Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity

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Developmental plasticity is found in most organisms, but its role in evolution remains controversial. Environmentally induced phenotypic differences may be translated into adaptive divergence among lineages experiencing different environmental conditions through genetic accommodation. To examine this evolutionary mechanism, we studied the relationship between plasticity in larval development, postmetamorphic morphology, and morphological diversity in spadefoot toads, a group of closely related species that are highly divergent in the larval period and body shape and are distributed throughout temperate areas of both the New and the Old World. Previous studies showed that accelerated metamorphosis is adaptive for desert-dwelling spadefoot toads. We show that even under common garden conditions, spadefoot toad species show divergent reaction norms for the larval period. In addition, experimentally induced changes in the larval period caused correlated morphological changes in postmetamorphic individuals such that long larval periods resulted in relatively longer hindlimbs and snouts. A comparative analysis of morphological variation across spadefoot toad species also revealed a positive correlation between the larval period and limb and snout lengths, mirroring the effects of within-species plasticity at a higher taxonomic level. Indeed, after ≈110 Ma of independent evolution, differences in the larval period explain 57% of the variance in relative limb length and 33% of snout length across species. Thus, morphological diversity across these species appears to have evolved as a correlated response to selection for a reduced larval period in desert-dwelling species, possibly diverging from ancestral plasticity through genetic accommodation.

allometry | genetic accommodation | life-history evolution | morphological diversity | larval period

Phenotypes result from genetic and environmental inputs to development, and consequently, organisms express different phenotypes in different environments (1–5). This developmental plasticity may buffer the effects of environmental variation and slow down the genetic response to selection, thus retarding evolutionary change (4, 6, 7). However, plasticity may also promote evolutionary diversification if environmentally induced developmental variants allow populations to persist under different environments and subsequently diverge into independently evolving lineages (4, 8–10). For instance, phenotypic plasticity enables organisms to withstand environmental heterogeneity or invade new environments (11–13), potentially involving novel phenotypes. Then, if the new environmental conditions persist long enough, selection may favor genetic changes affecting the frequency and/or expression of the developmental variants in lineages experiencing the novel conditions, a process referred to as genetic accommodation (4, 14, 15). Genetic accommodation of a novel phenotype could thus bridge microevolution and macroevolution through the action of selection on phenotypic plasticity so that trait differences among species reflect, at least in direction if not in magnitude, the observed within-species plasticity.

Although solid experimental and theoretical support exists for genetic accommodation within a lineage (15–19), few studies have described patterns of evolutionary diversification congruent with evolution through genetic accommodation (8, 20–23), nor has such congruence been explicitly examined in a phylogenetic context. The expectations for a genetic accommodation model of evolution are (*i*) ancestral plasticity, (*ii*) selection on the induced phenotype, (*iii*) genetic change and divergence of descendant reaction norms, and (*iv*) a common developmental mechanism for within-species plasticity and among-species variation.

Larval amphibian development is highly plastic, and it is regulated by thyroid hormone (TH) physiology (24–27). Such plasticity is adaptive, and, in some cases, it allows tadpoles to increase their developmental rate in response to environmental risks such as pond desiccation (28–30). However, altering the larval developmental rate often causes morphological, size-independent changes of the emerging metamorphs (31–37). Under unconstrained food availability, long larval periods generally result in larger juveniles with relatively (i.e., size-independent) longer hindlimbs and, to a smaller degree, longer heads (32, 33, 36, 38). Such environmentally induced changes in the larval period and the correlated changes in juvenile morphology could become fixed across lineages if they occupied divergent selection regimes (22).

The largest differences in the larval period among anuran species can be found within Pelobatoidea (New World spadefoot toads, *Scaphiopus* and *Spea*; Old World spadefoot toads, *Pelobates*; and parsley frogs, *Pelodytes*). *Pelobates* and *Pelodytes* breed in permanent or long-lasting ponds, and they have long larval periods, >180 days in some *Pelobates* species. *Spea* and *Scaphiopus* are adapted to much more ephemeral ponds, and they have dramatically reduced larval periods, as short as 8 days in *Scaphiopus couchii* [see supporting information (SI) Table 1] (39, 40). Even if reared in common garden experiments under identical conditions of water permanence and abundant food, these species differ broadly in their larval periods [*Scaphiopus* < *Spea* < *Pelodytes* < *Pelobates* (refs. 40 and 41)]. Short larval periods in *Scaphiopus* and *Spea* evolved after their divergence from *Pelobates* and *Pelodytes* in response to increasing ephemerality of their breeding sites during aridification of their habitat in North America (40). Additionally, several species in this group show adaptive plasticity in the larval period in response to pond duration (28, 30, 42, 43). Because of large differences in the

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Abbreviations: PGLS, phylogenetic generalized least-squares; TH, thyroid hormone.

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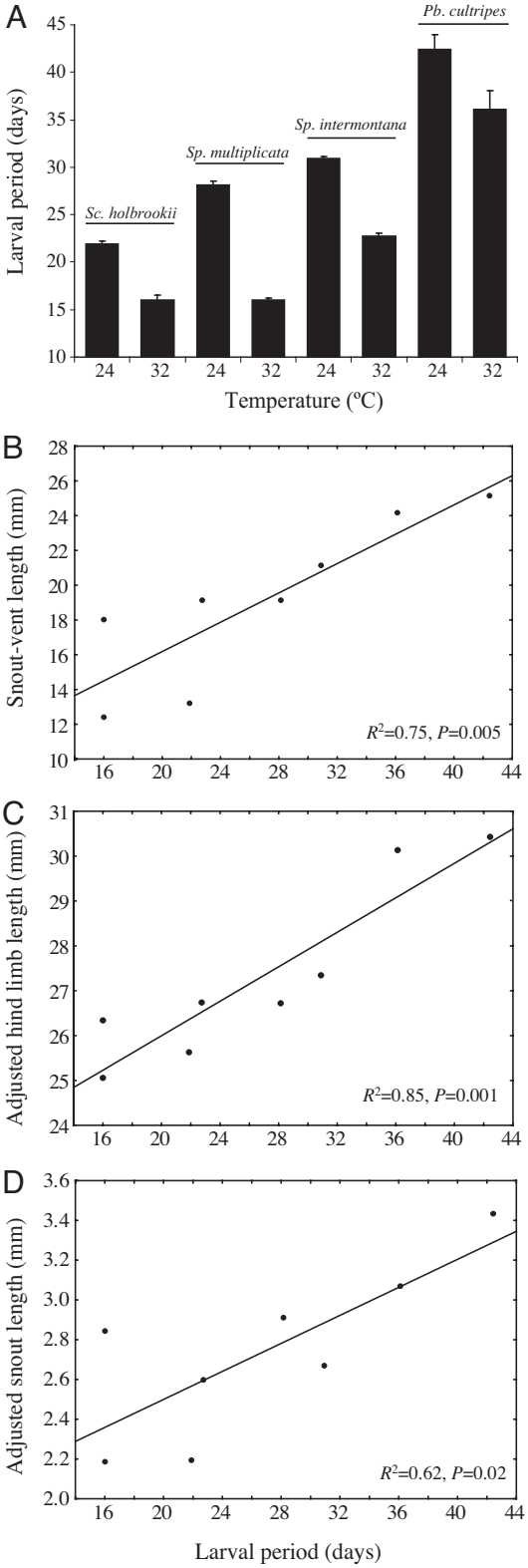

Fig. 1. Temperature affected larval developmental rate and the resulting morphology of metamorphs. (*A*) The average larval period was consistently shorter at high temperature, 24°C vs. 32°C, for all four species examined. The larval period was significantly correlated with body length (*B*), relative hindlimb length (*C*), and relative snout length (*D*). Adjusted means per species and experimental treatment for relative hindlimb and snout lengths were calculated as least-square means in analyses of covariance holding body length as a covariate. The larval period explained large proportions of the variance in morphometric traits.

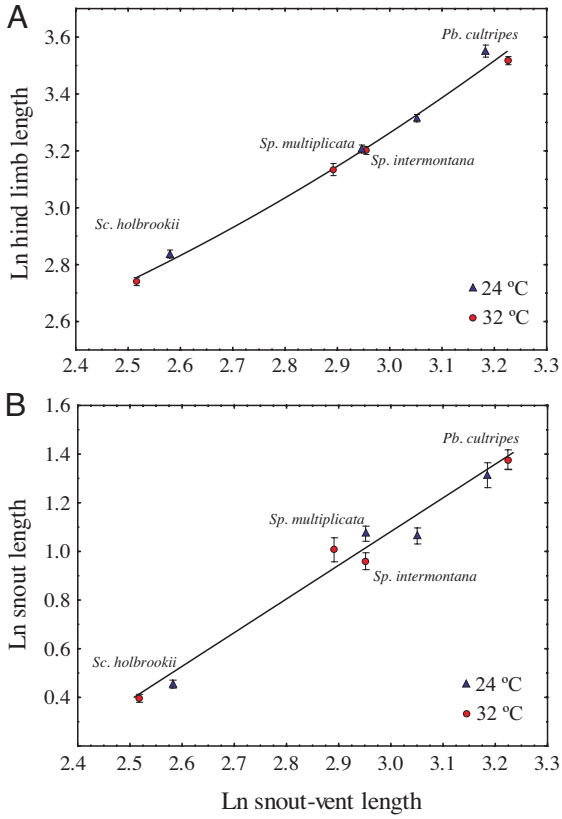

Fig. 2. We observed both plastic and evolutionary allometries [i.e., within species under different conditions and among species (ref. 2)] of hindlimb (*A*) and snout lengths (*B*) to body length. Triangles and circles indicate averages per species at either 24°C or 32°C, respectively, with error bars indicating ±SE. Evolutionary allometry was linear for snout, but it was slightly nonlinear for hindlimb. Sample sizes were 15 (*P. cultripes*), 21 (*Sp. multiplicata*), 28 (*Sp. intermontana*), and 97 (*Sc. holbrookii*) metamorphs per species.

larval period among species and the evidence for plasticity in the larval period within species, we used this group of species to test whether morphological diversity among taxa may be consistent with a correlated response to genetic accommodation of the larval developmental rate.

Results

Plasticity in the larval period and body shape were examined by measuring the effects of temperature on time to metamorphosis and morphology of juvenile *Pelobates cultripes*, *Spea multiplicata*, *Spea intermontana*, and *Scaphiopus holbrookii* reared individually as tadpoles at either 24°C or 32°C and preserved immediately after completion of metamorphosis (40). Temperature affected the larval developmental rate in all species (Fig. 1*A*) so that individuals reared at 32°C had significantly shorter larval periods (27.8% shorter on average ± 5.8 SE; $F_{1,156} = 240.59$, $P < 0.0001$). Temperature had a significant effect on body size as well; tadpoles reared at 32°C resulted in toadlets that were 6.3 ± 1.13% smaller ($F_{1,156} = 7.11$, $P < 0.01$) with 2.1 ± 0.3% shorter hindlimbs ($F_{1,150} = 4.76$, $P = 0.03$) and 3.9 ± 2.3% shorter snouts ($F_{1,150} = 8.76$, $P = 0.0002$) than those reared at 24°C. Importantly, the larval period duration was positively correlated with body length (Fig. 1*B*), relative hindlimb length (Fig. 1*C*), and relative snout length (Fig. 1*D*). In light of the correlation between the larval period and morphology, we examined allometric relationships between morphological variables. Allometry of snout length to body length was linear (2), whereas

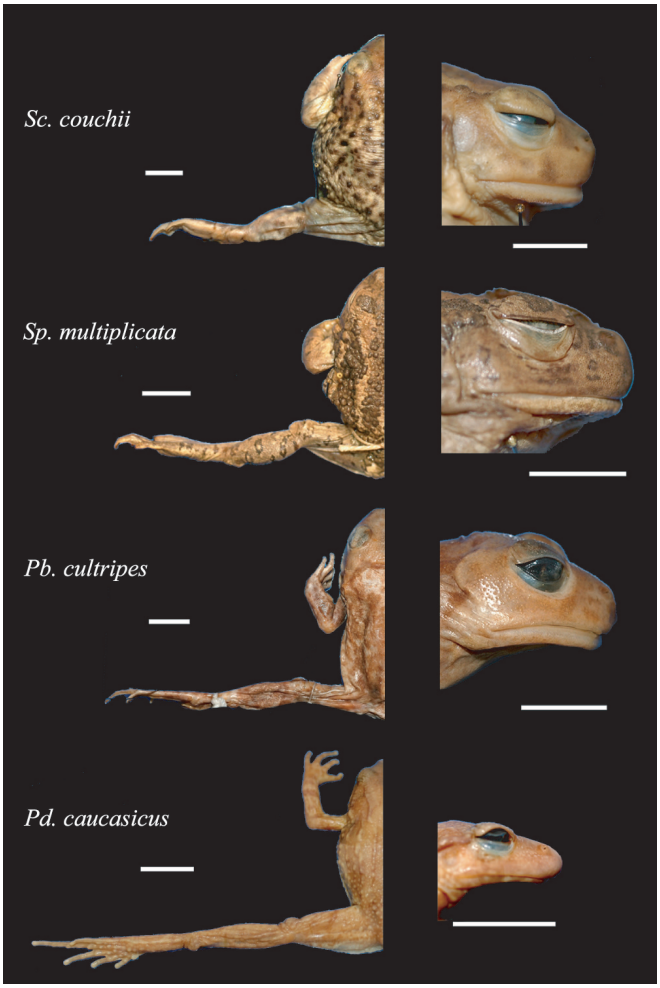

Fig. 3. Spadefoot toads and parsley frogs exhibit morphological variation across genera. Images have been rescaled so that snout–vent lengths are equal, to visualize size-free morphometric differences. Species with long larval periods (*Pelobates*, *Pelodytes*) show proportionately longer hindlimbs and longer snouts. (Scale bars, 1 cm.) The background was made uniform with Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA).

hindlimb showed slightly nonlinear allometry (2) with body length (Fig. 2 *A* and *B*).

Morphological diversity across taxa was quantified by obtaining the same morphological measurements (snout–vent, hindlimb, and snout lengths) from adult specimens of all species of spadefoot toads and parsley frogs in various scientific collections, except for *Spea hammondii* because of its lack of monophyly and uncertain taxonomic status of some of its populations (Fig. 3) (44). Specimens in the different collections had been collected at different times in different localities throughout the distribution of each species, reducing the likelihood of bias caused by microgeographic variation. A nested analysis of covariance showed that both hindlimb and snout lengths, corrected for overall body size, varied significantly across genera ($F_{3,348} = 97.63$, $P < 0.0001$ and $F_{3,348} = 78.43$, $P < 0.0001$, respectively for hindlimb and snout lengths) and also across species nested within genera ($F_{9,348} = 16.10$, $P < 0.0001$).

Because body shape differences within species were correlated with environmentally induced differences in the larval period, we tested for a correlation between the larval period and body shape across species. First, we conducted a Bayesian analysis on a subset of available Cyt *b* and 16S gene sequences in public databases (ref. 44; see *Materials and Methods*) to obtain a

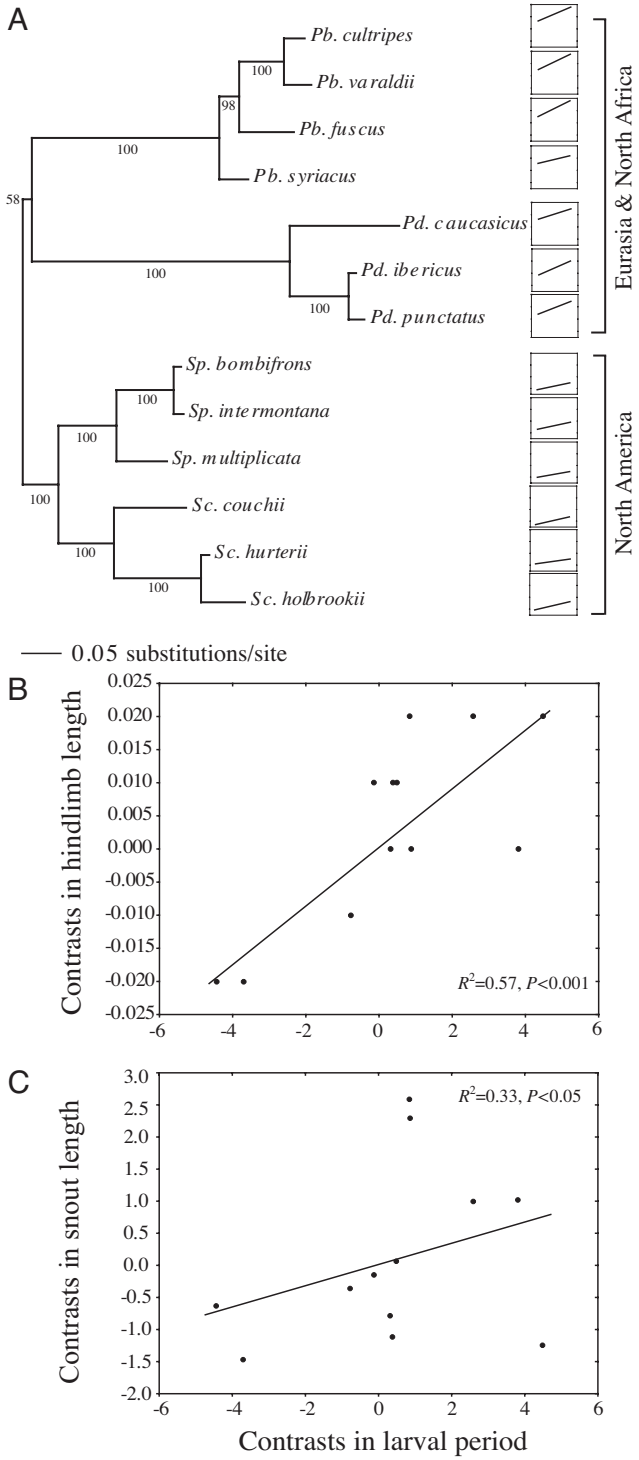

Fig. 4. Phylogeny and comparative analysis of the relationship between larval period and body shape. (*A*) The phylogenetic hypothesis for spadefoot toads and parsley frogs was reanalyzed from ref. 44, and it was used for our comparative analyses. Numbers under the branches indicate Bayesian posterior probabilities. The lines in the boxes on the right side show the range between the shortest and longest larval periods observed for each species throughout their distribution, obtained from the literature referenced in SI Table 1, and the range can be considered an approximation of species-level plasticity in the larval period across all larval conditions experienced. The *y* axes in all boxes were scaled to range from 0 to 140 days. (*B* and *C*) Regressions through the origin (65, 66) between standardized independent contrasts for the larval period and size-independent hindlimb length and snout length, respectively. Size-independent residuals of the morphometric traits were obtained from linear regression analysis against snout length before computation of the contrasts.

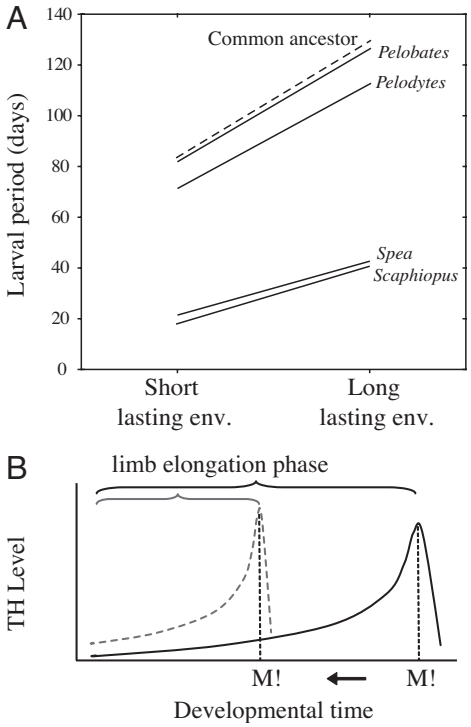

Fig. 5. Reconstructed reaction norm evolution and mechanistic model of morphological divergence. (*A*) Comparison between analytically reconstructed ancestral plasticity for the larval period (dashed line) and the average reaction norms for each genus. The reaction norms for the North American genera have shifted toward overall shorter larval periods, and they show less steep slopes, indicating that they have lost some plasticity. (*B*) New World lineages of spadefoot toads experience higher TH levels (dashed curve), achieving metamorphosis (M!) in a shorter time than Old World lineages (solid line). Such a heterochronic shift toward early metamorphosis reduces the opportunity for limb elongation, resulting in allometric differences in body shape, as implied by endocrine experiments within species (59, 60).

phylogenetic tree for subsequent comparative analyses (Fig. 4*A*). Then, we regressed size-corrected residuals of hindlimb and snout lengths against the minimum larval period for each species, obtained from published literature (SI Table 1), fitting a phylogenetic generalized least-squares model (PGLS, 45). Both hindlimb and snout lengths were significantly correlated with the minimum larval period ($r = 0.75$, $R^2 = 0.57$, $P < 0.01$; $r = 0.58$, $R^2 = 0.33$, $P < 0.05$, respectively; Fig. 4 *B* and *C*), whereas overall size (snout–vent length) was not ($r = 0.10$, $R^2 = 0.01$). Independent contrasts analyses (46) were also consistent with these results. The PGLS model also provided estimates for ancestral states of reactions norms of the larval period at the different nodes in the tree (47), confirming that the reduction in the larval period occurred after the split of the Old World and North American lineages (Fig. 5*A*) (40).

Discussion

Our data show that morphometric differences among species of spadefoot toads and parsley frogs reflect within-species morphological variation caused by plasticity in the developmental rate. We found that, as in other frog species (31–36), environmentally induced shifts in the larval period in four species of spadefoot toads affected their postmetamorphic shape as, within species, long larval periods correlated with relatively longer hindlimbs and, to a lesser extent, longer snouts. Similarly, across species, morphological variation was significantly correlated with differences in the minimum larval period, so that species with long larval periods have relatively longer hindlimbs and longer snouts (Fig. 3) than species with short larval periods, irrespective of their overall size and after accounting for phylogenetic relatedness. Such similarity in patterns of morphological variation within and between species and the relationship between the larval period and morphology suggests that diversity in spadefoot toads and parsley frogs has evolved to a large extent as a correlated response to evolutionary changes in the larval developmental rate.

The lineages of spadefoot toads and parsley frogs are old, and they have a long history of independent evolution under different environments because the split between Old World and New World lineages may be as old as 110 Ma (40, 44, 48). In that time, adaptation to ephemeral environments in the New World taxa shifted reaction norms for the larval period from an ancestral state similar to that of *Pelobates* to the derived state of *Spea* and *Scaphiopus*, with no overlap in their reaction norms even if reared under identical environmental conditions (Fig. 1*A* and ref. 38). In addition, there has been ample opportunity for selection to act on morphology. Relative limb length and head length have functional relevance for locomotion and feeding, and their plasticity in relation to shifts in life-history traits has been studied in detail (34, 49–52). Observed plastic changes in morphology within species were relatively minor (1–5% in length), and they were unlikely to have a high impact on locomotor performance or predator escape efficiency (31), and thus they were unlikely to have large effects on fitness and to be the direct target of natural selection. Rather, a large proportion of the variance in hindlimb length and snout length across all taxa is explained by the larval period alone, and it appears to be a correlated response to selection on the larval period. All taxa except *Pelodytes* are burrowers, and there are no major differences in diet or predator communities that would suggest differential selection among species for different relative limb and head lengths.

Hall (9) pointed out the impossibility of distinguishing between characters that arose through genetic assimilation of preexisting phenotypic variability (selection on the ability to produce the right phenotype, or adaptive plasticity) and characters that arose through selection of a mutation (selection on alleles of fixed effects) once the genetic assimilation process was completed. Although we agree with this observation, genetic assimilation, which is a special case of genetic accommodation (4), involves an initially plastic trait (environmentally induced) that becomes canalized or genetically fixed (4, 9, 16). In nature, a plastic trait may very likely retain a certain level of plasticity even after directional selection (10, 22) because some environmental heterogeneity will most likely persist, resulting in a shifted, rather than a flat, reaction norm. Indeed, in our study system, even though the larval period has diverged remarkably among lineages, all species have maintained a substantial level of plasticity in the duration of the larval period (Fig. 4*A*). The reaction norms show a shift toward overall shorter larval periods in the North American lineages, with only a slight decrease in plasticity. Analytical reconstruction of ancestral plasticity in the larval period (Fig. 5*A*) indicated that the last common ancestor of all spadefoot toads and parsley frogs was also plastic, with a similar reaction norm to *Pelobates*. Such plasticity in ancestors of New World spadefoot toads would have enabled them to survive in shorter duration ponds as North America became more arid.

Simple allometry explains to some extent the relationship between the larval period and morphological variation observed. Long larval periods under unconstrained growth often result in larger sizes at metamorphosis, and the relative increase in limb length and snout length may be in part the result of simple allometric relationships with overall body size (Fig. 2 *A* and *B*). However, *Pelodytes* species are smaller than *Spea*, but they have long larval periods, and they exhibit relatively long hindlimbs and snouts like *Pelobates*, suggesting that changes in the larval period

per se may also have some influence on morphology beyond a simple allometric relationship to body size.

The scenario of plasticity enabling an environmental induction of alternative phenotypes through differential expression of genetic variation already present in the population constitutes an alternative to the more traditional view that genetic change precedes phenotypic change. In both cases, genetic change is required for the evolution of the trait because in genetic accommodation, the regulation of the expression of the trait under the novel conditions changes over time so that reaction norms diverge beyond its ancestral state. A major difference between the fixed-trait mutation and the genetic accommodation hypotheses resides in their acceptance of the interchangeability of genetic change and environmental change as initiators of the process and the role given to preexisting, masked, genetic variation (4, 53, 54). Genetic accommodation requires ancestral plasticity, selection on the induced phenotype, genetic change controlling the expression of such phenotype causing divergence in the descendant reaction norms (9), and a common developmental mechanism for within-species plasticity and among-species variation. In our system, we find evidence for ancestral adaptive plasticity in the larval period, selection for a reduced larval period in New World taxa, and divergence of the descendant reaction norms. This pattern is congruent with the expectations of genetic accommodation, which thus becomes a more parsimonious hypothesis than fixed-trait mutation because it does not require the extra steps of novel mutation and its spread in population to initiate the divergence.

As for the regulatory mechanism, the amphibian developmental rate is highly conditioned by the environment and regulated by THs (24–27) so that intraspecific alterations in the larval period are often related to changes in TH concentration (55, 56). Across species, the evolution of short larval periods in *Spea* and *Scaphiopus* was achieved through changes in TH physiology, as they show increased tissue TH content and sensitivity compared with *Pelobates* (41). We suggest that, initially, environmental acceleration of larval development in New World spadefoot toad ancestors was likely achieved through increased TH levels (55, 56). Over time, direct selection would have favored genetic changes regulating TH levels and tissue sensitivity to TH, causing developmental acceleration in response to pond desiccation that would have further reduced the larval period in those lineages. However, because developmental processes are highly modular (57, 58) and because TH has different effects on different tissues at different times throughout development, accelerating development would cause allometric changes in morphology (shorter limbs and snout) in the absence of other genetic change. Indeed, endocrine experiments within species showed that relatively shorter legs were associated with larval periods that were experimentally shortened by high levels of exogenous TH (59, 60). Similarly in our case, short larval periods in *Scaphiopus* and *Spea*, resulting at least in part from elevated endogenous TH levels (41), imply truncated limb elongation through precipitation of metamorphosis, whereas long larval periods in *Pelobates* and *Pelodytes* imply extended periods of TH at lower titer that acts on limb elongation (Fig. 5*B*). Thus, the same endocrine mechanism, namely TH level regulation, may largely control larval period differences within and between species and have pleiotropic effects on morphology.

The expected genetic changes under each hypothesis are slightly different because mutationally induced variation would likely be driven by mutation on genes of major effect, whereas genetic accommodation is more likely to be driven by mutations and/or changes in frequency of modifier genes instead. Adaptive plastic traits such as developmental acceleration in spadefoot toad tadpoles requires both the ability to perceive a change in the environment and the ability to alter development accordingly, and they are very likely to be under polygenic control. West-Eberhard (4) suggests that correlated quantitative change in developmentally linked traits influenced by the same regulatory mechanism such as the correlated life-history and morphological changes observed indicate a complex, polygenic regulator of the phenotype, congruent with the kind of genetic architecture expected in the genetic accommodation hypothesis. However, a close examination of the genetic mechanisms affecting TH level regulation and its environmental sensitivity within and between species is needed to distinguish further between both hypotheses.

In conclusion, plasticity for the larval period is ancestral in this system and probably in most anurans. Selection for accelerated larval periods operated through continued aridification of the environment in the New World spadefoot toads, and a genetic response to selection must have occurred so that, even if brought under identical environmental conditions, reaction norms show divergence across species occupying different environments. These results link micro- and macroevolution of the larval period and morphology in spadefoot toads and parsley frogs, providing an example of congruence between within-species plasticity and phenotypic divergence among lineages in different environments, possibly evolved through correlated evolution and genetic accommodation.

Materials and Methods

Within-Species Plasticity. Buchholz and Hayes (40) studied variation in the larval period across most species of spadefoot toads and parsley frogs, rearing tadpoles from each species in the laboratory at different temperatures under ad libitum food availability. Individuals from those experiments were preserved at Gosner stage 46 (61), and their larval periods were recorded. We examined specimens reared at either 24°C or 32°C. To test for effects of temperature on the larval period, we fitted a general linear model to number of days from hatching to metamorphosis, nesting temperature within species using PROC MIXED (SAS, Cary, NC; ref. 62). Three morphometric traits were obtained from each individual with a caliper to the nearest 0.1 mm: snout-to-vent length, hindlimb length, and preocular head length (or snout length). To test for size-independent effects of temperature on morphology, we fitted a general linear model on each response variable (hindlimb and snout lengths) including snout–vent length as a covariate using PROC MIXED.

Phylogenetic and Comparative Analyses. In this study we included all extant taxa of spadefoot toads and parsley frogs within Pelobatoidea, except *Sp. hammondi* (SI Table 1), which was excluded because of a lack of monophyly and unresolved taxonomic status (44). We measured the same three morphological traits described in the previous section (snout–vent, hindlimb, and snout lengths) from a total of 362 adult toad specimens preserved in museum collections. We obtained the residuals from linear regressions between hindlimb and snout–vent lengths and between snout and snout–vent lengths to be used in subsequent comparative analyses (see below). We obtained larval period estimates for each species through a bibliographic survey, which provided both minimum and maximum larval periods observed per species (SI Table 1).

Published sequences of 16S rRNA (16S) (520 bp) and Cyt *b* (385 bp) (44) were obtained for all 13 target species except for *Sc. holbrooki*, whose Cyt *b* was unavailable. Sequence alignment and gap treatment followed Garcia-Paris *et al.* (44). To search for the best model of evolution that fit the data we used Model Test 3.7 (63). Following the Akaike Information Criterion, we selected a general time-reversible model with gamma parameter and proportion of invariant positions (GTR+I+G), and we subsequently conducted a Bayesian phylogenetic analysis with MrBayes 3.1.2 (64). The analysis used random starting trees and ran three "hot" and one "cold" Markov chains in duplicate, for 1,000,000 generations sampled every 10. The first 2,500 trees

were discarded as "burn in" based on standard deviations of the split frequencies. The 50% majority rule consensus tree of the remaining trees showed strict monophyly of all species included, and we therefore pruned the tree to keep a single branch per species for the comparative analysis. The resulting tree (Fig. 4*A*) was assumed as our phylogenetic hypothesis for comparative analyses. To test for correlations between minimum duration of the larval period and relative hindlimb and snout lengths, we used both PGLS and independent-contrasts approaches (45, 46) with Compare 4.5 (47). PGLS uses a two-parameter, exponential weighting matrix (45), and it allows for inclusion of within-species variation. Therefore, analyses were run by using the mean ± SE for each variable. The PGLS procedure estimates a parameter (α) that indicates the strength of the evolutionary constraint on character evolution. Low values of α (close to 0) indicate high phylogenetic constraint, and PGLS then approximates Felsenstein's contrasts method. In the models fitted to our data, the maximum likelihood estimate of α was $\alpha_1 = 3.25$ for hindlimb length and $\alpha_2 = 15.5$ for snout length, indicating a higher dependence on phylogenetic relationships in hindlimb length. PGLS also provided ancestral reconstruction of character states for the larval period from the weighted average of extant taxa under the assumption of a linear model and after 1,000 iterations.

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