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Morphological novelty emerges from pre-existing phenotypic plasticity

Nicholas A. Levis*, Andrew J. Isdaner and David W. Pfennig

Plasticity-first evolution (PFE) posits that novel features arise when selection refines pre-existing phenotypic plasticity into an adaptive phenotype. However, PFE is controversial because few tests have been conducted in natural populations. Here we present evidence that PFE fostered the origin of an evolutionary novelty that allowed certain amphibians to invade a new niche—a distinctive carnivore morph. We compared morphology, gene expression and growth of three species of spadefoot toad tadpoles when reared on alternative diets: *Scaphiopus holbrookii*, which (like most frogs) never produce carnivores; *Spea multiplicata*, which sometimes produce carnivores, but only through diet-induced plasticity; and *Spea bombifrons*, which often produce carnivores regardless of diet. Consistent with PFE, we found diet-induced plasticity—in morphology and gene expression—in *Sc. holbrookii*, adaptive refinement of this plasticity in *Sp. multiplicata*, and further refinement of the carnivore phenotype in *Sp. bombifrons*. Generally, phenotypic plasticity might play a significant, if underappreciated, role in evolutionary innovation.

An enduring problem in evolutionary biology is to explain how novel, complex phenotypes originate and diversify[1,2]. Although new phenotypes are generally assumed to arise exclusively from genetic changes, such as de novo or taxon-specific mutations[3,4], environmentally induced phenotypic change—that is, phenotypic plasticity—has long been proposed to play a role in initiating novelty[5–7]. This process—plasticity-first evolution (PFE)—occurs when: (1) a change in the environment triggers a change in phenotype via phenotypic plasticity; (2) different genotypes vary in the tendency and/or manner in which they respond to this environmental change; (3) selection favours certain responses (and, hence, genotypes) over others, thereby causing the degree and/or form of phenotypic plasticity to evolve; and (4) through this process, the pre-existing phenotypic plasticity is ultimately refined by selection into a fully functioning phenotype[5–7] (Fig. 1). However, PFE is controversial because few tests have been conducted in natural populations[8,9]. Here, we perform such a test and provide evidence suggesting that phenotypic plasticity preceded, and facilitated, morphological novelty.

To test the two critical predictions from PFE[7] that: pre-existing phenotypic plasticity was expressed in an ancestral lineage (Prediction 1) and subsequently refined by selection into a novel adaptive phenotype in a derived lineage (Prediction 2), one should contrast species that lack the novel phenotype with closely related species that possess it[7,10,11] (ideally, one should perform ancestral character reconstruction to establish that the focal phenotype is indeed a novel, derived trait[7]). In this framework, the former species serve as 'ancestor-proxies' because they exhibit the 'ancestral' state[7,10,11]. Both types of species should be reared in the environment normally experienced by the ancestor-proxy (the 'ancestral environment') and (separately) in the environment with which the novel phenotype is associated (the 'derived environment')[12,13]. Trait production and fitness can then be contrasted to test the above predictions[7]. (Note that because exposure to a novel environment is typically stressful[14], such exposure often alters development in ways that are not adaptive[10,15,16] (Fig. 1c). According to PFE theory[5–7], the derived environment causes the ancestor-proxy species to express plasticity in the focal phenotype (or its component traits), there is genetic variation in how this plasticity is expressed (as is nearly always the case[17,18]), and this variation—whether in the adaptive direction or not—serves as the raw material for natural selection to refine the plastic response in a fashion that is adaptive. We applied the above approach to examine the origins of a complex, adaptive phenotype in amphibians.

North American spadefoot toads of the genus *Spea* are among the few amphibians to have successfully invaded arid environments, partly because they have evolved a unique polyphenism[19]. Like most anurans[20], *Spea* tadpoles normally develop into an 'omnivore' morph, which has small jaw muscles, smooth mouthparts, numerous denticle rows and a long gut. This form eats detritus, algae and small crustaceans. However, if *Spea* tadpoles eat fairy shrimp or tadpoles[21,22], some individuals facultatively produce an alternative 'carnivore' morph (Fig. 2a). This complex, coordinated phenotype differs from the default omnivore morph behaviourally (carnivores are more active), developmentally (carnivores are younger at metamorphosis[19]) and morphologically (carnivores are characterized by large jaw muscles, notched mouthparts, few denticle rows, and a short gut; Fig. 2a). The evolution of this carnivore morph allowed *Spea* to invade an unexploited niche: rapidly drying ponds rich in fairy shrimp and other tadpoles[19] (Fig. 2b,c).

The carnivore morph is an evolutionary novelty restricted to *Spea*[12,21] (Fig. 2d). Furthermore, in certain derived populations, this morph has undergone canalization. In particular, owing to character displacement, *Sp. bombifrons* that occur in sympatry with *Sp. multiplicata* have secondarily lost carnivore–omnivore polyphenism and become nearly fixed for producing carnivores, regardless of their diet[23–25] (Fig. 2d).

We examined whether or not this evolutionary novelty arose via PFE by testing the above two critical predictions of the PFE hypothesis: (Prediction 1) pre-existing phenotypic plasticity was expressed in an ancestral lineage, and (Prediction 2) subsequently refined by selection into a novel adaptive phenotype in a derived lineage. This system is ideal for such a test. Indeed, previous work had suggested that pre-existing plasticity might be present in this system, that there was genetic variation in this pre-existing

Department of Biology, University of North Carolina, Chapel Hill, NC, USA. *e-mail: nicholasalevis@gmail.com

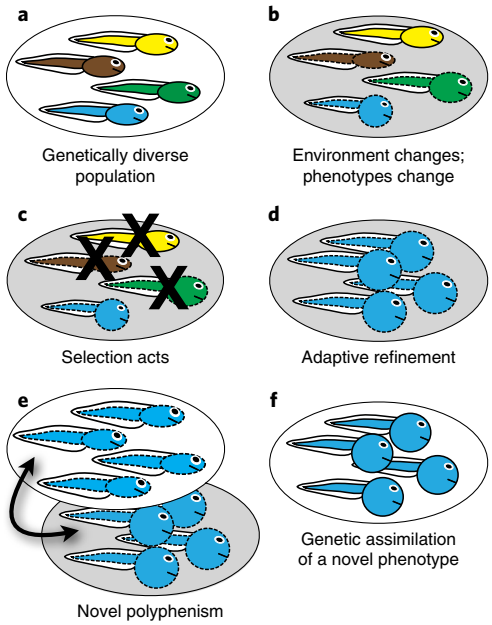

Fig. 1 | How plasticity can facilitate the evolution of a novel, complex phenotype. a,**b**, A genetically diverse population (**a**, different colours indicate different genotypes) experiences a novel environment (**b**, shading), which induces novel phenotypes (dashed lines), but genotypes differ in whether and how they respond to the novel environment (differences in shape). **c**, Selection acts on this formerly cryptic genetic variation (revealed by a change in environment) and disfavours genotypes that produce maladaptive or poorly adapted phenotypes. **d**, This leads to the adaptive refinement of the favoured phenotype (enlargement of the blue tadpole). **e**, If individuals produce either this novel phenotype or the ancestral phenotype depending on their environment, then the result is a novel polyphenism. **f**, Alternatively, selection might favour the loss of plasticity (that is, genetic assimilation), resulting in a novel phenotype that is produced regardless of the environment (indicated by the loss of dashed lines). Reproduced from ref. [54], Elsevier.

plasticity, and that this plasticity might have undergone adaptive refinement. One such study had found that the ancestor-proxy *Scaphiopus couchii* (a species that does not produce the carnivore morph; Fig. 2d) developed shorter guts when fed shrimp (a novel diet for *Sc. couchii*, but the normal diet of *Spea* carnivores) than when fed its normal diet of detritus[12] (a shorter gut is a component trait of the complex carnivore phenotype found in *Spea*; Fig. 2a). Moreover, *Sc. couchii* also exhibited greater heritability in gut length when fed shrimp versus detritus, suggesting that the derived stimulus (shrimp) uncovered cryptic genetic variation on which selection could have acted to promote the eventual evolution of the carnivore morph[26]. Finally, as evidence that this diet-induced plasticity might have undergone adaptive refinement, previous work also found that *Sp. bombifrons* (a species that produces carnivores) showed an increase in gut cell proliferation when fed shrimp versus detritus (gut cell proliferation is a measure of gut functionality); by contrast, no such increase was detected in *Sc. couchii*[12].

However, although these data are consistent with the hypothesis that the carnivore morph evolved via PFE, many vertebrates facultatively develop shorter guts when fed animals versus plants[12]. Therefore, to comprehensively evaluate PFE, we sought to determine if diet-induced plasticity is present in additional species and traits, including traits that are: (1) not ubiquitously plastic; (2) novel to the carnivore morph (for example, notched mouthparts; Figs. 2a) and (3) molecular features associated with production of the carnivore morph in *Spea* (that is, patterns of gene expression).

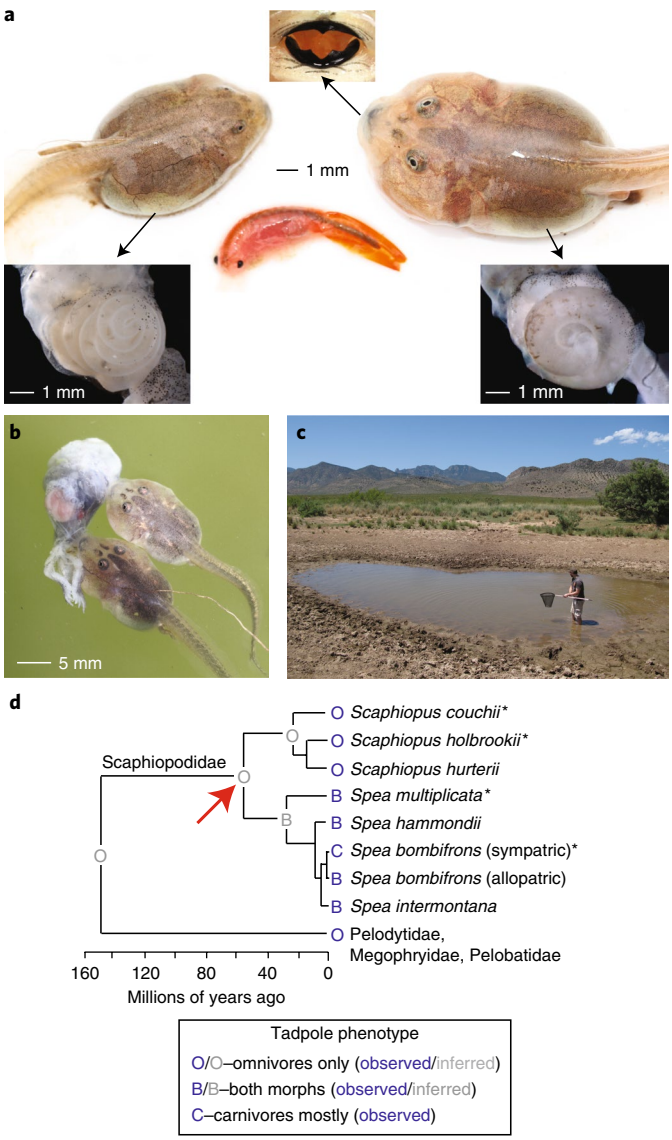

Fig. 2 | Ecology and evolution of the spadefoot toad resource-use polyphenism. a, *Spea* tadpoles normally develop into an 'omnivore' morph (left), but if they eat large animal prey, such as shrimp (middle), they produce a distinctive 'carnivore' morph (right), which is characterized by large jaw muscles, notched mouthparts (upper inset) and a short gut (lower inset). **b**, These features enable carnivores to prey on large animals (for example, other tadpoles). **c**, This protein-rich diet, in turn, hastens the carnivore's development, which allowed *Spea* to invade arid habitats where breeding ponds are highy ephermeral. **d**, In contrast to *Spea*, most frogs produce omnivores only[20], and it is therefore likely that the ancestor of Scaphiopodidae also did so (inferred phenotype based on the ancestral character state reconstuction in ref. [12]; time-calibrated phylogeny of North American spadefoot toads (family Scaphiopodidae) from ref. [21]). To study the origin of the carnivore morph, we used an omnivore-only producer, *Sc. holbrookii*, as a proxy for the last common ancestor of *Scaphiopus* and *Spea* (red arrow). Subsequent to the carnivore morph origin, *Sp. bombifrons* that occur sympatrically with *Sp. multiplicata* (*Sp. bombifrons* (sympatric)) have undergone genetic assimilation and produce mostly carnivores (asterisks: subjects of this study).

To conduct these tests of PFE, we leveraged the fact that different lineages of spadefoot toads appear to represent different stages in the evolution of the carnivore morph (Fig. 2d): from its possible initial induction (in *Scaphiopus*), to its refinement as part

of a novel polyphenism (in *Spea*), to its further refinement via genetic assimilation (in *Sp. bombifrons* that occur in sympatry with *Sp. multiplicata*). As we describe, our data suggest that PFE has indeed contributed to the origin of this evolutionary novelty.

Results

Evidence of pre-existing morphological and gene expression plasticity. To test Prediction 1 (evidence of pre-existing [ancestral] plasticity), we used *Sc. holbrookii* as a proxy for the ancestor of North American spadefoots (before the evolution of carnivore–omnivore polyphenism; red arrow in Fig. 2d). *Scaphiopus* is appropriate to serve as this ancestor-proxy because: (1) *Scaphiopus* is the closest extant outgroup to *Spea*[27]; (2) *Scaphiopus* is ecologically similar to *Spea*[28]; and (3) *Scaphiopus* does not express the polyphenism[12]. Specifically, *Scaphiopus* produces the omnivore morph only, and not the integrated set of traits that constitute the carnivore morph in *Spea* (Fig. 2a; Supplementary Table 1). This lack of polyphenism is the reconstructed state for the ancestor of *Spea*[12] and the state of most anurans generally[12,29] (Fig. 2d). By rearing *Sc. holbrookii* on both its normal diet (detritus) and (separately) on a novel diet of shrimp (the normal diet of *Spea* carnivores), we tested for pre-existing plasticity in both morphological and molecular phenotypes.

We began by testing for diet-induced plasticity in various trophic-related traits that constitute the carnivore morph in *Spea* (Supplementary Table 1). We found that the ancestor-proxy (*Sc. holbrookii*) did indeed exhibit diet-dependent plasticity in three of four morphological traits examined (Fig. 3a; Supplementary Table 2). Compared with detritus-fed tadpoles, shrimp-fed *Sc. holbrookii* tadpoles had fewer gut coils (they had shorter guts), fewer denticle rows and less notched mouthparts. However, whereas plasticity in gut coils and denticle rows was in the adaptive direction (based on trait function; Supplementary Table 1), plasticity in mouthparts was in the maladaptive direction (Fig. 3a; increased notching increases efficiency in capturing larger, more active prey[29] (Supplementary Table 1), and shrimp-fed *Sc. holbrookii* produced mouthparts with reduced notching). Perhaps because of this mismatch in carnivore trait integration, *Sc. holbrookii* grew more poorly on shrimp than on detritus (Fig. 3a). These findings agree with previous work that found diet-dependent plasticity in gut length in a different ancestor-proxy species (*Sc. couchii*[26]). Thus, consistent with PFE[7], ancestors of *Spea* likely harboured diet-dependent plasticity in some component traits of the carnivore morphology, but only a portion of this plasticity was adaptive.

As noted above, however, the presence of such pre-existing plasticity in the ancestor-proxy is relevant for PFE only if genetic variation for such plasticity was also present. Although we were unable to perform a robust test for cryptic genetic variation (CGV) in the observed diet-dependent plasticity in *Sc. holbrookii*, we tested if families differed in their responses to diet by treating family as a factor rather than a random effect. Every trait examined had significant between-family variation and/or a significant Family×Diet interaction (Supplementary Table 3). Thus, families may differ not only in the shape of their plastic responses, but also in the magnitude of trait expression. More rigorous tests are needed, but these data, together with the estimates of CGV in a different *Scaphiopus* (ancestor-proxy) species[26], point toward the plausibility of *Spea*'s ancestor harbouring heritable variation in responses to consumption of a novel shrimp resource.

Next, we tested for diet-induced plasticity in the expression of eight genes (Supplementary Table 4) that were previously found to be associated with carnivore–omnivore polyphenism (these genes are 'morph-biased' in expression level—that is, expressed more highly in one morph[30]—and therefore likely contribute to morph functionality) and also found to have undergone adaptive evolution in *Spea*[31]. Using the same experimental design as above, we also found diet-dependent plasticity in expression of five of eight candidate genes

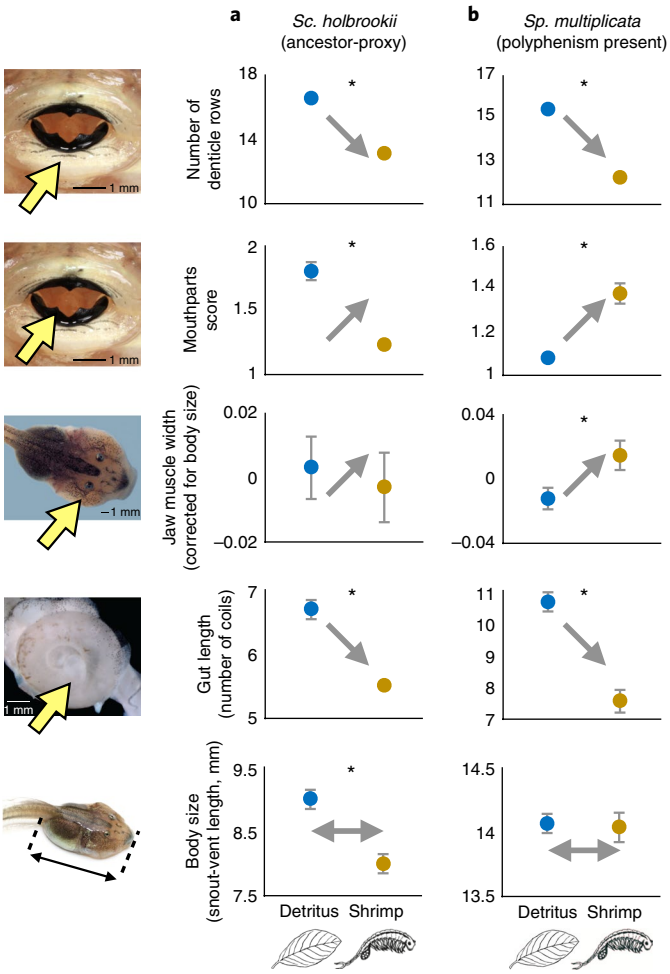

Fig. 3 | Diet-induced morphological plasticity of *Sc. holbrookii* and *Sp. multiplicata*. a, *Sc. holbrookii* reared on detritus (blue; $n=60$) or shrimp (brown; $n=60$). **b**, *Sp. multiplicata* reared on detritus (blue; $n=153$) or shrimp (brown; $n=149$). Points, means ± s.e.m. (individual data points are shown in Supplementary Fig. 2); asterisks, significant differences between diets; grey arrows, direction of adaptive plasticity (Supplementary Table 1). Photos show traits in *Spea* carnivores.

examined. However, as with morphology, much of this molecular phenotypic plasticity was not in the expected direction based on previous patterns observed in *Spea* (Fig. 4a; Supplementary Table 5). Indeed, only *tf*, an omnivore-biased immunological gene in *Spea* (upregulated in *Spea* omnivore tadpoles[30]), showed expression plasticity in the putatively adaptive direction in *Sc. holbrookii*: it was upregulated on detritus, as expected (Fig. 4a). In contrast, the four other genes that exhibited diet-induced plasticity in expression level showed a mismatch between morph-biased designation and diet: contrary to our expectations, these carnivore-biased metabolic (*pm20d2*), regulatory (*btf3* and *tbx15*), and structural (*col2a1*) genes had lower expression on shrimp than on detritus (Fig. 4a). Given that these were all carnivore-biased in *Spea*[30], this dietary mismatch may be indicative of a poorly coordinated response to consuming an animal diet. Finding such a mismatch between environment and gene expression is consistent with recent studies in guppies[16] and fruit flies[32], and it may potentiate rapid adaptation in a new environment (that is, a shrimp diet; see ref. [33] for more discussion). Thus, *Spea*'s ancestor likely did not exhibit an immediate, coordinated adaptive plastic response in gene expression as a result of its dietary transition to shrimp, possibly because exposure to this novel diet is stressful for non-carnivore species[26].

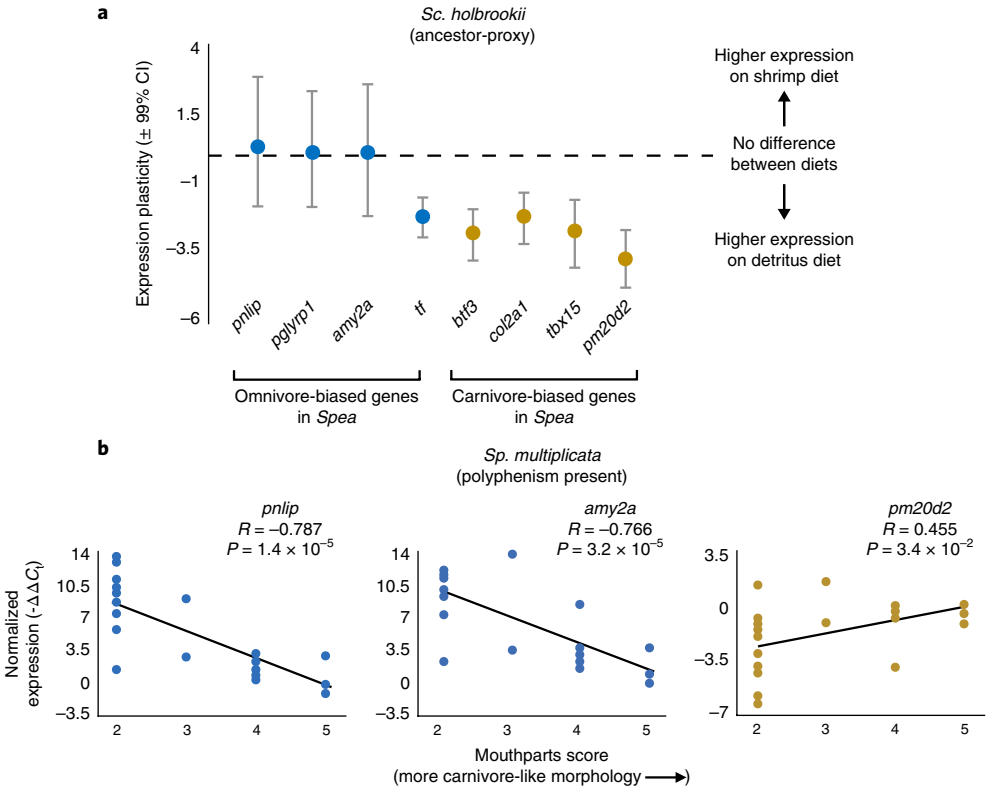

Fig. 4 | Diet-induced gene expression plasticity in *Sc. holbrookii* and *Sp. multiplicata*. a, Expression plasticity for omnivore- (blue) and carnivore-biased genes (brown) in *Sc. holbrookii* reared on detritus versus shrimp. Points are means ± 99% confidence interval generated from 5,000 bootstrap replicates. **b**, Relationship (Pearson product-moment correlation) between the degree to which naturally occurring *Sp. multiplicata* produced the carnivore morphology and the expression level of omnivore- (*pnlip* and *amy2a*; blue) and carnivore-biased genes (*pm20d2*; brown). Points are individual tadpoles ($n = 22$).

Evidence of adaptive refinement of morphological and gene expression plasticity. To test Prediction 2 (adaptive refinement of pre-existing plasticity), we performed similar experiments as above, but with *Sp. multiplicata*, a lineage that has evolved carnivore–omnivore polyphenism. Using this species, we tested for evidence of adaptive refinement in the morphological and gene expression plasticity that we previously documented in the ancestor-proxy, *Sc. holbrookii*.

We found support for Prediction 2. Specifically, unlike the diverse patterns of morphological plasticity observed in *Sc. holbrookii* (the ancestor-proxy), *Sp. multiplicata* (a species that has evolved carnivore–omnivore polyphenism) exhibited adaptive diet-dependent plasticity in all four traits examined (Fig. 3b; Supplementary Table 5). In particular, compared to detritus-fed tadpoles, shrimp-fed tadpoles had larger jaw muscles, more notched mouthparts and shorter guts; these traits aid in capture and digestion of shrimp (Supplementary Table 1). Likewise, compared with shrimp-fed tadpoles, detritus-fed tadpoles had more denticle rows (needed to rasp detritus from the pond bottom[29]). Indeed, as evidence that these diet-induced morphological changes are adaptive in *Sp. multiplicata* (and in further contrast to the ancestor-proxy species, *Sc. holbrookii*), *Sp. multiplicata* tadpoles grew and developed equally well on both shrimp and detritus (Fig. 3b; Supplementary Table 6). These findings are consistent with previous work using a different ancestor-proxy species[12], and they highlight how the evolution of carnivore–omnivore polyphenism enabled *Sp. multiplicata* to adaptively utilize alternative diets.

We further found evidence of adaptive refinement in gene expression in *Sp. multiplicata*. Because previous studies have shown morph-[30] and diet-dependent[31] gene expression using laboratory reared *Spea* tadpoles, we did not directly replicate these studies. Instead, we measured expression of three metabolic genes in free-living tadpoles from the wild (these genes would be expected to differ adaptively in expression, because they are involved in lipid, carbohydrate and protein metabolism). We began by confirming that all three genes were significantly associated with trophic morphology in these wild-caught tadpoles, and in the adaptive direction. In particular, both lipase (*pnlip*) and amylase (*amy2a*) were expressed more highly in omnivores, as predicted ($P = 1 \times 10^{-4}$ and $P = 3 \times 10^{-4}$, respectively; Supplementary Tables 7,8), whereas peptidase (*pm20d2*) was expressed more highly in carnivores, also as predicted ($P = 1.5 \times 10^{-3}$; Supplementary Tables 7,8).

Even more strikingly, at a finer morphological scale, the expression level of all three genes was significantly associated with the degree to which the focal individual produced the carnivore morphology (the latter was determined by the shape of the individual's keratinized mouthparts, which is diagnostic of overall morphotype[23]). For the two omnivore-biased genes (*pnlip* and *amy2a*), expression levels were significantly negatively associated with the extent to which individuals exhibited the carnivore morphology (likelihood ratio tests: $\chi^2 = 23.33$, $P = 1.4 \times 10^{-6}$ and $\chi^2 = 19.36$, $P = 1.1 \times 10^{-5}$, respectively; Pearson correlations: $R = -0.809$, $P = 5.3 \times 10^{-6}$ and $R = -0.765$, $P = 3.4 \times 10^{-5}$, respectively; Fig. 4b). By contrast, for the carnivore-biased gene (*pm20d2*), expression levels were significantly *positively* associated with the degree to which individuals produced the carnivore morphology ($\chi^2 = 5.23$, $P = 2.2 \times 10^{-2}$; $R = 0.460$; $P = 3.1 \times 10^{-2}$; Fig. 4b).

These significant associations between gene expression and fine-scale trophic morphology indicate that the expression of these genes has undergone adaptive refinement in *Sp. multiplicata*. The omnivore's detritus diet contains more fat and starch (metabolized by lipase and amylase, respectively) than the carnivore's diet[34]. By contrast, the carnivore's diet is more protein-rich (metabolized

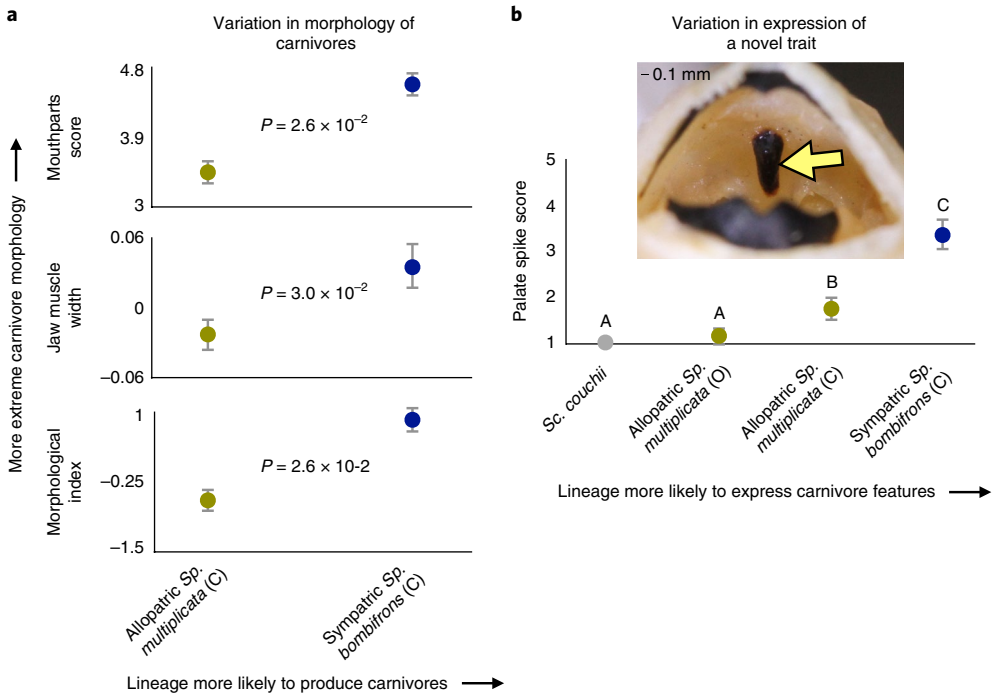

Fig. 5 | Evidence of refinement of the carnivore phenotype among wild-caught tadpoles of different lineages. a, Differences in magnitude of expression (according to a likelihood ratio test) of trophic traits of carnivores in a lineage in which carnivores are produced at low–intermediate frequencies as part of a polyphenism (allopatric *Sp. multiplicata*; $n = 82$) versus a lineage in which carnivores are produced at high frequencies (sympatric *Sp. bombifrons*; $n = 59$). **b**, Differences in magnitude of expression of a novel palate spike in lineages/morphs that differ in likelihood of expressing carnivore features ($n = 50$, 74, 82 and 59 for *Sc. couchii*, allopatric *Sp. multiplicata* (O), allopatric *Sp. multiplicata* (C) and sympatric *Sp. bombifrons* (C), respectively; O: omnivores; C: carnivores); different letters, values differed significantly. Photo: palate spike (score 5) in a sympatric *Sp. bombifrons* carnivore. In all panels, points are means ± s.e.m. (individual data points are shown in Supplementary Fig. 3).

by peptidase)[34]. However, not all carnivores (or omnivores) are alike in morphology: individuals within each morph class vary[35], and this variation in morphology is associated with variation in diet. Indeed, previous studies reported a significant positive relationship between the extent to which an individual exhibited the carnivore morphology and how many shrimp/tadpoles it had consumed, in both wild-caught[34] and lab-reared[21] animals. Thus, the more carnivore-like in morphology an individual is, the greater the need for the expression of genes that break down protein (for example, *pm20d2*), which is precisely what we found (Fig. 4b). Similarly, as an individual shifts its diet away from shrimp and toward algae and detritus and the more omnivore-like in morphology it becomes, the greater the need for expression of genes that can break down fats and starches (for example, *pnlip* and *amy2a*), which, again, is precisely what we found (Fig. 4b).

We take this tight coupling of diet, morphology, and gene expression as evidence of adaptive refinement. In addition, the co-variation of two carnivore-morph features that are unlikely to be directly causally linked (mouthpart score and *pm20d2* expression) suggests a coordinated response that points toward adaptive refinement. However, a comparison of the extent of covariation between trophic features and gene expression in *Scaphiopus* with the extent of covariation observed in *Spea* would be a useful future direction. Nevertheless, unlike the putatively non-adaptive gene expression plasticity we observed in *Scaphiopus* (for example, lower expression of *pm20d2* on shrimp and no change in *pnlip* or *amy2a* with diet; Supplementary Table 5), the expression of three metabolic genes appears to have undergone adaptive refinement in *Spea*.

Evidence of extensive refinement in a canalized lineage. We found even greater refinement of the carnivore phenotype in a lineage in which this morph is nearly fixed. As noted above, *Sp. bombifrons* that occur in sympatry with *Sp. multiplicata* have secondarily lost carnivore–omnivore polyphenism and become nearly fixed for producing carnivores[23–25] (Fig. 2d). Because sympatric *Sp. bombifrons* express the carnivore morph more frequently and thereby expose it to selection more often than other *Spea* lineages, PFE (and evolution by natural selection more generally) predicts that sympatric *Sp. bombifrons* should show the greatest refinement of the carnivore phenotype. As predicted, compared with wild-caught allopatric *Sp. multiplicata* carnivores (a lineage in which carnivores are produced less frequently as part of a polyphenism[19]), wild-caught sympatric *Sp. bombifrons* carnivores had more pointed keratinized mouthparts, larger jaw muscles and a greater morphological index (Fig. 5a). In other words, carnivores produced by sympatric *Sp. bombifrons* developed more exaggerated features overall.

While performing these comparisons, we identified a novel carnivore trait: a unique keratinized palate spike that is presumably used to spear and/or stabilize the carnivore's large shrimp and tadpole prey (Supplementary Fig. 1). We sought to determine if (as expected under PFE) this novel feature has also experienced adaptive refinement by comparing its expression among wild-caught tadpoles of sympatric *Sp. bombifrons*, allopatric *Sp. multiplicata* carnivores and omnivores, and *Sc. couchii* (*Sc. couchii* served as our ancestor-proxy for this analysis because more wild-caught tadpoles were available for this species than for *Sc. holbrookii*).

As predicted, the palate spike: (1) occurs only in *Spea*, (2) is produced as part of the carnivore–omnivore polyphenism and (3) shows its greatest development in sympatric *Sp. bombifrons* carnivores (Fig. 5b; Supplementary Fig. 1; Supplementary Table 9). Thus, selection appears to have refined individual carnivore features (Supplementary Table 1) and even favoured the evolution of a new feature (Fig. 5b) during the evolution of the complex carnivore phenotype. These data, along with previous evidence that the expression of the above candidate genes has undergone genetic

assimilation in sympatric *Sp. bombifrons*[31], demonstrate that, following its origin, the novel carnivore phenotype evolved in both form (traits became more exaggerated in certain lineages) and degree of plasticity (slopes of reaction norms have shifted[31]) when selection acted on both pre-existing plasticity and taxon-specific features.

Evidence of reduction of heritable variation in carnivore traits. Finally, as an extension of our analyses, we asked if any pre-existing heritable variation in diet-induced plasticity was subsequently reduced in *Spea*. As noted above, we found among-family variation in diet-induced plastic responses in *Sc. holbrookii* (Supplementary Table 3), and a previous study found evidence for the accumulation of cryptic genetic variation for components of the carnivore phenotype in *Sc. couchii* (significantly higher broad-sense heritability, H^2, when reared on a novel shrimp diet versus their standard detritus diet; Supplementary Table 10[26]). These data therefore suggest that the ancestor(s) to *Spea* may have similarly harboured heritable variation in trophic responses to a shrimp diet. In the present study, we found that such variation appears to have been purged in *Sp. multiplicata*. In particular, we found no significant differences in H^2 between diet treatments for any traits that we examined in *Sp. multiplicata* (Supplementary Table 10). This difference in accumulation of genetic variation between lineages is likely due to differences in the frequency with which these lineages expose shrimp-induced variation to selection: infrequently in *Scaphiopus* and frequently in *Spea*.

Discussion

Taken together with previous work[12,26,36], our results suggest that the ancestor of *Spea* likely possessed pre-existing plasticity in both morphological and molecular features, but that this plasticity was not necessarily coordinated nor fully adaptive. We speculate that when an ancestral population began consuming large animal prey (fairy shrimp and other tadpoles), this dietary shift uncovered selectable variation in morphology and gene expression[26]. Because some of this variation was adaptive (for example, producing a shorter gut would be adaptive when consuming a protein-rich diet), selection presumably favoured individuals that could switch between resources and thereby utilize a novel niche (short-duration ponds specifically and more arid environments generally). However, part of the adaptive refinement process likely consisted of overcoming non-adaptive plasticity, such as that observed among *Sc. holbrookii* in mouthpart morphology (Fig. 3a) and the expression of carnivore-biased genes (Fig. 4a). It is even possible that maladaptive plasticity facilitated subsequent adaptation to the novel environment (that is, consumption of the novel shrimp resource) by increasing the strength of directional selection[16,32]. Finally, following these initial changes and bouts of adaptation, some lineages (for example, sympatric *Sp. bombifrons*) underwent more extensive refinement when the carnivore morph became nearly fixed[24,25,31]. Thus, we have demonstrated that: (1) ancestral plasticity (and heritable variation therein) was likely present in both trophic traits and gene expression, and (2) these features were adaptively refined to generate an evolutionary novelty—a distinctive carnivore morph—that allowed *Spea* to invade an unexploited niche. Our study therefore provides vital support for two critical predictions of the PFE hypothesis from natural populations.

Finally, although PFE and mutation are often considered mutually exclusive pathways to novelty[3,4], our data suggest that novelty might arise when these two pathways act synergistically. In particular, as PFE unfolds (Fig. 1), novelty might typically entail the further elaboration of an initially environmentally induced phenotype via subsequent mutation. For example, the palate spike in *Spea* (Fig. 5b) might have arisen through mutation only after PFE was already underway. In support of this idea, we found no evidence that this spike could be induced by diet in our ancestor proxy, *Scaphiopus*. This lack of evidence for plasticity raises the possibility that this novel feature originated through *de novo* or taxon-specific mutation following the origin of the carnivore morph via PFE. Likewise, new mutations were likely needed to help overcome the putatively maladaptive plasticity seen in mouthpart formation (Fig. 3a) and gene expression (Fig. 4a)[37]. Additional studies exploring the extent of heritable variation in these plastic responses should illuminate whether or not new mutations were necessary for these responses to match those seen in *Spea*. Thus, during the evolution of the distinctive carnivore morph, PFE likely combined with taxon-specific mutations to create an even greater coordinated adaptive response.

In sum, our data provide support for PFE and thereby suggest that phenotypic plasticity might be critical in 'jump starting' evolutionary novelty. Although further tests in diverse systems are needed[7,38], our study reveals that phenotypic plasticity might play an underappreciated role in evolutionary innovation.

Methods

General approach. We sought to comprehensively evaluate the PFE hypothesis in spadefoots. For the focal lineage that possesses the novel trait (the carnivore morph), we chose *Sp. multiplicata*. For our ancestor-proxy lineage, we chose *Sc. holbrookii*. This species is an appropriate proxy for ancestral *Spea* (prior to the evolution of the carnivore–omnivore polyphenism), because *Scaphiopus*: (1) is the closest extant outgroup to *Spea*[27]; (2) produces omnivores only, the reconstructed state for the ancestor of *Scaphiopus* and *Spea*[12]; and (3) is ecologically similar to *Spea*. We reared each species in both the novel environment (shrimp diet, the diet of the derived carnivore morph) and the ancestral environment (detritus diet, the diet of the ancestral omnivore morph).

We then tested for both pre-existing plasticity and adaptive refinement. To determine if there was pre-existing plasticity, we examined reaction norms for both morphology and gene expression when *Sc. holbrookii* were exposed to the ancestral diet versus the derived diet. For morphology, we assessed the extent of ancestral plasticity for all component traits that comprise the novel carnivore phenotype (Supplementary Table 1). For gene expression, we measured expression levels of several candidate genes that have been implicated in being involved in carnivore production (Supplementary Table 4). To determine if there was adaptive refinement in morph functionality, we contrasted morphology, gene expression and growth—on both diets—of *Sc. holbrookii* and *Sp. multiplicata*. Finally, to evaluate if frequency of trait expression predicts the extent to which it is refined, we then contrasted production of carnivore features in *Sp. multiplicata* with those of a congener—*Sp. bombifrons*—that produces the carnivore morph more frequently.

Evaluating whether there is ancestral plasticity of morphology in *Sc. holbrookii*. We bred two pairs of *Sc. holbrookii* that had been part of an established laboratory colony at the University of North Carolina, Chapel Hill for 1–2 years. Breeding was induced by injecting adults with 0.04 ml luteinizing-hormone-releasing hormone (Sigma L-7134) at a concentration of 0.01 μg μl^{-1} and leaving pairs overnight in nursery tanks. Eggs from each sibship were kept in separate nursery tanks until hatching. Upon hatching, individuals were placed in separate, opaque, 90 ml plastic cups filled with dechlorinated water. Each hatchling was then haphazardly assigned to one of two diet treatments: (1) crushed fish food (hereafter 'detritus'), which simulates in form and nutrition the detritus on which *Spea* omnivores feed in natural ponds[39]; or (2) live brine shrimp (*Artemia*), which simulate the fairy shrimp (*Thamnocephalus* or *Steptocephalus*) on which *Spea* carnivores feed in natural ponds. Detritus-fed tadpoles each received 10 mg of detritus every three days and shrimp-fed tadpoles each received 2 ml of concentrated brine shrimp nauplii twice daily until these tadpoles were seven days old, at which time each was fed approx. eight live adult brine shrimp twice daily. After 11 days, we ended the experiment by killing tadpoles in a 0.1% aqueous solution of tricane methanesulfonate (MS-222), submerging a subset (21 tadpoles per family per diet) in RNAlater (ThermoFisher Scientific SKU AM7021), and preserving the rest (30 tadpoles per family per diet) in 95% ethanol.

To assess whether diet-dependent morphological plasticity was present in *Sc. holbrookii* tadpoles, we measured various trophic characters known to exhibit plasticity in *Spea* (Supplementary Table 1). We measured each tadpole's overall body size (snout–vent length; SVL) using hand-held digital calipers and determined its Gosner developmental stage[40]. We then measured the width of the jaw muscle (orbitohyoideus muscle; OH), counted the number of denticle rows (DR) and gut coils (GC), characterized the shape of the mouthparts (MP) on an ordinal scale from one (most omnivore-like) to five (most-carnivore-like)[23]), and measured the width of the lower mouthparts (LMP) using a micrometer and ×5 magnification. We standardized OH for body size (SVL) by regressing ln(OH) on ln(SVL).

To determine if there were diet-dependent differences in the above variables, we used likelihood ratio tests on linear mixed effects models. For all models, 'Family' was treated as a random effect and 'Diet' was the fixed effect. Gosner stage, DR, GC, MP and LMP were modelled using a Poisson distribution. Models were fit with maximum likelihood using the lme4 package[41] and likelihood ratio tests were performed using the anova function in R. The function fdrtool (package fdrtool)

was used to control for multiple testing. All analyses were performed using R version 3.4.0 with $\alpha = 0.05$.

Evaluating whether there is ancestral plasticity of gene expression in *Sc. holbrookii*. Next, we assessed whether diet-dependent gene expression plasticity was present in *Sc. holbrookii* tadpoles. To do so, we collected seven samples per diet per family (28 samples total), where each such sample consisted of three tadpoles from the same treatment pooled together. We extracted total RNA from these samples using a combination of TRIzol Reagent and the Ambion PureLink RNA Mini Kit (ref: 1218302531). Following extraction and treatment with DNase, we visually evaluated RNA quality on a denaturing TAE agarose gel[42] and determined RNA purity and concentration using a NanoDrop 2000 (Thermo Scientific) (Supplementary Table 11). We then reverse transcribed 600 ng of total RNA using the BioRad iScript Reverse Transcription Supermix for reverse transcription quantitative PCR (RT-qPCR) (cat. no. 1708841). Reverse transcription reactions consisted of 4 µl iScript RT Supermix, 600 ng of total RNA and enough nuclease-free water to bring the total reaction volume to 20 µl. These reactions ran according to the manufacturer's protocol.

For RT-qPCR, we focused on eight 'morph-biased' genes and a control gene (*actb*). These morph-biased genes were previously found to differ significantly in expression level between *Spea* carnivores and omnivores, even when these individuals were reared on an exclusive diet of shrimp (this was possible, because many individuals remain as omnivores even when fed shrimp). Thus, these differences in expression level were associated with different trophic morphologies per se and not dietary differences[30]. Of these genes, four were 'carnivore-biased' (that is, upregulated in carnivores relative to omnivores) and four were 'omnivore-biased' (upregulated in omnivores relative to carnivores). We also used an unbiased gene (*actb*) as a 'control' gene (Supplementary Table 4). Although these eight genes likely represent a small subset of genes that are differentially expressed between these morphs, and although none of these genes likely determines which morph an individual becomes (that is, none may be 'switch' genes), our goal was to measure expression levels of some of the genes that are crucial in morph functionality and that are therefore likely to undergo adaptive refinement during the evolution of carnivore–omnivore polyphenism. Essentially, the PFE hypothesis posits that adaptive refinement should occur at numerous loci (not just at 'switch loci'), as genes encoding diverse functions become finely tuned by selection to produce a fully functional phenotype[7]. Indeed, the eight morph-biased genes on which we focused have been implicated in key regulatory (*btf3* and *tbx15*), metabolic (*pm20d2*, *pnlip* and *amy2a*), structural (*col2a1*) and immunological (*pglyrp1* and *tf*) functions. Our control gene (*actb*) has a structural function.

We performed RT-qPCR on these nine (eight biased; one control) genes using 20 µl reactions of the BioRad iTaq Universal SYBR Green Supermix (cat. no. 172-5121) and its recommended cycle conditions for a standard run on a StepOnePlus thermocycler (Applied Biosystems cat. no. 4376600). Melt curve analysis was also performed for each well to evaluate primer specificity. Reaction components, conditions, and primer sequences are provided in Supplementary Tables 12, 13 and 14, respectively. For each gene, we ran all 28 samples in triplicate on a single plate.

We analysed these data two ways to determine the presence or absence of diet-dependent plasticity. First, we used the $\Delta\Delta C_t$ method[43]. This required first finding ΔC_t or the difference in mean C_t value between our gene of interest and *actb* (whose expression was invariant between diets; Supplementary Table 15). To obtain our values, we then calculated the difference between these ΔC_t values and a calibration sample, which was an arbitrarily chosen detritus-fed tadpole. Instead of using raw $\Delta\Delta C_t$ values for our analysis, we calculated the fold-change (here defined as RQ), in expression. To obtain expression fold-change, we used the formula: $RQ = 2^{-\Delta\Delta C_t}$. We compared these RQ values between diets for each gene using a Wilcoxon test with a chi-square approximation (a Kruskal-Wallis test). As above, we controlled for multiple testing with the function 'fdrtool' (package 'fdrtool').

Next, we determined the level of plasticity using a control gene-independent test. Here, we calculated the relative quantity from each individual's mean C_t value without standardizing to a control ($RQ = 2^{-C_t}$). Similar to a previous study[16], we defined plasticity as: $\text{plasticity} = \log \frac{\overline{x}RQ_{\text{shrimp}}}{\overline{x}RQ_{\text{detritus}}}$. We then used 5,000 iterations of nonparametric bootstrapping to generate 99% confidence intervals around these values. For this analysis, plasticity values (±99% CI) less than 0 indicate greater expression on detritus than on shrimp, plasticity values (±99% CI) greater than 0 indicate greater expression on shrimp than detritus, and plasticity values (±99% CI) equal to or overlapping with 0 have no differences between diet. Only genes that showed plasticity in our control-dependent ($\Delta\Delta C_t$) analysis *and* our control-independent (log ratio) analysis were considered to be diet-dependent in their expression.

Evaluating whether there is adaptive refinement of morphology in *Sp. multiplicata*. Although previous studies have demonstrated a significant relationship between trophic characters and diet in *Spea* (for example, refs [23,24,44,45]), we sought to corroborate these findings and (more importantly) determine if there is evidence for adaptive refinement of the ability to use alternative resources. To do so, we used individual *Sp. multiplicata* tadpoles from 17 families, which were derived from four separate populations in southeastern Arizona, USA. Using similar procedures to those described above for *Sc. holbrookii*, we reared these individuals individually on a diet of shrimp or detritus for 18 days. We subsequently measured three fitness proxies (mass, SVL and Gosner developmental stage) and four trophic characters (OH, DR, GC and MP) as above. Using likelihood ratio tests on linear mixed effects models, we determined if there was as significant effect of diet on any of the above responses. For all models, 'Population' and 'Family' were treated as random effects and 'Diet' was the fixed effect. Gosner stage, DR, GC and MP were modelled using a Poisson distribution. Models were fit with maximum likelihood using the lme4 package[41] and likelihood ratio tests were performed using the anova function in R. The function fdrtool was used to control for multiple testing.

In terms of our predictions, the results of the above-mentioned experiments with *Sc. holbrookii* revealed that this species had inconsistent diet-induced morphological plasticity and had lower fitness on a shrimp diet than on a detritus diet (Fig. 2). Therefore, we predicted that if the ability to utilize alternative resources has been adaptively refined in *Sp. multiplicata*, then we should find in this experiment that *Sp. multiplicata* consistently changed its trophic traits in an adaptive direction and had equivalent fitness on both diets. Such results would strongly suggest, in total, that the novel carnivore phenotype has undergone adaptive refinement during the evolution of the carnivore–omnivore polyphenism, as predicted by the PFE hypothesis.

Evaluating whether there is adaptive refinement of gene expression in *Sp. multiplicata*. We extracted RNA, synthesized cDNA, and normalized our data as above. However, before extraction, we measured each individual's MP score as a proxy for how carnivore-like that individual was. MP was used as a proxy for magnitude of the carnivore phenotype for two reasons: (1) this characteristic is robust and unaffected by freezing and treatment with RNAlater, and (2) we had no reason to suspect that the genes that were the focus of this experiment directly influenced the development of this characteristic. For this experiment, we focused on three metabolic genes (*pm20d2*, *pnlip* and *amy2a*) because they are likely among the first to experience changes in expression associated with production of alternative diet-dependent morphs and because their molecular functions are well-known.

To explore patterns among gene expression, phenotypic variation and morph in wild-caught *Sp. multiplicata*, we performed three analyses. First, because we observed that, for some genes, there were differences between morphs in the number of individuals that did not have detectable levels of gene product, we performed a two-tailed Fisher's exact test to quantitatively verify this observation. Specifically, for each gene, we assigned each individual as having ('yes') or not having ('no') a detectable level of gene product (based on the qPCR machine's output) and then determined if this yes:no ratio differed between morphs. For carnivore-biased genes, we expected there to be fewer omnivores with detectable levels of expression. In contrast, for omnivore-biased genes, we expected fewer carnivores with detectable levels of expression. Two individuals were omitted from all analyses because they had no detectable expression across all of our focal genes and because they had no expression of an additional non-metabolic gene that we used to confirm if low expression was biological or technical.

For the next analyses, we assigned a C_t value of 40 for all individuals that had undetectable levels of a given gene (excluding the two noted above). The value of 40 was chosen because we performed 40 cycles of amplification and it is ultimately a conservative estimate for the actual threshold cycle number that would be required to detect the levels of gene product in these 'undetected' samples.

Our second analysis consisted of evaluating the relationship between morphological variation in how carnivore-like individuals were (as indicated by their mouthparts score) and gene expression level ($-\Delta\Delta C_t$; here *gapdh* was used as the invariant control gene) using linear mixed-effects models and likelihood ratio tests as above. For this analysis, we used $-\Delta\Delta C_t$ to improve interpretation (larger values indicate greater expression of the gene of interest) and to improve normality prior to likelihood ratio tests. We used each gene's expression level as the fixed effect predicting mouthparts score because: (1) mouthparts score was a quantitative variable (unlike morph); and (2) it made more sense for a gene's expression to influence phenotype than vice versa. Therefore, 'Expression' was a fixed continuous variable and 'Population' was a random effect. For each gene, we performed a likelihood ratio test between a null model that contained only the random effect to the single-factor model (that retained the random effect). However, we also calculated the Pearson correlation between mouthparts score and each gene's level of expression to characterize their relationship without assigning causality.

Our third analysis was analogous to the second analysis above, except that we tested for differences between morphs rather than along the MP continuum. Therefore, 'Morph' was a fixed categorical variable and 'Population' was a random effect. We performed a likelihood ratio test as above. If there is no morph-biased gene expression then the null model is the best fit. By contrast, if the model containing morph as a fixed effect is the best model, then the alternative morphs have different levels of gene expression (there is morph-biased gene expression).

Evaluating whether there is adaptive refinement of morphology in sympatric *Sp. bombifrons*. We sought to determine if the carnivore morph had undergone further refinement following its establishment as an alternative morph in *Spea*. Because under PFE the frequency of expression of a trait should predict the degree to which it is adaptively refined[5,7], we compared the carnivore morph and

its component traits between *Spea* lineages that differ in frequency with which they produce carnivores (that is, polyphenic or fixed for carnivores). Specifically, we compared OH and MP of *Sp. multiplicata* carnivores from three populations that exhibit disruptive selection favouring production of both carnivores and omnivores[46] and that regularly produce intermediate frequencies of both morphs[23,24] with *Sp. bombifrons* carnivores from four populations that produce carnivores almost exclusively[24] and are under directional selection favouring indivduals that are more carnivore-like[45]. We also calculated a composite index of trophic morphology using previously described methods[45–48] and compared this between lineages. Briefly, we combined DR, MP and OH into a single multivariate shape variable (the morphological index; hereafter, 'MI') with a principal component analysis using a cross-correlation matrix (MI is PC1 of this analysis). In *Spea*, larger values of MI correspond to tadpoles that are more carnivore-like, with few denticle rows, highly keratinized mouthparts and large orbitohyoideus muscles. To ensure that our analysis was restricted to carnivores, we only used individuals that had an OH/SVL ratio ≥ 0.15 (ref. [49]). GC was not measured in these samples because the guts had been damaged or destroyed during previous processing.

According to PFE, the *Sp. bombifrons* lineages described above should be more carnivore-like than the *Sp. multiplicata* lineages. To test this prediction, we used likelihood ratio tests on linear mixed effects models, to determine if there was as significant effect of lineage type (polyphenic versus fixed for carnivores) on any of the above responses. For all models, 'Population' was treated as random effects and 'Lineage' was the fixed effect. DR and MP were modelled using a Poisson distribution. The function fdrtool was used to control for multiple testing.

During our quantification of the above traits in sympatric *Sp. bombifrons* lineages, we consistently noticed the presence of a keratinized 'spike' descending from the upper inside of the tadpoles' mouths (that is, descending from their palate; Fig. 2e inset). We quantified variation in this trait, which we termed the palate spike (PS), on an ordinal scale from 1 to 5. Individuals with a score of 1 either had no indication of a spike at all or, at most, had small flecks of pigment on the roof of the mouth. A score of 2 or 3 was given to individuals with a rough-edged patch of light brown pigment or if the patch had coalesced into a brown circle, respectively. A PS score of 4 meant that the circle had become substantially darker (almost black) and began to descend from the roof of the mouth. A score of 5 was given if the spike was longer and darker.

We first determined if PS was correlated with the morphological index determined above. Finding that this was a carnivore-biased trait (Supplementary Fig. 1), we then compared variation in this trait among wild-caught sympatric *Sp. bombifrons* carnivores, allopatric *Sp. multiplicata* carnivores and omnivores, and *Sc. couchii*. Note that *Sc. couchii* was used as an ancestor-proxy for this portion of our study (see Fig. 1 for phylogeny) because we were able to obtain adequate wild-caught samples of this species. Using these groups, we were able to determine if: (1) this trait has uniquely evolved in *Spea*, and (2) it has been refined in a manner consistent with the frequency of carnivore morph expression in different lineages.

Our analysis of PS started with a likelihood ratio test on a generalized linear mixed effects model fitted with a Poisson distribution that contained 'Group' (that is, *Sp. bombifrons*, *Sp. multiplicata* [C], *Sp. multiplicata* [O] or *Sc. couchii*) as a fixed effect and 'Population' as a random effect with a model that only contained the random effect. We then performed 10,000 iterations of a non-parametric analogue of ANOVA (randomized-residual permutation procedure; RRPP[50–52]) using the function advanced.procD.lm in the package geomorph to determine pairwise differences among groups. We expected that the more frequently a group expresses the carnivore phenotype, the greater the magnitude of its PS. Furthermore, if this trait is present in *Spea*, but not present in the ancestor-proxy *Sc. couchii*, then this would suggest that PFE has acted in concert with de novo mutations to refine the carnivore morph in *Spea*.

Estimating the extent of heritable variation in plasticity and plastic traits. For evolution to occur, traits must be heritable. To get a sense of the extent of heritability and heritable variation in the trophic traits we investigated, we performed two analyses. First, we estimated broad-sense heritability (H^2) of all the trophic and fitness-related traits in *Sp. multiplicata* using methods reported previously[26]. Specifically, for each diet, we fit a linear model with only sibship included as a random effect to obtain an estimate of trait variation among sibships (V_{AF})[26,53]. Then, by dividing double this value over the residual variance + V_{AF} we were able to estimate broad-sense heritability. We then generated 500 nonparametric bootstraps of the entire statistic to create 95% confidence intervals. Higher H^2 on a shrimp diet and non-overlapping confidence intervals is indicative of uncovering cryptic genetic variation (CGV) when tadpoles are reared on this diet[7,26]. Such evidence of CGV is expected for species that are not regularly exposed to the inducing cue (for example, *Scaphiopus*) and that do not regularly express the induced traits. Therefore, we compared the patterns of CGV (or lack thereof) in *Sp. multiplicata* with those previously published[26] for *Sc. couchii* reared under similar conditions. We would expect to find evidence of CGV in *Scaphiopus*, but not in *Spea*. This is because the *Spea* lineage regularly exposes variation associated with eating shrimp to selection, and the *Scaphiopus* lineage does not.

We also sought to gain insights on the heritable variation in *Sc. holbrookii*. However, because we only had two families to generate among-family variance estimates, any estimates of H^2 would be overcome with error (indeed, many of our estimates were >1). Therefore, we treated family as a factor rather than a random effect to test if families differ in their responses rather than estimate variance across families. To do so, we tested for a significant interaction between sibship and diet using ANOVA with type III sum of squares. In general, finding a significant 'Family' term or a significant interaction between 'Family' and 'Diet' would indicate that families differ in their plastic response. These observations coupled with the fact that a previous study found evidence of CGV in a different *Scaphiopus* species would suggest, at the very least, the plausibility of these responses being inherited.

Ethics statement. We have complied with all relevant ethical regulations and our study protocol was approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC ID 17-252.0-A).

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data is included as supplementary material and uploaded to the Dryad Digital Repository at https://doi.org/10.5061/dryad.pg0qd5c.

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Author contributions

N.A.L. and D.W.P. conceived and designed the study; N.A.L. and A.J.I. collected data; N.A.L. analysed the data; N.A.L. and D.W.P. wrote the manuscript. Photos in Figs. 2,3 were taken by D.W.P. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to N.A.L.

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Corresponding author(s): Nicholas A. Levis

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☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
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☐	☒	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	No software was used to collect data.
Data analysis	Data were analyzed using JMP Pro and R. Specific packages are indicated in the text.

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Data is included as supplementary material and uploaded to the Dryad Digital Repository. We declare no conflicts of interest.

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Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	We manipulated the diet of 120 tadpoles from a non-polyphenic species and 302 tadpoles from a polyphenic species and determined if there were diet-induced differences in 1) various trophic morphological characters, 2) growth, and 3) expression level of diet-related genes. In addition, we compared trophic morphology among wild-collected tadpoles from a non-polyphenic species (n = 50), two morphs of a polyphenic species (total n = 156), and a secondarily non-polyphenic species (n =82).
Research sample	For the focal lineage that possesses the novel trait (resource-use polyphenism), we chose Spea multiplicata. For our ancestor-proxy (non-polyphenic) lineage, we chose Scaphiopus holbrookii. This species is an appropriate proxy for ancestral Spea (prior to the evolution of the carnivore-omnivore polyphenism), because Scaphiopus: 1) is the closest extant outgroup to Spea ; 2) produces omnivores only, the reconstructed state for the ancestor of Scaphiopus and Spea; and 3) is ecologically similar to Spea. We reared each species in both the novel environment (i.e., shrimp diet, the diet of the derived carnivore morph) and the ancestral environment (i.e., detritus diet, the diet of the ancestral omnivore morph). To evaluate if frequency of trait expression predicts the extent to which it is refined, we then contrasted production of carnivore features in wild-caught tadpoles Sc. couchii, Sp. multiplicata, and Sp. bombifrons. The first species never produces carnivores, the second sometimes produces carnivores through diet-induced plasticity, and the third often produces carnivores regardless of diet.
Sampling strategy	Sample sizes were maximized using available laboratory space and were comparable with similar studies/systems.
Data collection	Data were collected by N.A.L. and A.J.I. by measuring tadpole morphology with digital calipers and measuring gene expression level using RT-qPCR.
Timing and spatial scale	Experimental morphological data were collected for Sp. multiplicata were collected in the summer 2015, and experimental morphological data for Sc. holbrookii were collected in the summer 2016. The later date for Sc. holbrookii was due to insufficient number of breeding pairs at the initial time point and limitations on space for experimental procedures. Gene expression data were collected in summer 2017 using samples that had been stored at -80C in RNAlater from the time of collection (2013 for Sp. multiplicata and 2016 for Sc. holbrookii) . Wild-caught morphological data were collected in summer 2017 using previously collected specimens stored in ethanol. These specimens were collected from the field in 2006, 2008, and 2016.
Data exclusions	Data were only excluded if a molecular technique (e.g. qPCR reaction) failed for all genes assessed. In these cases, the individual was omitted from analysis. Full detail is given in the methods of the manuscript.
Reproducibility	We had numerous replicates for each experiment performed, but have not taken steps to formally reproduce the entire study. Sample sizes ranged from 10 to14 replicates (for molecular assays) and 60 to >100 replicates for morphological measurements.
Randomization	As described in our methods, tadpoles from each family were randomly, and evenly, divided among treatment groups where applicable.
Blinding	Where possible, the investigators did not know the treatment group from which an individual was derived during morphological measurements. For RT-qPCR, blinding was not done because PCR plates needed to be designed up front.

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions	Field work only consisted of collecting tadpoles from naturally occurring ponds in the southwestern United States during the summer and following heavy monsoon rains.
Location	Ponds were located near Portal, AZ (lat: 31°54'49.14"N, long: 109° 8'29.34"W)
Access and import/export	These ponds have studied extensively for 20+ years. Access to ponds is annually coordinated with property owners and collection permits are obtained annually from the Arizona Game and Fish Department.
Disturbance	These tadpoles often develop in ponds used as a water source for cattle so they are used to frequent disturbance. However, we try to minimize our impact by not over-collecting at a given pond and only spending as much time in the water as necessary to collect samples.

Reporting for specific materials, systems and methods

Materials & experimental systems		Methods	
n/a	Involved in the study	n/a	Involved in the study
☒	☐ Unique biological materials	☒	☐ ChIP-seq
☒	☐ Antibodies	☒	☐ Flow cytometry
☒	☐ Eukaryotic cell lines	☒	☐ MRI-based neuroimaging
☒	☐ Palaeontology		
☐	☒ Animals and other organisms		
☒	☐ Human research participants		

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Laboratory animals were tadpoles of Scaphiopus holbrookii and Spea multiplicata.
Wild animals	Wild animals (Spea multiplicata, Spea bombifrons, and Scaphiopus couchii) were obtained from ponds near Portal, AZ 10-14 days after hatching using hand-held dip nets. Tadpoles were immediately euthanized in MS-222 and ultimately transported back to the University of North Carolina for measurements.
Field-collected samples	Field-collected samples were euthanized prior to any laboratory work performed on them. Samples were stored in 95% ethanol prior to use.