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Plasticity-led evolution: evaluating the key prediction of frequency-dependent adaptation

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Plasticity-led evolution occurs when a change in the environment triggers a change in phenotype via phenotypic plasticity, and this pre-existing plasticity is subsequently refined by selection into an adaptive phenotype. A critical, but largely untested prediction of plasticity-led evolution (and evolution by natural selection generally) is that the rate and magnitude of evolutionary change should be positively associated with a phenotype's frequency of expression in a population. Essentially, the more often a phenotype is expressed and exposed to selection, the greater its opportunity for adaptive refinement. We tested this prediction by competing against each other spadefoot toad tadpoles from different natural populations that vary in how frequently they express a novel, environmentally induced carnivore ecomorph. As expected, laboratory-reared tadpoles whose parents were derived from populations that express the carnivore ecomorph more frequently were superior competitors for the resource for which this ecomorph is specialized—fairy shrimp. These tadpoles were better at using this resource both because they were more efficient at capturing and consuming shrimp and because they produced more exaggerated carnivore traits. Moreover, they exhibited these more carnivore-like features even without experiencing the inducing cue, suggesting that this ecomorph has undergone an extreme form of plasticity-led evolution—genetic assimilation. Thus, our findings provide evidence that the frequency of trait expression drives the magnitude of adaptive refinement, thereby validating a key prediction of plasticity-led evolution specifically and adaptive evolution generally.

1. Introduction

Phenotypic plasticity is commonplace [1,2], but whether and how it impacts evolution is controversial [3–5]. An evolutionary process in which plasticity has long been implicated is the origins of novel, complex phenotypes (e.g. [2,5–10]).

According to the 'plasticity-led evolution' hypothesis (sometimes dubbed 'plasticity-first evolution' [11,12]), a novel complex phenotype first appears in a rudimentary form when the phenotype (or its components) is expressed via plasticity following a change in environment. Such environmental change is typically stressful, and organisms can mitigate this stress by using plasticity to facultatively produce a phenotype better matched to the new environment. If underlying genetic variation exists in either the tendency or manner in which individuals respond to this environmental change (as is nearly always the case [13]), then selection can act on these 'reaction norms' and improve the phenotype's functionality by altering the phenotype's form. Moreover, selection can also promote a change in the phenotype's regulation. Specifically, depending on whether or not plasticity is favoured [14,15], selection can favour either increased environmental sensitivity—which might ultimately maintain the new phenotype as part of a 'polyphenism' [1]—or decreased environmental sensitivity—which might ultimately cause the plasticity to be lost and the phenotype to become canalized through 'genetic assimilation' (*sensu* [16]). Essentially, plasticity-led evolution occurs when selection promotes an

adaptive change in an initially environmentally induced phenotype's form and/or regulation. Thus, plasticity itself can evolve (as has been long recognized (e.g. [13,15,17–19])), and, consequently, this evolution can facilitate the origin of a novel, complex phenotype.

Although laboratory studies support these ideas [16,20], and there are suggestive field studies (reviewed in [9,12]), many researchers remain sceptical of whether plasticity can facilitate evolution [3,4]. Such scepticism arises, in part, because the key criteria and predictions of the plasticity-led evolution hypothesis have not been made clear and evaluated in natural populations [3,4]. To address this concern, we [12] recently outlined four key criteria for testing this hypothesis, one of which (criterion 4) is that the focal trait should exhibit evidence of having undergone adaptive refinement as it is induced and exposed to selection repeatedly. Although this criterion is seldom validated, doing so is essential to rule out alternative explanations [12].

Moreover, of the few studies that have tested criterion 4 (cited in [12]), none have tested its critical, underlying prediction: that the rate and magnitude of phenotypic change should be positively associated with a phenotype's frequency of expression or use in a population [2,12,21,22]. This prediction is, in turn, rooted in two assumptions: (i) that individuals in ancestral lineages (where a rudimentary version of the focal trait is produced through plasticity) should express the trait less frequently than individuals in derived lineages (where the trait may be canalized); and (ii) that a trait in a population in which it is expressed (and exposed to selection) more frequently should evolve greater and more rapid refinement [2]. Essentially, during plasticity-led evolution, as an environmentally induced phenotype is recurrently produced (e.g. by persistent selection pressure favouring that phenotype), it will be exposed to selection more frequently and therefore have greater opportunity for adaptive refinement.

This notion that the frequency of trait expression drives the magnitude of adaptive refinement is a critical prediction not only of plasticity-led evolution, but also of evolution by natural selection more generally. Yet, 'frequency-dependent adaptation' has rarely been demonstrated empirically (but see [23–25]). (Note that frequency-dependent adaptation is a separate, albeit related, process from frequency-dependent *selection*, which arises when the fitness of an individual phenotype depends on its frequency in the population. Unlike frequency-dependent *adaptation*, frequency-dependent selection has been thoroughly studied; e.g. [26–28]). However, indirect support for frequency-dependent adaptation comes from studies: (i) using reciprocal transplants that demonstrate adaptation to local (i.e. frequently experienced) conditions and maladaptation to alternative conditions (e.g. [29–31]); (ii) of clinal variation in adaptation that have shown a pattern of changing phenotype ratios (including environmentally induced phenotypes) along the cline such that the greatest divergence occurs at the clinal extremes (e.g. [32–34]); and (iii) exploring adaptive radiation where generalist or plastic ancestors experience greater specialization over time (e.g. [35–38]).

Here, we perform an explicit empirical test of frequency-dependent adaptation. We do so by focusing on amphibian populations that have diverged in production of a novel, environmentally induced ecomorph. If the frequency of trait expression does indeed determine the degree to which that phenotype is refined by selection, then individuals from populations that produce this ecomorph more frequently should be superior competitors for the resource on which this ecomorph specializes. As we describe below, our findings are consistent with this expectation.

2. Material and methods

(a) Study subjects

We studied plains spadefoot toads, *Spea bombifrons*, from natural populations in the western USA. In many parts of its range, *S. bombifrons* has evolved a larval polyphenism in which it produces two, environmentally induced, resource-use ecomorphs (see the electronic supplementary material, figure S1): (i) omnivores, which are dietary generalists that feed mostly on detritus and small plankton, and which are normally produced by default; and (ii) carnivores, which are dietary specialists that feed on, and are induced by the consumption of, anostracan fairy shrimp or other tadpoles [39–45]. In most populations, omnivores are the more frequently produced of the two ecomorphs (e.g. earlier studies that sampled diverse sites in allopatry found an average of 20% carnivores and 80% omnivores [41–43]; see the electronic supplementary material, figure S2). However, in populations where *S. bombifrons* co-occurs with a congener (*Spea multiplicata*), these two species have undergone ecological character displacement, resulting in *S. bombifrons* producing nearly all carnivores (e.g. earlier studies found an average of 95% carnivores and 5% omnivores in sympatry [41–43]; see the electronic supplementary material, figure S2). We refer to these *S. bombifrons* populations that occur with and without *S. multiplicata* as 'sympatric' and 'allopatric', respectively. Because the two species have come into secondary contact following range expansion by *S. bombifrons* [46,47], sympatric populations represent the 'derived' state, whereas allopatric populations represent the 'ancestral' state.

For the experiments below, we created 10 full sibships of *S. bombifrons* by breeding adults that were recently collected from diverse populations in allopatry (electronic supplementary material, table S1 and figure S2), all of which probably experience ongoing gene flow [46]: Colorado (four sibships), northern Nebraska (one sibship), southwestern Nebraska (two sibships), Oklahoma (two sibships) and Texas (one sibship). These 10 sibships constituted our allopatric animals. We also created eight full sibships of *S. bombifrons* by breeding adults that were recently collected from six populations in the San Simon valley of southeastern Arizona (where *S. multiplicata* is present; electronic supplementary material, table S1 and figure S2), all of which probably experience ongoing gene flow [47]. These eight sibships constituted our sympatric animals. Breeding was induced by injecting adults with 0.04 ml luteinizing hormone-releasing hormone (Sigma L-7134) at a concentration of 0.01 $\mu g\ \mu l^{-1}$ and leaving pairs overnight in separate nursery tanks. The next day, adults were removed, and the eggs from each sibship were kept in these tanks until they hatched.

(b) Testing whether frequency of trait expression predicts its adaptive refinement

We predicted that sympatric tadpoles would be superior competitors for shrimp—and therefore grow more on shrimp—compared to allopatric tadpoles. This is because *S. bombifrons* produces carnivores (the ecomorph that specializes on shrimp) more frequently in sympatry than in allopatry (see §2a). Conversely, we predicted that allopatric tadpoles would be superior competitors for detritus compared to sympatric tadpoles, because *S. bombifrons* produces omnivores (the ecomorph that uses detritus) more frequently in allopatry than in sympatry (see §2a).

For these tests, we had to give each tadpole a population-specific mark (to differentiate it from its tankmate; e.g. see

[48]). We therefore needed to grow tadpoles to a sufficient size to receive these marks. To do so, we randomly selected 95 tadpoles from each of 15 sibships (eight allopatric, seven sympatric) and placed them in an outdoor wading pool (1.5 m diameter) for 4 days (water temperatures approx. 30°C). At the start, each pool received 50 ml of plant-based fish food. After returning the tadpoles indoors, we placed each sibship in clean water and fed them 400 mg of fish food. Twenty-four hours later, we measured the snout–vent length (SVL) of these tadpoles and created our experimental units.

Each experimental unit consisted of three tanks (18 × 13 × 8.5 cm, filled with 1.2 l of dechlorinated water) containing: (i) a single allopatric tadpole, (ii) a single sympatric tadpole, and (iii) two tadpoles, one of which was a sibling of the allopatric tadpole, and the other of which was a sibling of the sympatric tadpole. The first two were dubbed 'singleton tanks', whereas the third was dubbed a 'competition tank'. All tadpoles in each experimental unit were similar in SVL at the start (individuals varied by less than 2.5%). To distinguish between tankmates in the competition tanks, we injected pink elastomer [48] into the dorsal tail of one individual (equal numbers of allopatric and sympatric tadpoles were injected). All three tanks in each experimental unit were placed adjacent to each other.

Half of the competition tanks received daily 40 mg of crushed fish food (hereafter, 'detritus'), which simulates in form and nutrition the detritus on which *Spea* omnivores feed in natural ponds [44]. The other half received twice daily 100 live brine shrimp (*Artemia*), which simulate the fairy shrimp (*Thamnocephalus* or *Steptocephalus*) on which *Spea* carnivores feed in natural ponds. Preliminary tests indicated that these amounts of detritus and shrimp induced competition; i.e. food was completely eaten between feedings. Singleton tanks received half of these amounts; thus, the per capita amounts of food provided to singleton and competition tanks were identical. All tanks experienced 50% water changes every other day. We had 51 replicate units per diet. After 10 days, we ended the experiment by euthanizing tadpoles in a 0.8% aqueous solution of tricaine methanesulfonate (MS-222) and preserving them in 95% ethanol.

We evaluated the predictions outlined at the start of this section in three ways. First, we used likelihood ratio tests to compare a series of mixed models. 'Diet' (i.e. detritus or shrimp) and 'selective environment' (i.e. allopatry or sympatry) were fixed categorical variables and 'sibship', 'competitor sibship' and 'replicate' were random effects. We compared a null model that contained only the random effects to single-factor models that retained the random effects and included either diet or selective environment as a fixed effect, and to two-factor models (with and without an interaction term). The 'best model' was determined if it was significantly better than all other models according to likelihood ratio tests (performed using 'anova' in R). The biological interpretation of each model is described in the electronic supplementary material.

Second, we performed a type III sum of square analysis of variance (ANOVA) on the interaction model to corroborate our observations from the above test. We also calculated the effect size (Cohen's d) between diets for each selective environment to determine if tadpoles from sympatry have experienced greater divergence in growth between diets (i.e. greater growth on shrimp and/or reduced growth on detritus) than tadpoles derived from allopatry. If there was a significant interaction, we performed post hoc multiple comparisons tests by grouping selective environment with diet (i.e. allopatry.shrimp, allopatry.detritus, etc.) and using the 'pairwise.t.test' function with 'fdr' correction in R.

Finally, for each competition tank, we categorized each tadpole as the 'winner' of competition if it grew more than its tankmate. We then performed one-tailed Fisher's exact tests to determine if the number of winners differed between selective environments. One-tailed tests were used because we had the *a priori* prediction that there would be more allopatric winners on detritus and more sympatric winners on shrimp (this *a priori* prediction was based on patterns of trait expression in nature; see §2a). We also used Levene's tests to evaluate differences in the amount of variation in growth among selective environments and diets. If competitive differences between selective environments happen to be diet-dependent, then we would expect greater variation on one diet than on the other.

(c) Evaluating mechanisms of adaptive refinement

The results of the previous experiment revealed that (see §3a): (i) tadpoles from sympatry grew more than tadpoles from allopatry on shrimp, and (ii) tadpoles from allopatry grew more than tadpoles from sympatry on detritus. Based on previous work [41,42,44,48–52], we evaluated five, non-mutually exclusive mechanisms that could explain these differences between selective environments in competitive ability. We specifically tested whether tadpoles from the two selective environments have diverged in: (i) intrinsic growth rate, (ii) time budgets, (iii) trait integration, (iv) shrimp capture ability, or (v) trophic morphology. Each test is described in the electronic supplementary material.

(d) Testing for genetic assimilation of trophic morphology

Finally, we tested if tadpoles from sympatry developed more carnivore-like features, even in the absence of the cue that normally induces the carnivore morphology (ingestion of live shrimp or tadpoles). Finding such a pattern would suggest that trophic morphology has been genetically assimilated in sympatry. To perform this test, we randomly selected 10, two-day old tadpoles from each sibship in §2b (we selected these tadpoles before they had been fed). We euthanized and preserved tadpoles as in §2b. We then measured SVL and the width of the jaw muscle (orbitohyoideus muscle; OH), which is diagnostic of ecomorphology [39]. We standardized OH for body size (SVL) by regressing log OH on log SVL [44,53]. We then compared these size-corrected OH and SVL values between allopatry and sympatry using a likelihood ratio test and linear mixed effects models (fitted with maximum-likelihood in the R package 'lme4'). Specifically, we used a likelihood ratio test (through the 'anova' function in R [54]) to compare a null model only containing the random effect 'sibship' with a full model that retained this random effect and also included 'selective environment' as a fixed effect.

3. Results

(a) Testing whether frequency of trait expression predicts its adaptive refinement

At the start of the experiment, tadpoles from the two selective environments did not differ in body size (likelihood ratio test between null model and selective environment model: $\chi^2 = 0.81$, $p = 0.3681$; type III sum of squares ANOVA: $\chi^2 = 1.1548$, $p = 0.2825$). At the end of the experiment, however, tadpole growth showed a significant diet by selective environment interaction (table 1*a*,*b*). That is, the magnitude of diet-dependent growth differed across selective environments. A multiple comparisons test revealed that tadpoles from the two selective environments had comparable growth on a detritus diet, but that sympatric tadpoles grew more than allopatric tadpoles on a shrimp diet (table 1*c*). This difference on a shrimp diet created a significantly greater slope between diets for sympatry than for allopatry (figure 1*a*). Consistent

Table 1. Results from competition experiment, including (*a*) summary statistics from our model selection procedure; (*b*) results from our ANOVA on the interaction model; and (*c*) distance between group means in growth and their associated *p*-value following false discovery rate correction (in parentheses). ((*a*) and (*b*) indicate that the interaction between diet and selective environment was significant; (*c*) shows that this interaction was driven primarily by shrimp-fed sympatric tadpoles (Sym.shr) growing more than shrimp-fed allopatric tadpoles (Allo.shr), while detritus-fed tadpoles had comparable growth across both selective environments (Allo.det and Sym.det).)

(*a*) model selection				
model	**AIC**	**logLike**	χ^2	***p*-value**
null	832.99	−411.50	—	—
diet	790.71	−389.35	44.29	2.84×10^{-11}
selective environment	834.75	−411.37	0.00	1.000
diet + selective environment	792.50	−389.25	44.25	2.90×10^{-11}
diet : selective environment	788.36	−386.18	6.14	0.013
(*b*) type III sum of squares ANOVA				
term	χ^2	***p*-value**		
intercept	53.14	3.11×10^{-13}		
diet	20.98	4.64×10^{-6}		
selective environment	0.04	0.841		
diet : selective environment	6.34	0.012		
(*c*) multiple comparisons test				
group	**Allo.det**	**Allo.shr**	**Sym.det**	
Allo.shr	1.31 (0.001)	—	—	
Sym.det	0.11 (0.766)	1.42 (3.7×10^{-4})	—	
Sym.shr	2.24 (2.6×10^{-8})	0.93 (0.016)	2.35 (1.1×10^{-8})	

with this difference in slope, we found that effect size between diets (Cohen's d) was greater for sympatry (Cohen's $d = 1.206$) than for allopatry (Cohen's $d = 0.722$). This pattern matches our prediction for sympatric tadpoles: they exhibited greater adaptive refinement (improved growth achieved through superior competitive ability) than allopatric tadpoles on the diet that is frequently consumed in sympatry (shrimp).

Furthermore, when we categorized each tadpole as 'winner' or 'loser' (depending on whether or not it grew more than its competitor), sympatric tadpoles were more often the winner on shrimp (35 sympatric winners versus 16 allopatric winners; $p = 0.0002$), whereas allopatric tadpoles were more often the winner on detritus (31 allopatric versus 20 sympatric winners; $p = 0.0236$; figure 1*b*). This result is consistent with the observation that the slopes of two selective environments intersect near the detritus category (figure 1*a*). These results also support our prediction (see §2b): sympatric tadpoles were superior competitors on shrimp, and allopatric tadpoles were superior competitors on detritus.

Finally, a shrimp diet yielded greater variation in growth than a detritus diet ($\sigma^2 = 4.64$ versus 2.58, respectively; $p = 0.0067$), and there was greater variation in growth for sympatric tadpoles than allopatric tadpoles ($\sigma^2 = 5.14$ versus 3.67, respectively; $p = 0.0397$). However, when tadpoles were grouped by diet and selective environment simultaneously, the differences in variation only approached significance ($p = 0.0724$). Generally, these results, again, suggest that there is a greater effect of competition on shrimp (i.e. greater growth variance), and that sympatric tadpoles had a greater difference in growth between diets than allopatric tadpoles (i.e. greater variance for sympatry).

(b) Evaluating mechanisms of adaptive refinement

Sympatric and allopatric tadpoles did not differ in: (i) intrinsic growth rate on alternative diets (electronic supplementary material, table S2); (ii) time spent resting, swimming, eating or active (electronic supplementary material, table S3); or (iii) trait integration (electronic supplementary material, table S4). These two groups did differ in: (iv) time to eat shrimp; and (v) certain trophic traits. Regarding time to eat shrimp, sympatric tadpoles captured and consumed shrimp faster than allopatric tadpoles ($\chi^2 = 5.11$, $p = 0.0238$; figure 2). Also, as expected, there was significantly lower variance in shrimp capture time for sympatric tadpoles than for allopatric tadpoles ($\sigma^2 = 3340$ versus 20824, respectively; $p < 0.0001$).

Regarding trophic traits, as predicted, sympatric tadpoles had significantly more carnivore-like mouthparts than allopatric tadpoles (mean ± s.e.m. mouthparts scores = 2.8 ± 0.2 versus 1.8 ± 0.1 for sympatric and allopatric tadpoles, respectively; electronic supplementary material, table S5). For jaw muscle (OH) width, there was a significant diet by selective environment by treatment interaction. Delving into this interaction revealed that, for tadpoles reared in competition, sympatric tadpoles did not differ between diets, but allopatric tadpoles did (electronic supplementary material, table S6). Specifically, sympatric tadpoles showed consistently large OH widths across diets (thereby providing

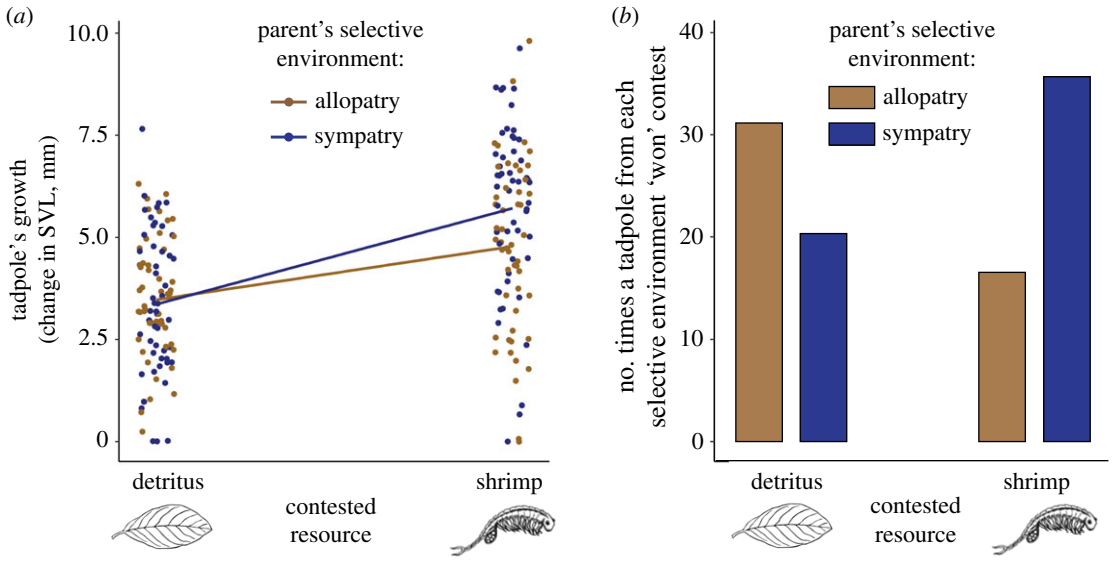

Figure 1. Evidence of frequency-dependent adaptation. Tadpoles from sympatric populations (where carnivores are produced frequently): (*a*) grew more on and (*b*) won more contests over, the resource for which carnivores are adapted—shrimp—than did tadpoles from allopatric populations (where carnivores are produced infrequently). By contrast, tadpoles from allopatry (*b*) won more contests over detritus, a resource for which omnivores are adapted. (Online version in colour.)

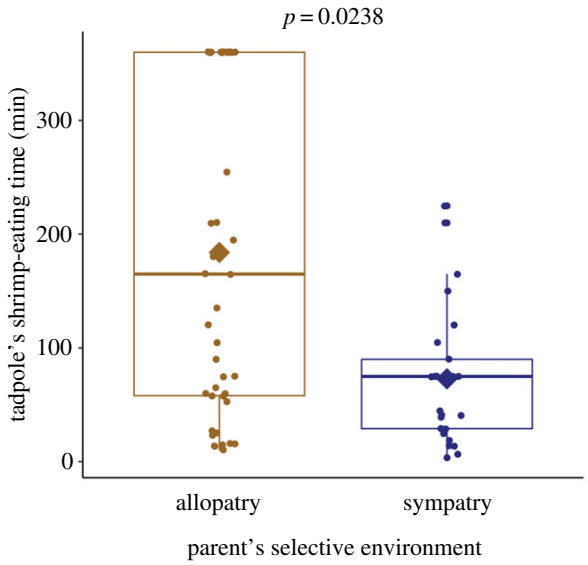

Figure 2. A mechanism of frequency-dependent adaptation. Tadpoles from sympatric populations (where carnivores are produced frequently) ate shrimp faster than tadpoles from allopatric populations (where carnivores are expressed relatively infrequently). Diamonds, group means. (Online version in colour.)

evidence of canalization in this trait; see also §3c), but allopatric tadpoles showed plasticity (larger OH on shrimp than on detritus). A multiple comparison test confirmed this pattern: detritus-fed allopatric tadpoles had significantly smaller OH widths than all other groups (electronic supplementary material, table S6B). When we focused on singletons, sympatric tadpoles had significantly larger OH widths than allopatric tadpoles, but there was no diet by selective environment interaction (electronic supplementary material, table S7). In contrast with the patterns for mouthparts and jaw muscles, sympatric tadpoles had significantly more *omnivore*-like denticle rows than allopatric tadpoles (8.3 ± 0.6 versus 4.5 ± 0.3 for sympatric and allopatric tadpoles, respectively; electronic supplementary material, table S5). Gut length did not differ between diets, selective environments or treatments.

(c) Testing for genetic assimilation of trophic morphology

On average, sympatric tadpoles had significantly larger OH widths (0.041 ± 0.012) than allopatric tadpoles (-0.029 ± 0.010; $\chi^2 = 6.58$, $p = 0.0103$; figure 3). Because this difference was already apparent in tadpoles that had not experienced the dietary cue(s) that normally induce carnivores, and because these sympatric tadpoles represent the derived state (see §2a), this finding suggests that sympatric tadpoles have undergone genetic assimilation in trophic morphology.

4. Discussion

A key prediction of plasticity-led evolution, and of evolution by natural selection generally, is that the frequency of a trait's expression will determine the degree to which its functionality is improved by selection [2,12,21,22]. In particular, compared to individuals from populations that express a particular phenotype infrequently, those from populations that express this phenotype more frequently should produce a superior version of the phenotype [12,18,25,32,55–58]. We tested this expectation of frequency-dependent adaptation experimentally by using *S. bombifrons* tadpoles from natural populations that have diverged in the frequency with which they produce an environmentally induced carnivore ecomorph.

Our results were consistent with frequency-dependent adaptation. Specifically, compared to tadpoles from allopatric populations (which express the carnivore ecomorph relatively infrequently; see §2a), those from sympatric populations (which express the carnivore ecomorph frequently): (i) were superior competitors for shrimp, a resource for which carnivores are specialized [59] (figure 1 and table 1); (ii) were more efficient at capturing and consuming shrimp (figure 2); (iii) showed less variation in shrimp-capturing ability; (iv) had more exaggerated carnivore features (electronic supplementary material, table S5); and (v) were more carnivore-like prior to experiencing an environmental cue—shrimp ingestion—that normally induces production of the carnivore

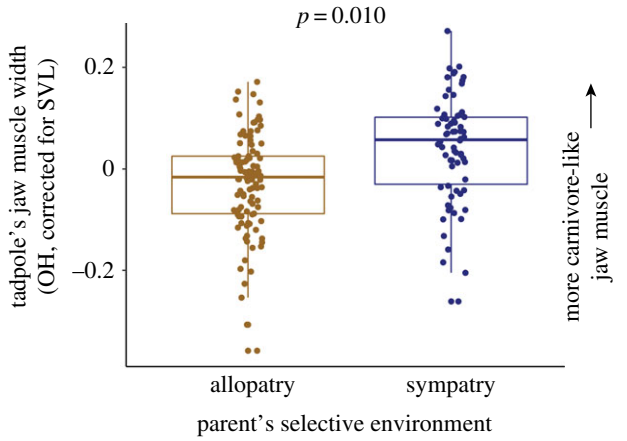

Figure 3. Evidence of genetic assimilation of trophic morphology. Even in the absence of a dietary cue that normally induces carnivores, tadpoles from sympatric populations (where carnivores are produced frequently) developed more carnivore-like jaw muscles (OH) than tadpoles from allopatric populations (where carnivores are expressed relatively infrequently). (Online version in colour.)

ecomorph [39,45,60] (figure 3). We also found evidence that the *omnivore* ecomorph has undergone adaptive refinement in populations where this phenotype is expressed more frequently. Compared to tadpoles from sympatric populations (which seldom express the omnivore ecomorph; see §2a), those from allopatric populations (which express the omnivore ecomorph frequently) were superior competitors for detritus, a primary resource of the omnivore ecomorph [59] (figure 1*b*). These results therefore suggest that neither selective environment produces tadpoles that are intrinsically superior across both diets. Instead, the selective environment that produces a given ecomorph more frequently (carnivores in sympatry, omnivores in allopatry) appears to produce a competitively superior version of that ecomorph. Thus, our data provide empirical support from natural populations for frequency-dependent adaptation.

Regarding possible mechanisms of this frequency-dependent adaptation, we found no evidence that tadpoles from the two selective environments differed in: (i) intrinsic growth rate (electronic supplementary material, table S2); (ii) time spent resting, swimming, eating or active (electronic supplementary material, table S3); or (iii) trait integration (electronic supplementary material, table S4). Sympatric tadpoles did, however, eat shrimp faster (figure 2) and exhibited less variation in shrimp-eating time than allopatric tadpoles. Thus, the competitive advantage of sympatric tadpoles in using shrimp (table 1 and figure 1) could be explained, in part, by sympatric tadpoles being better at capturing and consuming shrimp.

Differences between selective environments in tadpole trophic morphology probably also contributed to the sympatric tadpoles' competitive advantage on shrimp. Sympatric tadpoles had larger jaw (OH) muscles and mouthparts than allopatric tadpoles (electronic supplementary material, table S5). Both traits aid in the capture of large, mobile prey, such as fairy shrimp and tadpoles [50,61,62]. Indeed, previous work found a similar pattern. One such study [63] compared the morphology of experimentally reared tadpoles from sympatry versus allopatry and found that the former were more likely to express the carnivore morphology; the former also had significantly different jaw muscle (OH) allometry (the slope of the relationship between OH width and body length was steeper for tadpoles derived from sympatry than for those from allopatry). Another study [25] found that wild-caught tadpoles from sympatric populations were more carnivore-like in their morphology than wild-caught tadpoles from allopatric populations. Together with the present study, these studies suggest that populations which express the carnivore morph more frequently produce more exaggerated carnivore features *and* that those exaggerated features improve fitness. Thus, more frequent trait expression predicts greater magnitude of adaptive refinement.

As noted above, we found that sympatric tadpoles produced larger (more carnivore-like) jaw muscles than allopatric tadpoles, even prior to cue exposure (figure 3). This result implies that sympatric tadpoles: (i) may be primed to eat shrimp from early development (larger jaw muscles are needed to eat shrimp); and (ii) do not need an environmental cue to develop the carnivore morphology. This result further suggests adaptive refinement of the carnivore ecomorph in sympatry relative to allopatry. At a mechanistic level, because tadpoles from sympatry start out more carnivore-like, they may have greater difficulty overcoming a potential trade-off in the ability to switch between morphs [64], and thus have greater difficulty developing as omnivores. Regardless of the exact mechanistic cause, this early phenotypic bias may be adaptive, given that selection favours carnivore production in sympatric populations of *S. bombifrons* [53].

Our finding that sympatric tadpoles do not need an environmental cue to produce carnivore-like jaw muscles suggests that the ancestors of these tadpoles might have undergone genetic assimilation of trophic morphology. Although genetic assimilation has previously been demonstrated in laboratory experiments [16,65,66], and theory supports its role in enabling populations to adapt to rapidly changing environments [6,10,67–72], its relevance to natural populations has been questioned (e.g. [3,4]). Interestingly, we also found evidence of genetic assimilation in jaw musculature from our competing tadpoles (electronic supplementary material, table S6). In this case, sympatric tadpoles had larger (more carnivore-like) jaw muscles in both diet treatments and exhibited the flat reaction norm characteristic of genetic assimilation [12,73]. Similarly, Levis *et al*. [74] found evidence of genetic assimilation in patterns of gene expression. Whereas gene expression profiles of allopatric tadpoles differed between detritus and shrimp diets, those of sympatric tadpoles did not. Furthermore, a transcription factor (*btf3*) exhibited loss of diet-dependent expression plasticity, and a peptidase gene (*pm20d2*) showed an overall decrease in expression in sympatry relative to allopatry, suggesting possible improved efficiency [74]. These studies, combined with those of other natural systems (e.g. [36,37,55,75–79]), point to the generalizability—and possible importance—of genetic assimilation.

Additional studies are needed, however, to identify the mechanisms underlying any such genetic assimilation [72]. For instance, the gene expression differences mentioned above are consistent with genetic assimilation, but they could also be caused by persistent epigenetic changes (e.g. see [80]). To distinguish between genetic assimilation and 'epigenetic assimilation' as mechanisms underlying constitutive expression of a phenotype will require investigating

whether constitutively expressed phenotypes are associated with DNA sequence changes versus epigenetic 'tags' (e.g. methylation).

Returning to frequency-dependent adaptation, why should the frequency of trait expression drive the magnitude of its adaptive refinement? Frequency-dependent adaption is expected to occur for at least two, non-mutually exclusive, evolutionary reasons. First, differences in the size of subpopulations that express the phenotype should lead to differences in both: (i) the strength of selection relative to that of genetic drift and (ii) the number of variants exposed to selection per generation [2,81]. A rough analysis suggests that the subpopulation of *S. bombifrons* carnivores in sympatry is at least twice as large as that in allopatry (electronic supplementary material, appendix S1). All else being equal, this difference across selective environments in numbers of carnivores suggests that selection should be at least twice as effective at acting on sympatric carnivore subpopulations than on allopatric carnivore subpopulations. Although the exact selection coefficients and effective populations sizes are unknown, selection favouring extreme carnivores in sympatry is probably stronger than selection favouring carnivores in allopatry: sympatric populations are under strong directional selection, whereas allopatric populations are under weak disruptive (i.e. quintic rather than quadratic) selection [53]. Thus, the recurrent exposure of a relatively larger population size of carnivores in sympatry may have played a causal role in the adaptive evolution of this phenotype. Both factors—recurrence of phenotype expression and large population producing the phenotype—are probably needed for rapid adaptation, and the relative importance of each factor warrants further study.

A second reason frequency-dependent adaptation should occur is that as a trait's frequency of expression increases, so should the bias in the direction of selection on non-specific modifiers of that trait [2,82]). That is, selection on loci that show antagonistic pleiotropy among alternative phenotypes should favour those alleles that are best suited to the most frequently expressed phenotype [2]. This bias in modifier accumulation can alter the fitness consequences associated with different phenotypes in a population, and it can even cause such fitness effects to diverge among populations that diverge in the frequency at which these phenotypes are expressed. Indeed, a re-assessment of data from previous studies of this system [43,44] suggests that the covariance between carnivore morphology and body size (a proxy for fitness [48,52,53]) is nearly twice as large for sympatric tadpoles than for allopatric tadpoles (0.04013 versus 0.02103). Using the Price equation [83], this suggests that sympatric populations might accumulate modifications to the carnivore phenotype nearly twice as fast as allopatric populations. Interestingly, for a difference in carnivore morphology of the magnitude observed between allopatric and sympatric populations to arise, it would take approximately 70 spadefoot generations (electronic supplementary material, appendix S2), which corresponds to the estimated time (approx. 150 years) that these two selective environments have been separated and diverged in ecomorph production [46,47]. Thus, differences in modifier accumulation, as a result of biases in phenotype production, may drive patterns of genetic and phenotypic divergence within (and potentially between) species.

In conclusion, our findings provide evidence that the frequency of trait expression drives the magnitude of adaptive refinement. Thus, our results thereby support a key prediction of both plasticity-led evolution and adaptive evolution.

Ethics. The University of North Carolina's IACUC approved all the procedures. Field collections were conducted under Scientific/Education Collecting Permits AZ SP594327 and SP615759, CO 15HP995, NE 574, OK 6650 and TX SPR-0316-094.

Data accessibility. Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.cq408t7 [84].

Authors' contributions. N.A.L. and D.W.P. conceived and designed the study and drafted the manuscript. N.A.L. collected and analysed data. Both authors gave final approval for publication.

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