parameters such as canopy cover, the presence and type of emergent or floating vegetation, and the openness or structure beneath the water surface (Touchon 2012; Touchon and Vonesh 2016). Given that the efficacy of tadpole phenotypes may depend on pond characteristics such as background vegetation or water turbidity (Eterovick et al. 2010; Polo-Cavia and Gomez-Mestre 2017), each

distinct habitat may be more or less advantageous to certain tadpole morphologies (Kopp et al. 2006). Adult frogs likely move among ponds during the breeding season and juveniles are also likely to disperse after metamorphosis. Dispersal among ponds would likely swamp a strong effect of localized selection in a single environment. (3) Predators may be sufficiently spatially and temporally heterogeneous as to render the variety of different morphological responses adaptive over longer time scales. Like many tropical anurans, female *D. ebraccatus* breed multiple times throughout the rainy season (as often as every 12 days; Wells 2007), which lasts for approximately six months in central Panama where our study was conducted (Touchon 2012). There are many ponds in our field site, some of which never contain fish (Touchon and Vonesh 2016), whereas others contain fish only after large rainstorms result in flooding from nearby streams (J. Touchon, pers. obs.). Such heterogeneity may result in particular plastic morphological responses being more or less advantageous at different times or in different places. Thus, if the predator community changes sufficiently between breeding bouts or between ponds, each genotype should persist over longer time scales.

It was beyond the scope of this experiment to assay survival of different genotypes when placed with different predators, but this will be an essential aspect of future research to understand the adaptive nature of predator-induced plasticity. Given the substantial variation in baseline (i.e., control) phenotypes of *D. ebraccatus* tadpoles, and those exposed to predators, it should be possible to design experiments that permit us to isolate the effects of tail morphology and coloration in the absence of predators, thereby permitting studies of how each contributes to improving prey survival. Only with such experiments we will more completely understand the role of genetic variation in phenotypic plasticity in organismal evolution.

AUTHOR CONTRIBUTIONS

J.C.T. and J.M.R. conceived and executed the study. J.C.T. analyzed the data. J.C.T. and J.M.R. wrote the manuscript.

ACKNOWLEDGMENTS

We thank the Smithsonial Tropical Research Institute (STRI) for logistical support and the Autoridad Nacional del Ambiente de Panamá (ANAM) for research permit SC/A-8-06. This research was conducted under Boston University IACUC protocol # 05-022. We thank K. Warkentin, K. Zamudio, I. Gomez-Mestre, M. Hughey, M. Caldwell, S. Poo, and A. Estrada for assistance with designing and implementing the experiment and R. Espinoza for helpful comments on the manuscript. This work was funded by grants from National Science Foundation (DEB-0508811), Boston University, and STRI to JCT and National Science Foundation (DEB-0506043) and Cornell University to JMR.

DATA ARCHIVING

The doi for our data is https://doi.org/10.5061/dryad.2n6h627.

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Associate Editor: A. Crawford
Handling Editor: M. Noor

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Full results of predator-induced plasticity models.
Table S2. Model selection results: Heritability.
Table S3. Model selection results: Variance components.
Figure S1. The distributions of differences in phenotypes between randomly selected groups of tadpoles from a unique family and their half-sib relatives or an unrelated set of tadpoles.