Road to evolution? Local adaptation to road adjacency in an amphibian (*Ambystoma maculatum*)

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Natural history and site selection

The spotted salamander (*Ambystoma maculatum*) is widely distributed throughout eastern North America, with a range extending from southern Quebec to the southeastern United States [31]. Within the study region, spotted salamanders breed in late March or early April, when adults migrate from upland terrestrial habitat into ephemeral wetlands to reproduce. In this study region, females oviposit egg masses containing approximately 100 embryos per mass. Embryos develop over 8-10 weeks before hatching, and continue to develop as aquatic larvae throughout the summer until they metamorphose into terrestrial juveniles [32].

I used National Wetland Inventory Maps [33] and visual driving searches to identify roadside pools located less than 10 m from a paved road within the Yale Myers Forest region (Fig. 2 inset). In spring 2009, I selected for experiment the five roadside pools believed to be most influenced by runoff as indicated by specific conductance values. I then assigned to each roadside pool a woodland pool, located at least 200 m from the nearest paved road, and thereby yielding five unique pool pairs. To control for confounding variation, I selected woodland pools that minimized inter-pair distance, while at the same time maximized similarity in pool size, forest canopy cover, and emergent vegetation. Reciprocal transplants were conducted within each of these five pairs (Fig. 2).

Reciprocal transplant experiment

In the days leading up to breeding (signaled by the appearance of conspecific egg masses in like pools located in central and southern Connecticut), I monitored pools daily for the arrival of new egg masses. From each pool, I collected a subset of embryos from egg masses less than 48 hours old, with a target of 10 egg masses per pool. I selected egg masses that were spatial distributed (if possible) [34] and conspicuously large and distinct so as to avoid sampling masses that might have originated from the same female. From each egg mass, I carefully dissected out two clusters of 10 embryos. One cluster was stocked into one of five experimental enclosures in the origin pool while the other was relocated (suspended in a small enclosure containing pool water, and incubated within an iced cooler) to the transplant pool and also assigned to one of five experimental enclosures. Each pool contained five experimental blocks, with each block containing one local enclosure and one transplanted enclosure. I targeted stocking 10 embryos from each of two egg masses per enclosure, yielding one unique pairing of egg masses (hereafter "clutch pair") that was replicated across, but not within pool pairs. This design was chosen to maximize family level diversity while maintaining an additional level of replication at the clutch pair level, while at the same time balancing logistics of resources and spatial constraints within pools. Enclosure assignment was haphazard for each clutch pair. Because two woodland pools presented fewer than 10 oviposited clutches ($n = 6$, $n = 8$ respectively), a total of 88 enclosures each were stocked with 20 embryos (from 44 unique clutch pairs) while 12 enclosures each were stocked with 10 embryos (from 6 unique clutch pairs). Thus, across 10 pools, I stocked a total of 100 enclosures, 88 of which contained 20 embryos each, while 12 contained 10 embryos each.

Each enclosure consisted of a 14 l plastic container containing six 7 cm diameter ventilation holes on the sides and a 25 x 13 cm hole in the lid. I screened over the side ventilation holes with noseum mesh (ca. 97 holes per cm^2) affixed with Gorilla Glue around the edges, thus creating a barrier to aquatic predators, yet facilitating water flow. Mesh was also affixed to the top

ventilation hole using Gorilla Glue and Duct Tape. The inside of each enclosure was fitted with a piece of hardware cloth that acted as cradle to support egg clusters off the bottom of the enclosure. Two pieces of closed cell blue foam (ca. 2.5 x 5 x 36 X cm) were secured to the long sides of the enclosures with Silicone II and stainless steel screws. This provided flotation such that embryos were submerged but suspended at a height comparable to that of naturally oviposited egg masses.

Size and developmental stage of eggs and hatchlings

Immediately after stocking eggs into field enclosures, I placed egg masses on ice and returned them to the laboratory in New Haven, CT. There, I dissected approximately five eggs from each egg mass and placed them in a glass petri dish in which photographs were captured with a digital camera attached to a dissecting stereomicroscope. Vitelline membranes surrounding embryos were left in place. I used ImageJ software [35] to estimate the two-dimensional surface area of each embryo represented by a best-fit ellipse. I also estimated the developmental stage of each egg mass from this sample [36]. At the end of the experiment, I set aside a subset of surviving hatchlings from each enclosure (i.e. clutch pair) for a separate experiment and analysis. From the remaining survivors, I then preserved a target of three, haphazardly selected individuals. For each of these individuals, I used a dissecting stereomicroscope to assess snout-vent length (SVL) and developmental stage [37].

Characterizing roadside and woodland environments

In each pool, I measured seven environmental characteristics associated with amphibian distribution and performance [29]. Specific conductance, dissolved oxygen, pH, and wetland depth were measured twice (20 April and 22 May 2009) during the experiment, while temperature was measured every thirty minutes using deployed temperature loggers. I also collected water samples to assay the concentration of chloride ions using liquid chromatography. All water parameter measurements were taken 10 cm below the surface at the location of the deepest point in each pool. In roadside pools, specific conductance was also measured at the base of the water column because a strong halocline spans the water column. I therefore chose to analyze and report specific conductivity as the mean value taken from the top and bottom of each roadside pool. To estimate the influence of forest canopy cover, I captured leaf-off hemispherical photographs (on 1 April 2011) at each of five locations per pool (2 m from shore at each of the four cardinal compass points plus the approximate center of pool). I used HemiView software [38] to estimate global site factor (GSF)—a measure of solar radiation reaching the water surface see [39]. GSF was calculated for each of two dates spanning the experiment (10 Apr; 16, May). From the overall temperature dataset, I calculated average temperature over each of two time periods, 06 – 28 April 2009, and 29 April – 20 May 2009.

Statistical analyses for reciprocal transplant responses

All statistical analyses were conducted in R V. 2.13 [40]. I used the *MCMCglmm* package [41] to analyze survival as a bivariate response of successes and failures, characterized by a binomial distribution (using the "multinomial2" family). I used weak, non-informative, parameter expanded priors. To analyze growth and developmental rates, I used the R package *lme4* [42]. While *lme4* utilizes a frequentist framework, MCMC methods provides a more conservative method of inference due to the uncertainty associated with estimating degrees of freedom in hierarchical models. I therefore evaluated *lme*4 models using the function *pvals.fnc* in the

languageR package [43], which uses MCMC methods to compute p-values and confidence intervals. Growth and developmental rates were specified as exponential functions based on initial and final values of size and developmental stage, respectively, in relation to the number of days elapsed from stocking until the end of the experiment (e.g. [ln(final size)-ln(initial size)]/period). Initial size was defined as embryo diameter calculated from estimates of embryo area, while final size comprised hatchling snout-vent length (SVL). Developmental stages were assayed for embryos and hatchlings with two different keys because no single key provides continuous classification from egg to metamorphosis: The Harrison key [44] describes 46 developmental stages from a single cell embryo to development of the fourth finger bud while the Watson and Russell key [37] decribes 22 stages from development of the forelimb bud to metamporphosis, overlapping the final 10 stages of the Harrison key. To provide a continuous measure of developmental rate, I adjusted final developmental stage of hatchlings by 36—the number of stages spanning fertilization to the appearance of the forelimb bud, as described by the Harrison key [44]. Collectively, this approach provided a continuous developmental staging system from ovum to metamorphosis.

In all models, I specified deme and the experimental grow-out environment, along with their interaction (i.e. G x E), as fixed effects. A complimentary set of models was evaluated with egg size as a covariate. In the most parameterized model, the following random effects were included: pool pair, clutch pair, and experimental block. In the least parameterized model, only pool pair was included. I evaluated model fit for each combination of random effects (Table S1) and selected for inference the model with the lowest DIC or AIC values (for bivariate and univariate models, respectively). Models were run for 3,000,000 iterations, with a burnin period of 300,000, and a thinning interval of 500. Model performance was evaluated by examining traces of the posterior distributions of the location effects and covariance matrices. Models in which the interaction effect was found not to be significant were refit with only main effects (i.e. G + E), and the random effect structure was re-evaluated using the same procedure as above Table S2). Developmental and growth rate response variables were averaged by enclosure, while survival was measured as the number of successes and failures per enclosure.

Because of low or zero survivorship in roadside pools, only 79 out of the 100 enclosures yielded surviving hatchlings that were preserved for estimating developmental and growth rates. Of these, 46 enclosures were from woodland pools, while 33 were from roadside pools. These data well represent the growth and developmental rates of roadside and woodland demes across the G x E interaction. However, in this case, they are poorly suited to inclusion with survival for a multivariate analysis. This is because enclosures that had low or zero survival had no corresponding measure of growth or developmental rates. Such enclosures were most frequently those housing the woodland deme within a roadside pool, thus skewing the data. I therefore chose not to conduct a multivariate analysis of the combined response of growth rate, developmental rate, and survival.

Statistical analyses for environmental characteristics

I used *MCMCglmm* to conduct multivariate analysis of variance (MANOVA) to test whether the seven environmental variables differed with respect to environment. I specified environment (i.e. E) as the fixed effect and individual pool as the random effect. I log-transformed specific conductance to improve normality of the multivariate distribution. No pairs of variables

exhibited strong correlation (correlation < 0.53 among all pairwise comparisons), and so all variables were retained in the analysis. Following a significant multivariate response (posterior mean = -1.45; 95% CI = -1.70 – -1.17; $P_{mcmc} < 0.001$), I used *lme4* to conduct univariate mixed model analyses of the effect of environment (fixed effect) on each response variable. I included both pool and survey date as random effects. Inclusion of both random effect terms was supported by AIC evaluation in each model.

Table S1. Model selection results. A set of candidate models were composed for the analysis of each response variable. The model with the lowest Deviance Information Criterion (DIC) or Akaike Information Criterion (AIC) was chosen for inference. DIC was utilized in the models for survival because they were composed in a Bayesian framework, whereas AIC was utilized in models for growth and developmental rates, which were composed in a frequentist framework. There was a significant G x E interaction in the model for survival, but not in the models for growth rate or developmental rate. Thus, the latter two models were refit and reported only with main effects (i.e. G + E).

Response variable ~ fixed effects	***Random effects***	***DIC† or AIC***	
		Without egg size covariate	With egg size covariate
Survival ~ G x E	pair+clutch+block	1407.219†	1407.638†
	pair+clutch	1411.810†	1411.778†
	pair+block	1407.696†	1408.24†
	pair	1411.571†	1411.692†
Growth rate ~ G + E	pair+clutch+block	-160.3	-153.0
	pair+clutch	-158.0	-151.1
	pair+block	-162.3	-155.0
	pair	-160.0	-153.1
Developmental rate ~ G + E	pair+clutch+block	-557.9	-544.6
	pair+clutch	-538.5	-523.3
	pair+block	-545.3	-530.1
	pair	-540.5	-525.3

Table S2. Parameter estimates, confidence intervals and p-values of models selected for inference. All models were composed with and without egg size as a covariate.

Model	*Parameters*	*Estimate*	*Lower CI*	*Upper CI*	*Pmcmc*
		Without egg size covariate / With egg size covariate			
Survival ~ G x E					
	Intercept	0.905 / 0.061	-1.252 / -3.022	3.030 / 3.225	0.296 / 0.954
	Deme	-1.070 / -1.201	-1.883 / -2.036	-0.321 / -0.338	0.008 / 0.004
	Environment	1.473 / 1.470	0.472 / 0.452	2.444 / 2.460	0.002 / 0.004
	G x E	1.116 / 1.134	0.131 / 0.104	2.102 / 2.127	0.028 / 0.036
	Egg size	NA / 0.128	NA / -0.236	NA / 0.475	NA / 0.471
Developmental rate ~ G + E					
	Intercept	0.080 / 0.081	0.077 / 0.074	0.083 / 0.088	< 0.001 / < 0.001
	Deme	-0.002 / -0.002	-0.004 / -0.004	0.000 / 0.001	0.107 / 0.093
	Environment	0.004 / 0.004	0.002 / 0.002	0.006 / 0.006	< 0.001 / < 0.001
	Egg size	NA / 0.000	NA / -0.001	NA / 0.001	NA / 0.891
Growth rate ~ G + E					
	Intercept	2.099 / 2.013	2.043 / 1.898	2.157 / 2.130	< 0.001 / < 0.001
	Deme	0.009 / -0.002	-0.022 / -0.035	0.042 / 0.032	0.507 / 0.917
	Environment	-0.007 / -0.008	-0.037 / -0.039	-0.024 / 0.021	0.642 / 0.580
	Egg size	NA / 0.014	NA / -0.002	NA / 0.031	NA / 0.081

Table S3. Model outputs showing contrasted effects of G x E interaction on survival. The model was composed with the original interaction effect of G x E reformulated as one main effect (referred to as G x E†) comprising four levels: (1) Roadside deme grown out in roadside environment (R in R); (2) roadside deme grown out in woodland environment (R in W); (3) woodland deme grown out in woodland environment (W in W); and (4) woodland deme grown out in roadside environment (W in R). The initial model for contrasts specified W in W as the reference level. In order to compute all contrasts, the model was run a second time with R in R as the reference level. Like parameters vary slightly between models because estimates were generated in a randomization framework.

Model	***Parameters***	***Estimate***	***Lower CI***	***Upper CI***	***Pmcmc***
Survival ~ G x E† (ref. level = W in W)		Without egg size covariate / With egg size covariate			
	Intercept	2.415 / 1.500	0.163 / -1.981	4.581 / 4.664	0.043 / 0.352
	R in R	-1.526 / -1.409	-2.574 / -2.540	-0.475 / -0.283	0.004 / 0.017
	R in W	-0.046 / 0.066	-0.810 / -0.797	0.774 / 0.927	0.889 / 0.892
	W in R	-2.593 / -2.602	-3.575 / -3.620	-1.593 / -1.683	< 0.001 / < 0.001
	Egg size	NA / 0.123	NA / -0.244	NA / 0.478	NA / 0.494
Survival ~ G x E† (ref. level = R in R)					
	Intercept	0.866 / 0.110	-1.255 / -3.070	3.056 / 3.255	0.319 / 0.931
	R in W	1.475 / 1.478	0.478 / 0.544	2.455 / 2.479	0.004 / 0.004
	W in R	-1.065 / -1.184	-1.849 / -2.056	-0.280 / -0.353	0.007 / 0.007
	W in W	1.526 / 1.406	0.528 / 0.369	2.635 / 2.526	0.004 / 0.010
	Egg size	NA / 0.125	NA / -0.224	NA / 0.516	NA / 0.489