

DURATION AND CONSISTENCY OF HISTORICAL SELECTION ARE CORRELATED WITH ADAPTIVE TRAIT EVOLUTION IN THE STREAMSIDE SALAMANDER, *AMBYSTOMA BARBOURI*

Jonathan M. Eastman,¹ John H. Niedzwiecki,² B. Paul Nadler,³ and Andrew Storfer^{1,4}

¹*School of Biological Sciences, Washington State University, Pullman, Washington 99164*

²*Biology Department, Belmont University, Nashville, Tennessee 37212*

³*Department of Computer Science, University of Northern Iowa, Cedar Falls, Iowa 50613*

⁴*E-mail: astorfer@wsu.edu*

Received January 4, 2008

Accepted May 12, 2009

A fundamental challenge in evolutionary biology concerns estimating the extent to which ecological trade-offs may impose constraints on adaptive evolution. Novel ecological stressors may limit adaptive evolution of naive lineages that have experienced historically different selective regimes. Regarded as recently derived from a pond-breeding ancestor, streamside salamanders face the novel and strong selection pressure of breeding in streams with fish predators. A statistical phylogenetic approach was used to test whether adaptive evolution of antipredator performance phenotypes in streamside salamanders was positively associated with: (1) estimated per-lineage duration of coexistence with predatory fish; and (2) consistency of this predator selective-regime within lineages. Average durations of fish contact were computed for each salamander lineage on a set of chronograms. Selection consistency was determined by estimating the number of ecological transitions between fish and fishless states using stochastic character mapping. Historical selection in streamside salamanders can be generally characterized as unstable, apparently punctuated by the stochastic loss and recolonization of predatory fish in most lineages. We found that the efficacy of antipredator phenotypes in salamanders is strongly related to historical duration, as well as consistency, of selection imposed by predatory fish.

KEY WORDS: *Ambystoma texanum*, conflicting selection, historical constraint, local adaptation, niche evolution, phylogenetic constraint, trait evolution.

A central challenge in evolutionary biology is to understand the extent to which historical constraints influence the capacity of populations to evolve in response to current selection pressures. Phylogenetic history can constrain contemporary evolution via trade-offs that result from adaptation to a selection regime that differs from current environmental conditions (Wilson 1975; Lande 1979; Sih et al. 2000; Orzack and Sober 2001). Adaptive evo-

lution may otherwise be constrained functionally (Edwards and Naeem 1993), developmentally (Derrickson and Ricklefs 1988), environmentally (Newman 1992), or genetically (Wright 1951; Slatkin 1985, 1987; Schluter 2000). In contrast, genetic canalization for example (Kawecki 2000) may facilitate current adaptation by maintaining traits that are presently adaptive (i.e., exaptations sensu Gould and Lewontin 1979). Thus, understanding the course

of adaptive trait-evolution among lineages necessitates examination under an explicitly historical context (Lande 1979; Estes and Arnold 2007).

If environments exert stable and consistent selection pressures (i.e., niche stability) on ecologically relevant traits, lineages should evolve toward local adaptive optima (Fisher 1930; Haldane 1930; Wright 1932; Kawecki and Ebert 2004). However, variable selection induced by niche instability can constrain population mean fitness if phenotype–environment trade-offs exist and affected lineages fail to evolve phenotypic plasticity (Lynch 1987; Kawecki 2000; Huerta-Sanchez et al. 2008). Trade-offs preventing fitness maximization across all temporally encountered environments for particular genotypes, should be observable through comparative phylogenetic analysis if one were able to: (1) assess the historical variability of selective pressures on lineages; and, (2) estimate the trait optima associated with the different selective regimes. Despite the temporal lability of selection imposed by environmental variation expected in nearly every biotic system, few studies have tested whether fluctuating historical selective pressures constrain adaptive evolution in contemporary populations.

One of a pair of sister salamander taxa (streamside salamander, *Ambystoma barbouri*; smallmouth salamander, *A. texanum*) is well-suited for testing the effects of phylogenetic and ecological history on phenotypic trait evolution. The stream-breeding *A. barbouri* is hypothesized to have diverged during the late Pleistocene from the pond-breeding *A. texanum* due to climatic warming, pond drying, and thus decreased availability of pond-breeding habitat (Petranka and Sih 1987; Kraus and Petranka 1989). Putatively monophyletic, streamside salamanders are en-

demic to a narrow geographic range centered in Kentucky with few peripherally isolated populations in adjacent areas (Kraus and Petranka 1989; Fig. 1). In contrast, smallmouth salamanders have a widespread and largely contiguous geographic range throughout much of eastern United States (Kraus and Petranka 1989; Petranka 1998). It is currently presumed that *A. texanum* is the progenitor of *A. barbouri* (Kraus and Petranka 1989) because pond-breeding is the ancestral state for Ambystomatidae (Petranka 1998) and more widely distributed taxa are presumed to have greater speciation probabilities than narrowly distributed species (Darwin 1859; Rosenzweig 1995; Demastes et al. 2007; Eastman et al. 2007).

The invasion of ephemeral stream habitats (Maurer and Sih 1996) exposed *A. barbouri* to a new selective regime (Sih et al. 2000). In comparison to more permanent ponds, highly ephemeral stream habitats impose strong selection for rapid metamorphosis (Kats et al. 1988). As predicted, empirical laboratory and field studies support that apparently adaptive high larval activity rates are positively correlated with high feeding and metamorphosis rates (Petranka and Sih 1987; Maurer and Sih 1996; Sih et al. 2000). However, some populations of *A. barbouri* breed in more permanent streams that support predatory fish. In these habitats, selection favors reduced larval activity and consequent predator avoidance (Kats et al. 1988; Sih et al. 2000, 2003). Thus, streamside salamander larvae face conflicting selection pressures in different streams (Storfer and Sih 1998; Storfer et al. 1999; Sih et al. 2000). *Ambystoma barbouri* thereby comprises two apparent ecotypes, whereby selection favors relatively high activity levels in ephemeral streams and low activity levels in fish-bearing streams.

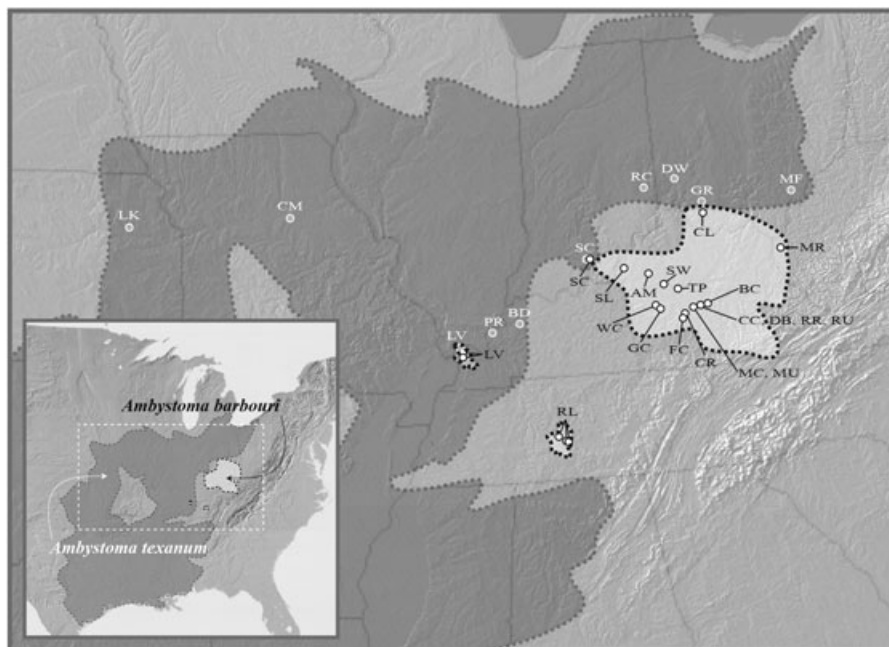


Figure 1. Sampling localities and geographic ranges of *A. barbouri* and *A. texanum*.

Generally, streamside salamanders are observed to have poor antipredator avoidance behavior, and it is suggested this is attributable to contemporary gene flow between fish and fishless habitats (Storfer and Sih 1998; Storfer et al. 1999). It has also been hypothesized that *A. barbouri* may be phylogenetically constrained in their response to the novel selective force of fish predation due to its relatively recent divergence in a genus with a long evolutionary history of pond-breeding in fishless habitats (Petranka 1998; Sih et al. 2000).

Herein, we test two hypotheses to discern whether phylogenetic history, and specifically the history of selection in each deme, explains observed variation in antipredator behavior among populations of *A. barbouri*. We test whether the efficacy of antipredator performance in *A. barbouri* is positively correlated with lineage-specific evolutionary duration of coexistence with fish, as well as consistency of historical selection induced by predatory fish. We distinguish between “duration” and “consistency” of selection because, for example, a lineage that has coexisted with fish for half its existence and fish introduction occurring only once should be better adapted than another also coexisting for half its existence with fish, but with recurrent fish introductions and losses. As such, estimating duration of coexistence alone may not accurately reflect the evolutionary history of a particular deme. We assume that a phenotype-by-environment negative correlation exists such that any single phenotype is not optimally fit in both selective regimes (i.e., fish-associated versus fishless), a result supported by multiple empirical studies (Kats et al. 1988; Storfer and Sih 1998; Sih et al. 2000). We attempt to measure adaptive “lag load” (defined as the extent of deviation from optimal trait values; Bürger and Lynch 1995; Kawecki 2000) for fish-association in lineages of the streamside salamander. We predict that contemporary lineages having experienced both longer durations of coexistence with fish and consistent selection are expected to be closer to adaptive optima (Björklund 1996; Hansen et al. 2008) and therefore ought to exhibit more effective antipredator phenotypes than those with shorter durations and/or fluctuating selection regimes.

Methods

SAMPLING LOCALITIES AND TISSUE ACQUISITION

We collected tissue samples from individual *A. barbouri* and *A. texanum* across their respective ranges (Fig. 1; Table 1) between 1999 and 2004. Both geographic forms (eastern and western; Kraus and Petranka 1989) of *A. texanum* were sampled, with sampling effort most concentrated in the eastern portion of its range, where this species is sympatric with *A. barbouri*. Collections for *A. barbouri* covered the entire species range, including peripherally isolated populations in western Kentucky, eastern West Virginia, and central Tennessee (Fig. 1). Tail clips (ca. 1 cm²) or whole larval specimens were preserved in 95% ethanol and stored

Table 1. Locality data and accession numbers for phylogenetic analysis of mtDNA sequences.

Species	Accession	Haplotype	North	West
<i>A. barbouri</i>	EU980549	AM	38.43	84.97
	EU980578	BC-a	37.89	84.38
	EU980583	BC-b	37.89	84.38
	EU980584	CC	37.89	84.39
	EU980589	CL	39.52	83.98
	EU980574	CR	37.79	84.55
	EU980587	DB	37.89	84.37
	EU980586	FC	37.77	84.57
	EU980545	GC	37.97	84.83
	EU980592	LV-a	37.37	88.46
	EU980590	LV-b	37.37	88.46
	EU980580	MC	37.88	84.45
	EU980591	MR	38.33	82.47
	EU980575	MU-a	37.88	84.45
	EU980582	MU-b	37.88	84.45
	EU980570	RL-a	35.82	86.48
	EU980571	RL-b	35.82	86.48
	EU980572	RL-c	35.82	86.48
	EU980585	RR-a	37.90	84.39
	EU980581	RR-b	37.90	84.39
	EU980588	RR-c	37.90	84.39
	EU980579	RU	37.90	84.39
	EU980577	SC-a	38.65	85.61
	EU980553	SC-b	38.65	85.61
	EU980548	SL	38.49	85.32
	EU980576	SW	38.20	84.75
	EU980573	TP	38.19	84.58
EU980546	WC-a	38.02	84.84	
EU980547	WC-b	38.02	84.84	
<i>A. texanum</i>	EU980561	BD-a	37.40	87.40
	EU980567	BD-b	37.40	87.40
	EU980568	BD-c	37.40	87.40
	EU980558	CM	38.76	92.20
	EU980550	DW-a	40.24	84.66
	EU980552	DW-b	40.24	84.66
	EU980589	GR-a	39.74	84.01
	EU980551	GR-b	39.74	84.01
	EU980569	LK	38.93	95.23
	EU980562	LV-a	37.37	88.46
	EU980563	LV-b	37.37	88.46
	EU980564	LV-c	37.37	88.46
	EU980565	LV-d	37.37	88.46
	EU980556	MF-a	39.43	81.47
	EU980557	MF-b	39.43	81.47
	EU980559	PR-a	37.21	88.12
	EU980560	PR-b	37.21	88.12
EU980566	PR-c	37.21	88.12	
EU980554	RC	39.90	84.92	
EU980555	SC	38.69	85.67	
<i>A. bishopi</i>	EU517608			
<i>A. californiense</i>	AY659995			
	NC006890			
<i>A. cingulatum</i>	EU517584			
<i>A. mexicanum</i>	AJ584639			
	AY659991			
<i>A. tigrinum</i>	AY659992			
	NC006887			
	EU980544			

at -20°C upon return to the laboratory. Taxonomic identification was determined in the field based on morphological characteristics following Kraus and Petranka (1989).

Total genomic DNA was isolated from each of 78 tissue samples using either a modified phenol–chloroform extraction protocol (Sambrook et al. 1989) or Puregene DNA extraction kits (Gentra Systems, Plymouth, MN). Maximal lengths of 913 nucleotides (bp), comprising both the mitochondrial control region and an adjacent intergenic spacer (ca. 350 bp), unique to the ambystomatids, were obtained by polymerase chain reaction (PCR) amplification using primers (DL3 and THR) developed by Shaffer and McKnight (1996). We performed PCR amplification as in Storfer et al. (2004). After successful amplification, products were purified using vacuum filtration with Montage SEQ₉₆ kits (Millipore, Billerica, MA). Purified PCR products were cycle-sequenced using BigDye Terminator 3.1 (Applied Biosystems, Foster City, CA; ABI), sequenced with an ABI 377 automated sequencer. We obtained additional sequences electronically from GenBank for *A. cingulatum*, *A. bishopi*, and for each of three species within the tiger salamander complex (*A. californiense*, *A. mexicanum*, and *A. t. tigrinum*; Table 1) to be used as outgroups. An additional haplotype from a larval *A. t. tigrinum*, collected by JN, was also included as an outgroup. All sequence editing was performed first with visualization of electropherograms using SEQUENCE ANALYSIS (ABI) and alignment was performed in CLUSTALX (version 1.83.1; Thompson et al. 1997) using default parameters; newly generated sequences were deposited in GenBank (for accession numbers, see Table 1).

COMPARATIVE ANALYSIS

Figure S1 presents a flow diagram of methods used, the details of which directly follow. Briefly, a statistical phylogenetic framework was used to evaluate the correlation between observed antipredator trait values for populations of *A. barbouri* with estimated measures of historical consistency and duration of selection toward optimal antipredator phenotypes.

At 14 localities, four phenotypic traits related to antipredator performance (Storfer and Sih 1998; Storfer et al. 1999) were scored for *A. barbouri*, including: (1) efficacy of antipredator-feeding trade-off using *Daphnia magna* as food and chemical cues from green sunfish (*Lepomis cyanellus*) as a predator (both *A. barbouri* and *A. texanum* exhibit antipredator responses to green sunfish cues as if the fish were present, see Kats and Dill 1998); (2) length of time surviving in the lethal presence of green sunfish; (3) responsiveness to a simulated predatory attack; and (4) extent of cryptic coloration, measured as a function of melanophore pigmentation of individuals (Storfer and Sih 1998; Storfer et al. 1999).

All experiments used streamside salamander larvae at roughly three weeks of age, reared from wild-collected eggs. Data

from the feeding experiment were calculated as the difference in number of *D. magna* eaten in presence versus absence of the predator, *L. cyanellus*. Survival data were recorded as the average number of individuals from a given locality surviving the lethal presence of the predatory *L. cyanellus*, averaged across 20 time points. Response to physical perturbation was performed using a tap-test to simulate a predator attack; the number of taps sufficient to provoke an escape response of a larva was recorded. To estimate crypsis, the average number of melanophores was quantified from photographic images of the dorsocranial region of individual larvae; larvae with fewer melanophores better match the substrate in streams with sunfish (Storfer et al. 1999). Data from these performance assays were log-transformed and constant of unity was added to raw data before transformation to avoid negative values. Further, values from the perturbation test and the coloration assay were represented as the inverse of the raw data, i.e., $\text{Ln}(\frac{1}{x+1})$, to facilitate interpretability of results from subsequent analyses.

TOPOLOGY GENERATION

A set of credible chronograms was generated in BEAST (version 1.4.6; Drummond and Rambaut 2007) to test trait-evolution hypotheses for *A. barbouri*, later conducted in BAYESTRAITS (Pagel 1999; Pagel et al. 2004) using the generalized least-squares approach. A relaxed molecular-clock method, with log-normally distributed evolutionary rates, was used for chronogram estimates under a coalescent model of constant “population” size (Drummond et al. 2006) using BEAST, aided by BEAUTI for input-file generation (both version 1.4.6; Drummond and Rambaut 2007). Nearly all priors were uninformative and were left as default; however, following Yang (1996) and Sullivan and Swofford (2001), the proportion of invariant sites was constrained to be zero. Sampling involved a total chain length of 5×10^6 generations, with subsampling every 10^3 generations. The first 3001 samples were discarded as burnin, well after stationarity had been reached, as assessed qualitatively by change in likelihood scores using TRACER (version 1.4; Drummond and Rambaut 2007). For node constraints, a uniform prior distribution on the divergence of *A. californiense* from other members of the tiger salamander complex (*A. mexicanum* and *A. tigrinum* in this study) was used to calibrate chronogram estimates. This range (3.0 to 6.0 MYA) subsumes the estimates given by Wakabayashi and Sawyer (2001) for the secondary orogenic event of the Sierra Nevada range in California, the presumed evolutionary catalyst initiating the divergence of *A. californiense* from *A. tigrinum* (Shaffer and McKnight 1996). Given the lack of resolution of phylogenetic relationships within the Ambystomatidae, a wide range of values (0 to 33 MYA) was used as a uniform prior on the root node to account for this uncertainty (see Shaffer and McKnight 1996). The crown group is thought to have originated in the Oligocene (Larson et al. 2003), thus informing the lower bound. It should be noted that

absolute divergence times are inconsequential to analyses conducted herein, although we provide a conservative upper bound on the estimate for the evolutionary origin of *A. barbouri*. To assess sensitivity of topological estimates to choice of tree prior, a secondary analysis in BEAST was performed, differing only by its specification of a Yule-speciation tree prior.

Although presumed monophyletic based on a set of reproductive, behavioral, and morphological characters (Kraus and Petranks 1989), previous phylogenetic analyses by Shaffer et al. (1991) provided little resolution of the relationship between *A. barbouri* and *A. texanum*. A topological hypothesis of monophyletic *A. barbouri* was tested from the posterior distribution of trees from the BEAST run. This ensured that comparisons of trait evolution were made within a single group of closest common ancestry. The posterior probability for a clade containing all sampled *A. barbouri* haplotypes was evaluated using a constraint-filter in PAUP* (version 4.0b10; Swofford 1991).

DURATION AND CONSISTENCY OF SELECTION

Lineage-specific durations, in absolute time of exposure to predatory fish (hereafter termed dwell times), and historical consistency of this selective pressure were estimated in SIMMAP (version 1.0; Bollback 2006). The state of a binary ecological trait was determined for each terminal node: either fish-associated versus fishless larval-habitat. Some localities of the streamside salamander support green sunfish (*L. cyanellus*), bluegill sunfish (*L. macrochirus*), or green-bluegill hybrids; populations of the small-mouth salamander are not known to coexist with these aquatic predators (Sih et al. 2000). Ecological trait-evolution was fitted with a continuous-time Markov model in SIMMAP (i.e., fish or fishless). A gamma prior distribution ($\alpha: 3.0, \beta: 2.0$) was placed on the parameter describing transition-rate between character states (Huelsenbeck et al. 2003; Bollback 2006). The sum of durations in the character-state associated with exposure to fish was tabulated for each path from root to each terminal node, so to estimate historical duration of selection induced by predatory fish within each lineage. A random sample of 200 trees from the stationary phase of the BEAST analysis was used, upon which stochastic trait-mappings were generated. Ten realizations from the prior distribution of ecological-state frequencies (a symmetrical beta distribution, $\alpha: 1.0$) were drawn per tree sample. For each tree in the sample, 10 realizations from prior distributions were used; dwell times were computed as the average durations across the 2000 simulated histories in SIMMAP. SIMMAP dwell times were interpreted by the Java program, TREDWE, written ad hoc (available from www.wsu.edu/~storfer/eastman/software). TREDWE enables users to extract the evolutionary duration (i.e., dwell time) of each character state for all paths (root-to-tip) present in the mutational histories generated by SIMMAP. Values for dwell times were log-transformed for subsequent analysis.

Measures of selection consistency were determined in part by the total number of transformations between ecological states along each path. Per lineage lengths were interpreted by TREE-STAT (version 1.1; Rambaut and Drummond 2007) and were averaged across the 2000 simulated character histories, generated in SIMMAP. To develop a quantitative measure of the consistency of selection toward optimal trait values, dwell time was divided by the log-transformed transitions between ecological states (fishless or fish). Thus, a lineage that had never been exposed to fish (both dwell time and transitions equal zero) would be expected to exhibit the most ineffective antipredator performance.

ASSOCIATION OF SELECTION WITH ANTIPREDATOR PERFORMANCE

Estimates of the duration (i.e., dwell times) and consistency of selection were regressed against measures of antipredator phenotypes in BAYESTRAITS. The random sample retained from the BEAST analysis (200 ultrametric trees) was pruned to leave only *A. barbouri* for which antipredator performance data were available (see Fig. 2). The variance-covariance matrix of the continuous data is informed by a commensurate correction for “phylogenetic heritability” (see Freckleton et al. 2002) via the parameter, λ (range: $\lambda \geq 0$; Pagel 1999; Pagel et al. 2004). Lineages with longer periods of shared evolutionary history are expected to exhibit similar trait-values (Felsenstein 1985), and this parameter describes the degree to which trait-values are associated solely due to phylogenetic relationship (Pagel 1999; Pagel et al. 2004).

Evolutionary regression of antipredator performance on both selection measures (i.e., consistency and duration) was performed in BAYESTRAITS under a Brownian motion model “A,” adequate for modeling trait evolution due either to genetic drift or to variable selection (Housworth et al. 2004). MCMC sampling involved 5.5×10^6 generations, where the chain was sampled every 10^3 generations; the first 501 samples were discarded as burnin. A one-tailed *t*-test was performed to assess whether the regression slopes were significantly positive. The standard deviation of the slope was determined by the raw data, whereas the slope (β), corrected for phylogenetic nonindependence, was determined in BAYESTRAITS. The slope, simply, was the ratio of the trait-covariance (e.g., between dwell time and antipredator performance) to the variance of the variable along the abscissa (e.g., dwell time; Pagel 1999).

Results

TOPOLOGY ESTIMATES AND PHYLOGENETIC HYPOTHESES

Monophyly of *A. barbouri* with respect to *A. texanum* is supported with the exception of two haplotypes (i.e., SC-b and GR-a; Fig. 2) that may be a result of interspecific hybridization. If these two

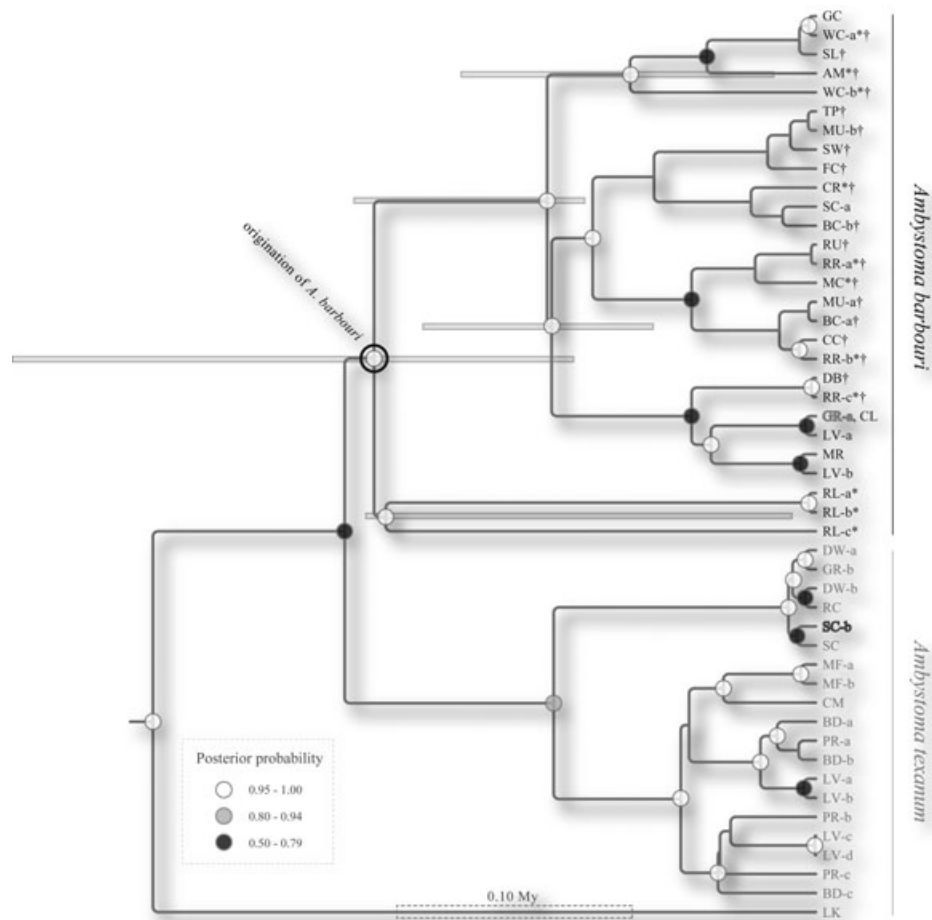


Figure 2. Tree estimates from Bayesian analyses conducted in BEAST. Posterior support for internal nodes denoted by arbitrary groupings of relative support (exceeding 0.49 posterior probability). Dark-shaded terminal taxa represent samples from *A. barbouri*; those light-shaded are *A. texanum*. Bolded taxon names (GR-a and SC-b) represent putative hybrids between the two species. Locality designations correspond to those in Figure 1. *, collection acquired from a fish-bearing locality; †, sample used in regression analyses using data for antipredator performance. Outgroups (see text) have been removed. Node-associated bars represent 95% highest posterior densities for the primary and well-supported lineages within *A. barbouri*. For the sake of clarity, variance associated with estimated heights of other nodes is not depicted.

haplotypes are permitted to be positionally unconstrained in the constraint filter-test implemented in PAUP, a large proportion of posterior trees sampled by BEAST (0.9665) are consistent with the monophyly of *A. barbouri*.

Our choice of a coalescent prior in the BEAST analysis appears to exert little influence on tree estimates. Using TREE-STAT, node heights (in units of time) were compared between the analysis performed under a coalescent prior (see Methods) and one performed under a Yule speciation prior. Natural-log-transformed values from these different analyses were highly correlated ($p_{\rho} \neq 0, 54 \text{ df} < 0.0001$), and slope was not significantly different from 1.0 ($p_{\beta} = 1, 54 \text{ df} = 0.8812$).

TRAIT EVOLUTION

Character-state reconstruction of the node shared by all sampled *A. barbouri* suggested streamside salamanders likely originated

in habitats containing predatory fish (SIMMAP $p_{\text{pos}} = 0.9227$), not more recent than 0.11 MYA as determined by the posterior distribution of credible node heights from BEAST. However, fish-introductions appear to have occurred several times in the evolutionary history of *A. barbouri* in part because streamside salamander lineages currently in coexistence with fish lacked any posterior support as a monophyletic group, as determined in PAUP using a constraint filter-test (BEAST $p_{\text{pos}} < 0.0001$). Further, ecological trait histories simulated in SIMMAP suggested a strong bias toward loss of the fish-associated character-state in streamside salamanders. On average, 1.92 transitions with $CI_{\alpha=0.05} [1.57, 2.26]$ from fishless to fish-associated character states were estimated across each simulated tree-history; average tree-wide transitions between fish-associated to fishless states was 13.88 with $CI_{\alpha=0.05} [13.26, 14.49]$. The stochastic loss of predatory fish is expected to be approximately sevenfold more frequent than is

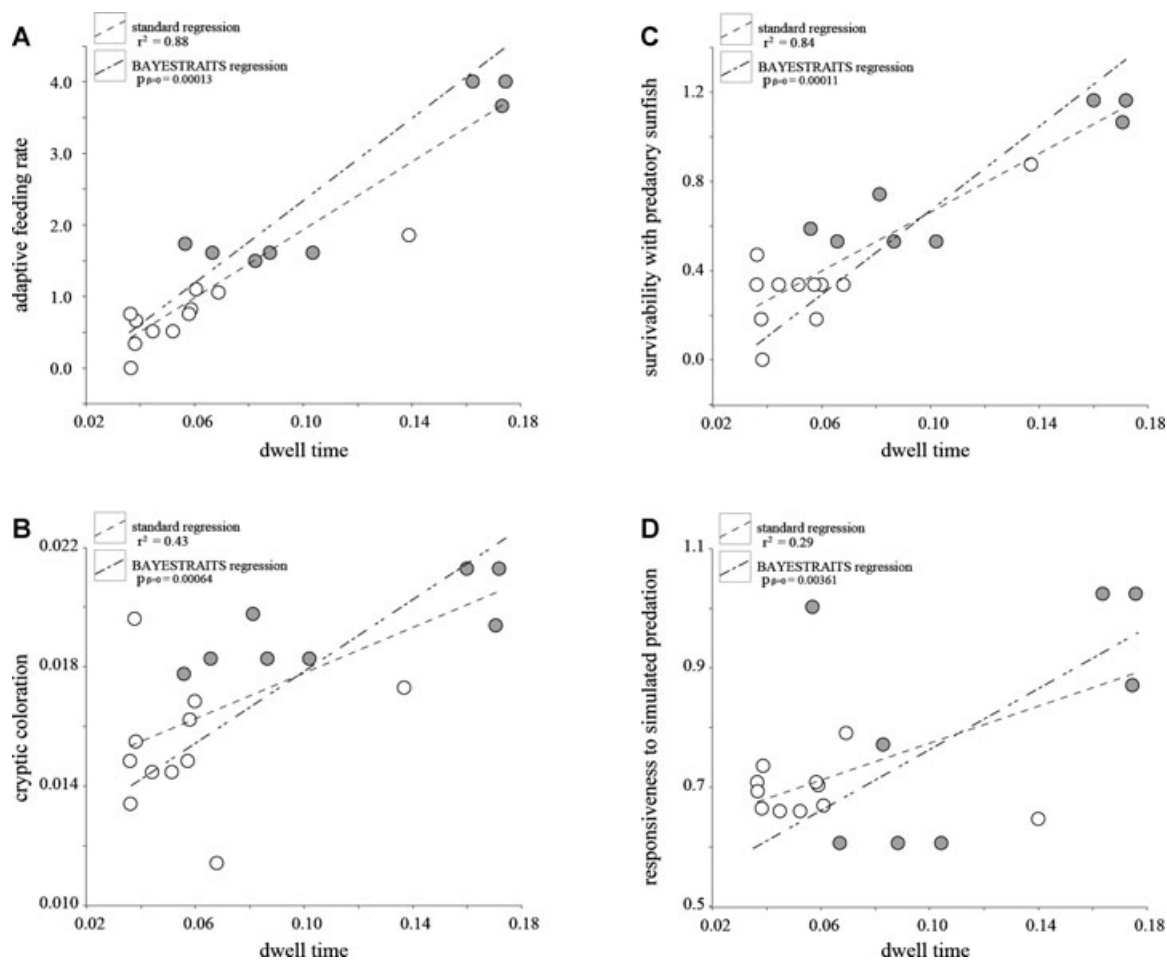


Figure 3. Regression analyses of four measures of antipredator performance against estimates of dwell time within lineages of the streamside salamander. Dwell times were estimated by stochastic mapping of character histories in SIMMAP, by treating fish-association of salamanders, or lack thereof, as an ecological trait evolving by Markov process. Estimates of absolute durations of evolutionary contact with predatory fish were recorded for each lineage and were used for regression analysis. Using BAYESTRAITS, all evolutionary regression coefficients were determined to be significantly positive ($p_{0.05(1),17} < 0.005$). Open symbols correspond to animals from fishless localities; closed symbols represent animals from habitat that presently supports predatory fish.

novel introduction to (or recolonization of) these predators in populations of *A. barbouri*.

Strong support was estimated for positive relationships between the efficacy of antipredator phenotypes with both selection consistency and lineage-specific durations of predator coexistence (dwell times) from BAYESTRAITS evolutionary regression (Figs. 3 and 4; Table 2; all BAYESTRAITS regression analyses, $p_{\beta=0, (1)17df} < 0.004$). For measures along the axis of ordinates, larger values imply more effective antipredator performance (Storfer and Sih 1998; Storfer et al. 1999). Results indicate sizable differences in both antipredator performance, as well as ecological history within lineages of the streamside salamander (Figs. 3 and 4). Coefficients of determination were larger for standard regression analyses of selection consistency (compare Fig. 3 with Fig. 4). Yet, the predictive power provided by the inclusion of the number of transitions between ecological states in this mea-

sure of historical selection appears to be small, relative to the sole consideration of dwell time. In all cases, estimates of phylogenetic heritability of trait values (λ) were nonzero, suggesting the influence of phylogenetic effect in shaping phenotypes expressed by individuals of contemporary populations (Table 2).

Discussion

Results from this study point to the general conclusion that the extent to which lineages are adapted to contemporary selective pressures is strongly associated with both duration and consistency of a particular selective regime. Specifically, our results suggest that niche stability and consistent selection within lineages facilitates adaptation of *A. barbouri* to predatory fish, as a diverse theoretical literature would predict (Wilson 1975; Lande 1979; Harvey and Pagel 1991; Orzack and Sober 2001; Reznick

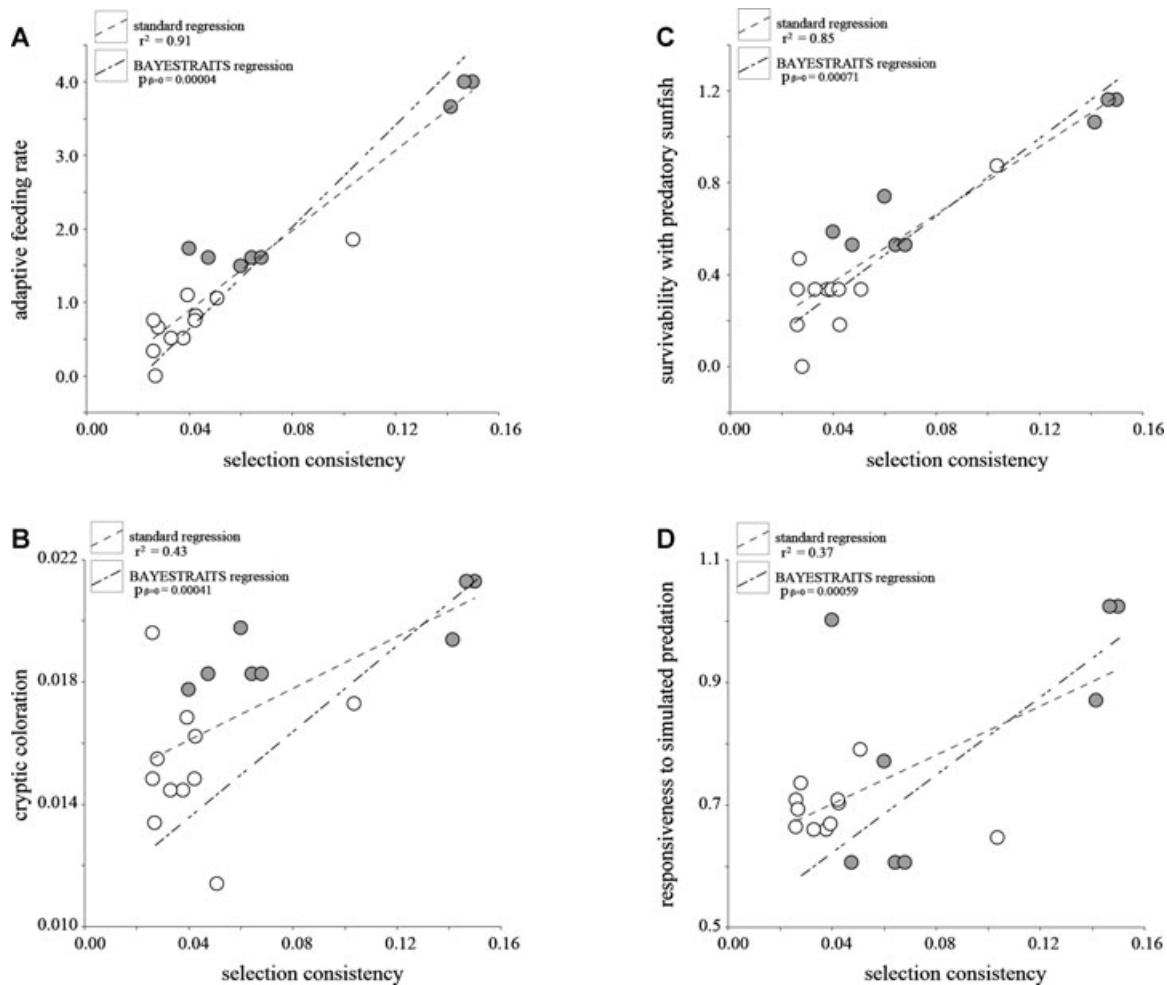


Figure 4. Regression analyses of four measures of antipredator performance against estimates of selection consistency. Total number of lineage-wide transitions between fishless and fish-exposed character states was used to scale per-lineage dwell times (as in Fig. 3), to quantify selection consistency. Both dwell times and ecological state-transitions were stochastically generated in SIMMAP on a set of credible topologies. All regression coefficients were significantly positive ($p_{0.05(1), 17} < 0.001$). Symbols are same as in Figure 3.

and Ghalambor 2001), but few empirical works have tested (e.g., Michaud 2006). Much as Hansen et al. (2008) has provided evidence for lag loads owing to fluctuating multivariate selection, herein we present empirical evidence for the adaptive evolutionary costs associated with lineages experiencing fluctuating selection pressures due to a dichotomous ecological trait.

Depending on whether *A. barbouri* breed in the presence or absence of predatory fish (Kraus and Petranka 1989), divergent suites of phenotypic characteristics are selectively advantageous (Storfer and Sih 1998). Higher activity rates, correlated with both high feeding and developmental rates, enable larvae to metamorphose quickly enough to escape a fishless, highly ephemeral stream (Kats et al. 1988). Fish-bearing streams (or stream reaches) are less ephemeral than fishless habitats (Sih et al. 1992; Storfer and Sih 1998), thereby relaxing the requirement for rapid metamorphosis. Further, lower activity rates benefit salamanders that are susceptible to predation from visual predators such as green

sunfish (Kats et al. 1988; Storfer and Sih 1998; Sih et al. 2000). Thus, evolution of each ecotype appears to be influenced by the existence of separate adaptive optima for larval characteristics among fish and fishless lineages.

Lineages that have never experienced predatory fish ought to be relatively close to an adaptive peak associated with highly ephemeral, fishless breeding-habitat (i.e., rapid developmental, feeding, and activity rates; Storfer and Sih 1998; Storfer et al. 1999). In contrast, lineages that have been in contact with predatory fish for most their evolutionary duration ought to have trait values corresponding to a separate adaptive peak (i.e., high larval survivability and reduced feeding rates in presence of predatory fish, generally reduced developmental and activity rates, effective background matching, and high responsiveness to predatory attack as measured in previous studies; Storfer and Sih 1998; Storfer et al. 1999). As predicted, all four measures of antipredator performance were positively correlated with dwell time of exposure

Table 2. Estimated regression coefficients and phylogenetic heritabilities (λ) for four measures of antipredator performance and two predictor variables: duration of selection within lineages (i.e., dwell time) and selection consistency in lineages toward effective antipredator performance. One-tailed *t*-tests were performed to assess whether the regression slopes were significantly positive. The standard error of each slope was determined by the raw data, where slope ($\hat{\beta}$), corrected for phylogenetic heritability, was estimated in BAYESTRAITS. For each analysis, $v=17$ and $\alpha=0.05$. *Marginal likelihood of each model was determined by the harmonic mean of likelihoods across all states in the post-burnin period of analysis in BAYESTRAITS.

	Selection duration		Selection consistency	
	λ : 0.578	λ : 0.631	λ : 0.631	λ : 0.631
	LnL.*: 140.83	LnL.*: 143.89	LnL.*: 143.89	LnL.*: 143.89
	$\hat{\beta}P_{0.05(1),17}$		$\hat{\beta}P_{0.05(1),17}$	
Survivability	7.947	0.00011	7.295	0.00071
Responsiveness	2.163	0.00361	3.099	0.00059
Feeding	27.960	0.00013	35.029	0.00004
Crypsis	0.053	0.00064	0.063	0.00041

to predatory fish (Fig. 3) and with the historical consistency of this selective pressure (Fig. 4). The four measures of antipredator performance are likely nontrivial components of fitness, as indicated by other studies (e.g., Storfer and Sih 1998; Sih et al. 2000). The antipredator behaviors and extent of crypsis measured are correlated with survivorship of larvae in lethal presence of predatory fish (Storfer and Sih 1998; Storfer et al. 1999), and both are strong predictors of metamorphosis for salamanders in streams with predatory fish (Sih et al. 2000). The absence of phenotypic plasticity in our four measures of antipredator performance is suggested by common garden experiments, indicating a genetic component to the measured traits and thereby suggesting the presence of distinct trait optima (Storfer and Sih 1998; Storfer et al. 1999).

Early *A. barbouri* seem to have evolved in contact with predatory fish (SIMMAP $p_{\text{pos}} = 0.9227$). In addition, it is likely that stochastic loss and recolonization of fish populations has typified the history of *A. barbouri*, given the strong support for nonmonophyly of salamander lineages in contact with predatory sunfish (Fig. 2). Although phylogenetic relatedness likely influences adaptive trait evolution in this system (Table 2), patterns of historical selection peculiar to each lineage are able to surmount this phylogenetic effect (Figs. 3 and 4). These results are in apparent conflict with those of Sih et al. (2003), which suggested *A. barbouri* had evolved for certain traits in the “wrong direction” upon evolutionary exposure to predatory fish. In contrast, the efficacy of antipredator behavior in *A. barbouri* is significantly positively associated with duration (i.e., dwell time), as

well as consistency of apparent coexistence with fish. It is important to note that dwell time and selection consistency should be treated distinctly. That is, although dwell times may be equivalent among any two given lineages, one lineage may have a single fish introduction, whereas the other lineage may have multiple introductions and losses. The former lineage is expected to be closer to its adaptive optimum than the latter. Accordingly, including the number of ecological transitions appears to be more predictive of the efficacy of antipredator phenotypes than considering dwell time alone (Figs. 3 and 4).

These results dovetail previous work suggesting gene flow between fish and fishless habitats constrains antipredator performance in contemporary populations of streamside salamanders (e.g., Storfer and Sih 1998; Storfer 1999; Storfer et al. 1999; Sih et al. 2000; Garcia et al. 2003). That is, historical constraints also influence local adaptation of *A. barbouri* to predatory fish, but contemporary gene flow from fishless to fish-supporting localities also can swamp local adaptation by reducing the efficacy of antipredator behaviors and cryptic coloration (Storfer and Sih 1998; Storfer et al. 1999).

This study also suggests the possibility for hybridization between *A. texanum* and *A. barbouri* as a constraint on adaptive evolution. Preliminary molecular evidence suggests hybridization between *A. texanum* and *A. barbouri* because two mitochondrial haplotypes (SC-a, *A. barbouri* and GR-a, *A. texanum*; Fig. 1) are found in well-supported clades of the other species. *A. texanum* and *A. barbouri* are either sympatric or parapatric in these areas (Petranka 1982; Fig. 1), and *A. texanum* evolved in the absence of predatory fish (Sih et al. 1992). Hybridization has been suggested with previous work at site SC-a (Kraus and Petranka 1989). These results are further supported with morphological data showing melanophore counts and larval size at hatching are intermediate between *A. barbouri* and *A. texanum* at this site (J. H. Niedzwiecki, unpubl. data). Much as gene flow between populations with conflicting selection pressures in *A. barbouri* has likely constrained local adaptation to predatory fish (Storfer and Sih 1998; Storfer et al. 1999), gene flow between ecologically diverged species may cause fitness reductions in hybrids whereby intermediate phenotypes perform poorly (Mayr 1954; Templeton 1986; Coyne and Orr 2004).

A few aspects of our study warrant caveats. First, ancestral state reconstruction should be treated with caution because incomplete sampling of all major lineages within *A. barbouri* is likely, despite nearly complete geographic sampling (Fig. 1). Much as in interspecific comparative analyses, differences in lineage-longevity resulting from the possession of a particular trait may bias reconstructed ancestral states (Schluter 2000). Second, whereas this study investigated historical patterns under which adaptive trait values of contemporary populations are constrained, the current selective regime should also be considered. The

apparent nonrandom distribution of contemporary habitat-type (fishless vs. fish-bearing) along axes of antipredator performance indicates the importance of contemporary selection pressures (Figs. 3 and 4). If current selective agents were inconsequential to the efficacy of antipredator phenotypes, one would expect that data would not be separable by current habitat-type, as seems to be the case. A notable exception appears with measures of behavioral responses to simulated predation (Figs. 3D and 4D); some of the least responsive larvae were collected from fish-bearing localities. Third, the reliance on a single locus to estimate credible intraspecific phylogenies may result in inaccurate tree reconstruction. However, it seems quite unlikely that the use of additional genetic loci would be cause for major revision of our main results. That is, our Bayesian analyses incorporated an extent of uncertainty in use of our data to reconstruct gene trees and historical selection regimes. Nonetheless, we must be more tentative with regard to putative evidence of hybridization. Lineage sorting notoriously muddles the detection of hybridization through phylogenetic methods, especially given only a single locus (Moore 1995; Arnold 1997).

In addition to the above concerns, although we assume here that a set of phenotypic traits is statically optimal for larval streamside salamanders in the presence or absence of predatory fish, it is quite possible that these optima have shifted over the course of evolutionary contact between salamanders and fish (Roff 2000; Schluter 2000). However, the significant positive relationships between antipredator performance and measures of selection seem to indicate that trait optima have not markedly changed (Figs. 3 and 4). Although some traits could be under perpetual directional selection, developmental constraints, antagonistic pleiotropic effects, or genetic correlations likely provide limitations on the evolution of such traits (Futuyma 1998).

CONCLUSIONS

Indicated by two ecotypes, populations of the streamside salamander appear to be under divergent selection, and the adaptive evolution of the species is strongly influenced both by contemporary processes (e.g., gene flow; Storfer and Sih 1998; Storfer 1999; Storfer et al. 1999; Sih et al. 2000; Garcia et al. 2003), as well as historical constraints. A historically unstable niche represents an important constraint on the adaptive evolution of antipredator phenotypes among lineages of *A. barbouri* experiencing periodic fish introductions and losses (Figs. 3 and 4). That is, our study suggests consistency, as well as duration of selection imposed by predatory fish is important in shaping adaptive trait evolution in the streamside salamander. In these analyses, we provide new software as a general means of interpreting and using estimates of lineage-specific durations within a particular character-state (i.e., dwell times) from SIMMAP (provided in a Java application called “TREDWE” for interpreting the nexus-formatted output of

stochastically mapped character histories from SIMMAP; available at www.wsu/~storfer/eastman/software). This method may prove especially useful where dwell times are expected to bear heavily on the states or values of other traits.

Future work may test the relationship between historical patterns of selection and both behavioral and developmental traits associated with the fishless selective regime. Indeed, the rate of loss of fish is substantially larger than the estimate of transitions from fishless to fish-bearing larval habitat, suggesting that selection intensity on streamside salamander larvae may be somewhat weakened. If we are to assume disjointed adaptive peaks associated with each ecotype, historical selection-consistency and duration of the selective regime are expected to be equally important in the adaptive evolution of populations from fishless, but highly ephemeral, larval habitat.

ACKNOWLEDGMENTS

We thank N. Barton, J. Baumsteiger, J. Bollback, J. Cotter, J. Demastes, L. Harmon, J. Huelsenbeck, J. Kerby, V. Minin, B. O’Meara, S. Spear, T. Spradling, M. Takahashi, and several anonymous reviewers for helpful comments that greatly improved the quality of this manuscript. Funding for this research was provided by the National Science Foundation (DEB-0548415) to AS.

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Associate Editor: J. Huelsenbeck

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Figure S1. Overview of phylogenetic comparative methods used herein. See text for details.

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