

STRONG SELECTION BARRIERS EXPLAIN MICROGEOGRAPHIC ADAPTATION IN WILD SALAMANDER POPULATIONS

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Microgeographic adaptation occurs when populations evolve divergent fitness advantages across the spatial scales at which focal organisms regularly disperse. Although an increasing number of studies find evidence for microgeographic adaptation, the underlying causes often remain unknown. Adaptive divergence requires some combination of limited gene flow and strong divergent natural selection among populations. In this study, we estimated the relative influence of selection, gene flow, and the spatial arrangement of populations in shaping patterns of adaptive divergence in natural populations of the spotted salamander (*Ambystoma maculatum*). Within the study region, *A. maculatum* co-occur with the predatory marbled salamander (*Ambystoma opacum*) in some ponds, and past studies have established a link between predation risk and adaptive trait variation in *A. maculatum*. Using 14 microsatellite loci, we found a significant pattern of genetic divergence among *A. maculatum* populations corresponding to levels of *A. opacum* predation risk. Additionally, *A. maculatum* foraging rate was strongly associated with predation risk, genetic divergence, and the spatial relationship of ponds on the landscape. Our results indicate the sorting of adaptive genotypes by selection regime and strongly suggest that substantial selective barriers operate against gene flow. This outcome suggests that microgeographic adaptation in *A. maculatum* is possible because strong antagonistic selection quickly eliminates maladapted phenotypes despite ongoing and substantial immigration. Increasing evidence for microgeographic adaptation suggests a strong role for selective barriers in counteracting the homogenizing influence of gene flow.

KEY WORDS: Adaptive divergence, *Ambystoma maculatum*, *Ambystoma opacum*, evolution, gene flow, migration, natural selection, predator–prey interactions.

Natural populations can face a wide range of environmental conditions, even across short distances on the landscape. This environmental heterogeneity can induce natural selection for different local trait optima in each habitat. Local adaptation occurs when populations evolve traits that confer higher fitness in the local environment than in foreign environments, regardless of distance (Kawecki and Ebert 2004) and has been observed in many taxa and

across a wide range of spatial scales (Hereford 2009). The ability for populations to diverge adaptively in response to local environmental conditions depends on both the strength of selection within a habitat and the level of gene flow between populations occupying dissimilar habitats (Wright 1969; Lenormand 2002; Garant et al. 2007). Whereas gene flow can increase the genetic variation within the recipient population, high levels of gene flow



can also impede adaptive divergence by introducing maladaptive phenotypes (King and Lawson 1995; Storfer et al. 1999; Hendry et al. 2002; Rosenblum 2006; Bolnick and Nosil 2007).

Even though gene flow can overwhelm local adaptation, an increasing number of examples suggest that adaptive divergence can occur over the spatial scales at which focal organisms disperse. So-called microgeographic adaptations have now been documented across a range of systems (Jain and Bradshaw 1966; Selander 1970; De Meester et al. 2002; Hargeby et al. 2004; Skelly 2004; Kavanagh et al. 2010). These microgeographic adaptations suggest either very strong selection that counteracts gene flow, or much lower gene flow than expected based on dispersal ability. Gene flow can be lower than expected if landscape barriers impede movement, if individuals preferentially select certain habitats, or if selective barriers prevent maladapted individuals from colonizing and reproducing (Ehrlich and Raven 1969; Sork et al. 1999; Nosil et al. 2005; Edelaar et al. 2008). The position of habitats on the landscape can also influence patterns of adaptive divergence, as the spatial arrangement of populations determines the level of gene flow and the relative similarity of selection regimes that exchange genes if environmental variation is spatially correlated (Epperson and Li 1996; Stone et al. 2011; Urban 2011). Therefore, it is critical to look at natural selection, gene flow, and the spatial context of populations concurrently to gain a more complete understanding of the mechanisms underlying microgeographic adaptation in nature.

Understanding the genetic mechanisms underlying ecological divergence requires knowing which genes are under selection within a habitat. However, the genes of interest are rarely identified for most species, and therefore studies often use neutral genetic markers to estimate gene flow (Gaggiotti et al. 1999; Thibert-Plante and Hendry 2010). Whereas genetic loci under selection will indicate divergent selection concurrent with adaptive divergence, neutral loci should only show a signal of divergence once impediments to gene flow have arisen (e.g., selection against migrants; Nosil et al. 2005). Past studies have demonstrated that neutral genetic divergence can accompany relatively rapid ecological divergence among populations, especially when selection is strong (Senar et al. 2006; de Leon et al. 2010; Thibert-Plante and Hendry 2010; Funk et al. 2011; De Luna et al. 2012).

In the present study, we explore the roles of selection, gene flow, and the spatial arrangement of ponds on patterns of microgeographic adaptation in natural populations of the spotted salamander (*Ambystoma maculatum*). In the study area, *A. maculatum* co-occur with a predatory salamander species (the marbled salamander—*Ambystoma opacum*) in some, but not all temporary ponds. Larvae of the fall-breeding *A. opacum* grow to a size that allows them to prey intensively on larvae of the spring-breeding *A. maculatum* after egg hatching (Stenhouse 1985; Petranka 1998). However, this relationship shifts during the developmental pe-

riod as *A. maculatum* larvae increase in size and the gape-limited *A. opacum* eventually becomes physically unable to consume *A. maculatum*. Previous common environment experiments suggest that *A. maculatum* populations have adapted more intense foraging behavior when they face high *A. opacum* predation risk in their natal habitat (Urban 2007a). Demographic models indicate that this increased foraging activity should allow larvae to grow into a size refuge earlier from the strongly gape-limited *A. opacum* (Urban 2007b).

We estimated the relative contributions of antagonistic selection, gene flow (as indicated by neutral genetic differentiation), and the spatial arrangement of populations on the landscape to patterns of three genetically determined traits in *A. maculatum* that are under selection from *A. opacum* predation (Urban 2010). We used long-term field surveys to estimate differences in predator-induced natural selection among populations and data from common environment experiments to estimate the trait variation among populations attributed to genetic effects. We also assessed genetic divergence across all populations using 14 microsatellite loci and applied discriminant analyses to the genotypic data. We used spatially explicit analyses and general linear models to identify the factors that were significantly associated with trait variation across the focal populations. The life history of the focal species and the distribution of populations over a small area allowed us to assess the relative importance of the effects of selection, gene flow, and landscape context on microgeographic adaptation in spotted salamander populations.

Methods

STUDY AREA AND NATURAL HISTORY

The focal populations of *A. maculatum* inhabit 11 temporary ponds along the isolated Northford Ridge in southern Connecticut (study area = 2 km²; Fig. 1). These ponds are located within a large protected area dominated by broadleaf deciduous and mixed secondary forest. This pond complex is created by relatively impervious basalt bedrock and includes ponds of varying size and hydroperiod. Focal ponds are separated by a mean minimum distance of 116 m (Fig. 1).

Ambystoma maculatum is a widely distributed pond-breeding amphibian species in eastern North America. Spending most of their life in the surrounding terrestrial habitat, adults return to temporary ponds each spring to mate and lay eggs. Small aquatic larvae emerge from the egg mass around eight weeks after breeding and develop rapidly to metamorphosis within four months. Their initial small size makes *A. maculatum* larvae vulnerable to predation by *A. opacum* larvae, a fall-breeding species that serves as a voracious, yet gape-limited predator. Whereas all of the focal ponds support *A. maculatum* populations, heterogeneity

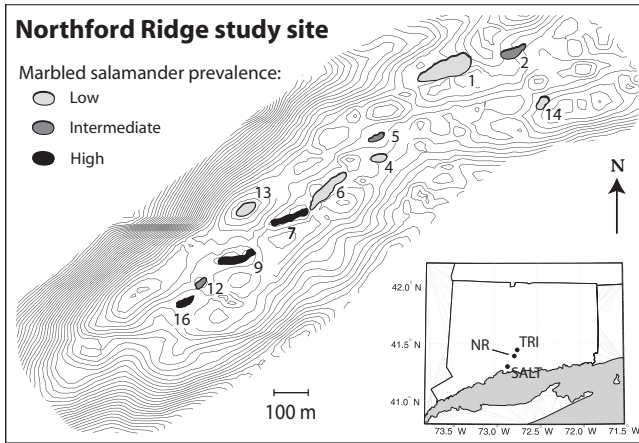


Figure 1. Topographic map (2 m contour intervals) of the Northford Ridge study site. The network of ponds is displayed with pond shading representing marbled salamander predation risk (i.e., *Ambystoma opacum* prevalence across eight years). These same predation risk categories are used to assign individuals in Figure 2. The inset map shows the outlying populations used to assess genetic divergence within the context of regional gene flow (SALT, Saltonstall; TRI, Trimountain).

in pond habitat leads to variation in the presence and density of *A. opacum* among ponds. *A. opacum* larval survival depends, in part, on freezing depth during the winter. For this reason, *A. opacum* is found consistently in some ponds and at high densities, and infrequently in others and at low densities (Fig. 1).

FIELD SURVEYS

We conducted area-constrained sampling over eight years to estimate the occurrence of larval *A. opacum* within each pond across years. Surveys used standardized dip-net sweeps across the entire pond basin, with sampling effort proportional to pond size (Skelly and Richardson 2010). We also estimated pond permanence (i.e., hydroperiod) across three years as the mean number of days from the first *A. maculatum* breeding activity until the last day water was observed in the pond. Hydroperiod determines the duration of the larval stage and imparts a strong selection pressure on amphibian populations breeding in temporary ponds (Ryan and Winne 2001; Loman 2002; Karraker and Gibbs 2009).

A. opacum prevalence during this time frame varies from 38% to 100% among ponds. We use *A. opacum* prevalence to estimate selection rather than other measures such as *A. opacum* density because we have better long-term data on occurrence and a more limited dataset on *A. opacum* density. However, the long-term prevalence dataset correlates well with sparser data on mean density ($\rho = 0.69$). *A. opacum* prevalence is not significantly correlated with important pond characteristics, including pond size, canopy cover, temperature, or the prevalence of other important predatory species, including beetle larvae (*Dytiscus verticalis*) and

newts (*Notophthalmus viridescens*). However, *A. opacum* prevalence is explained by winter pond temperatures (Herstoff and Urban, in review), as *A. opacum* cannot overwinter in ponds that freeze completely in winter.

TRAIT VARIATION AMONG POPULATIONS

Previous common garden experiments suggest that *A. maculatum* populations exhibit genetically determined phenotypic differences that vary depending on a population's co-occurrence with *A. opacum* (Urban 2007a). In these experiments, we removed eggs from natural ponds soon after being laid and raised them in a common environment. Several traits in *A. maculatum* are known to be inducible by *A. opacum* cues (Urban 2008). Therefore, we added cues from *A. opacum* to all containers to look at differences in induced foraging responses among populations.

We analyzed three *A. maculatum* traits that were determined to be under selection by *A. opacum* predation. Assays of *A. maculatum* survival during *A. opacum* predation trials reveal selection for *A. maculatum* larvae that are large and have larger tail fins (Urban 2010). In addition, rapid foraging is thought to be under selection because of strong gape-limited predation by *A. opacum* (Urban 2007a, 2007b). In this study, we analyze previously published data from a common environment experiment that evaluated foraging, growth, and morphological trait variation among the 11 populations (Urban 2007a, 2010). Prior work evaluated this trait data in relation to selection, but not in relation to more complex spatial gradients or neutral genetic variation. Correlations between adaptive and neutral genetic variation can suggest the mechanisms that shape evolutionary dynamics in a natural system (Leinonen et al. 2008; Nosil et al. 2009; Agudo et al. 2012). Because trait data were not available for one focal population (NR 4), only genetic analyses were conducted with this population.

Five *A. maculatum* egg masses were collected from each study population within 48 h of being deposited by females to minimize environmental effects. Eggs were kept in 19-L plastic containers within an outdoor enclosure. Five larvae were haphazardly chosen from each family group (i.e., egg mass) after hatching and housed individually in 1-L plastic experimental containers. Each population was represented by 25 individuals, for a total of 245 experimental individuals; only four masses were collected from one of the focal ponds (NR5). Experimental containers were housed in a temperature-controlled room ($14.3^{\circ}\text{C} \pm 0.5^{\circ}$ SD) with temperatures and photoperiod corresponding to natural spring conditions in this region. Containers were moved every two days to minimize any influence of spatial location within the room. Every two days, experimental individuals were fed equal numbers of size-matched *Daphnia magna*. To estimate the response to predation risk, 25 mL of water was added to enclosures every other day that had been conditioned by *A. opacum* larvae

feeding on larval *A. maculatum* in 68-L bins and filtered using 70- μm Nitex mesh to prevent additional food provisioning.

Surveys of feeding behavior were conducted weekly, including one day and one night observation. The feeding activity of each individual larva was recorded 30–60 times during each observation period. Feeding attempts were defined as a rapid forward lunging movement accompanied by mouth suction. Mean foraging rate was obtained by dividing the number of feeding attempts by the total time of observation. The same observer recorded a total of 320 observations for each individual across 32 survey periods. The experiment was ended after five weeks, the time at which *A. opacum* risk decreases in natural populations as *A. maculatum* reach a size refuge (Urban 2007a). Survival of *A. maculatum* larvae was high throughout the experiment (99.6%).

In addition to foraging rate, we also measured body size and relative tail fin size at the end of the experiment—two morphological traits linked to performance differences in previous larval amphibian research (Fitzpatrick et al. 2003; Van Buskirk et al. 2003; Storfer and White 2004). Body size was measured as the square root of the sum of squared distances among 28 morphological landmarks and their centroid (Bookstein 1991; Urban 2010). Tail fin size was measured as the area of the parabola bounded by a rectangle created with tail fin depth and length as the major axes (Fitzpatrick et al. 2003) and corrected for overall body size by using the residuals after regression on total body size. We did not detect any significant remaining nonlinearity or correlations between size-corrected tail fin area and centroid size, and we found no evidence that populations differed from a common allometric relationship (Urban 2010).

For studies investigating adaptation, the general recommendation is to raise individuals under laboratory conditions for multiple generations. Females of the spotted salamander do not reach maturity for three years or later (Petranka 1998), which makes raising successive laboratory generations impractical in the short term. However, we can exclude some of the most common origins of maternal effects. The most common maternal effects are offspring habitat, postnatal care, and differential provisioning of offspring (Lynch and Walsh 1998; Mousseau and Fox 1998). We removed eggs from breeding sites soon after laying to limit any effect of habitat choice, and spotted salamanders do not provide parental care for their offspring. Egg size is another common avenue through which females can alter offspring traits, especially in amphibians (Kaplan 1998). In previous research, *A. maculatum* egg size did not vary significantly among study ponds or in relation to marbled salamander predation risk (Urban 2007a). We measured these same traits in a recent experiment, and mean traits correlated strongly with those measured in the earlier experiment. The previous experiment was conducted six years earlier, suggesting the long-term stasis of population trait differences over two to three generations (M. Urban, unpubl. data). Importantly, the same

population trait means were observed even in ponds that had been colonized by *A. opacum* in the meantime, suggesting that maternal effects related to a mother's assessment of predation risk also do not influence trait variation. These pieces of evidence indicate that measured trait differences among populations are, in large part, genetically determined.

GENETIC VARIATION AMONG POPULATIONS

We collected genetic samples from each of the Northford Ridge populations in 2009, and supplementary samples were collected in 2011 for populations with low larval survival from the original sample. A single egg was removed from each *A. maculatum* egg mass present within a pond (up to 50 eggs per pond), and eggs were reared to hatching in the laboratory. Female spotted salamanders often deposit a large primary egg mass and a smaller satellite mass (Petranka 1998). We did not collect eggs from satellite masses to avoid duplicate sampling within sibling groups. We also collected samples from two outlying populations to compare genetic signals among the suite of experimental ponds to baseline genetic differentiation that exists between ponds unlikely to share direct gene flow. The two outlying populations are Saltonstall (SALT—12.1 km south of the focal area centroid) and Trimountain (TRI—4.5 km north; Fig. 1).

Genomic DNA was extracted from the tail fin tissue of each sampled individual using the Qiagen DNeasy extraction kit. DNA was used in polymerase chain reactions (PCRs) where protocols differed among primers (Table S1). We amplified 10 tetra- and four dinucleotide repeat microsatellite loci. PCR products were identified using capillary electrophoresis on an ABI 3730 DNA Analyzer. GENEMAPPER v3.7 was used to score alleles and MICROSATELLITE TOOLKIT v3.1 (Park 2001) was used to check for scoring errors. In total, 326 individuals were genotyped and analyzed with a mean of 25 individuals per pond.

Deviations from Hardy–Weinberg equilibrium for each locus were estimated in FSTAT v2.9.3 (Goudet 1995), with significance assessed using 10,000 randomizations. The degree of linkage disequilibrium among loci was also assessed using FSTAT. Null allele frequencies were obtained using MICRO-CHECKER v2.2.3 (van Oosterhout et al. 2004) and loci exhibiting consistent null allele frequencies greater than 0.15 were excluded from later analyses (Chapuis and Estoup 2007). Genetic differentiation was estimated using three indices: standard F_{ST} (Weir and Cockerham 1984), G'_{ST} standardized based on heterozygosity levels (Hedrick 2005), and Jost's D measure of relative differentiation based on allelic diversity (Jost 2008). F_{ST} was calculated in FSTAT and SMOGD was used to calculate G'_{ST} and Jost's D (Crawford 2010). Each estimate of genetic divergence has its advantages (Heller and Siegmund 2009; Meirmans and Hedrick 2011; Whitlock 2011), so we performed analyses with each. From this point forward we will refer to estimates of genetic differentiation as a measure of gene

flow; however, we note that we do not measure gene flow directly but rather rely on an indirect measure (Wright 1931; Whitlock and McCauley 1999).

Analyses based on microsatellite markers assume that loci are not affected by natural selection. We verified that none of our loci showed evidence of nonneutral behavior using the *F_{dist}* approach implemented in *LOSITAN* (Beaumont and Nichols 1996; Antao et al. 2008). This method is based on the assumption that genetic differentiation between populations experiencing divergent selection pressures should be higher for loci under selection than for the rest of the genome. Using genotypic data, the program creates a null distribution of *F_{ST}* values and identifies loci with higher or lower pairwise *F_{ST}* values (than predicted by the null distribution) as being candidate loci for selection. We performed the analysis with 25,000 simulations, a false discovery rate of 0.10, and both the stepwise mutation and infinite alleles models.

We used discriminant analysis of principal components (DAPC) to assess the level of neutral genetic divergence between populations. DAPC provides a description of the genetic clustering using coefficients of the alleles (loadings) in linear combinations that produce the largest between-group and smallest within-group variances in these loadings (Jombart et al. 2010). We also incorporated a *k*-means clustering component to estimate the most supported number of distinct genetic groups based on Bayesian Information Criterion (BIC). Simulation and empirical studies indicate that DAPC performs as well or better than other individual-based clustering methods (e.g., *STRUCTURE*), particularly when more complex structuring processes are operating (Jombart et al. 2010; Klaassen et al. 2012). We conducted DAPC analyses on (1) all 13 populations sampled, including the 11 Northford Ridge focal populations and two outlying populations, and (2) only the Northford focal populations. DAPC and *k*-means clustering were conducted using guidelines in the *adegenet* package in R 2.14 (R Development Core Team 2011; Jombart and Ahmed 2012).

To test whether genetic clustering of individuals was associated with differences in predator selection among populations, we included *A. opacum* prevalence as a pond-level variable in further DAPC analyses. We categorized ponds based on *A. opacum* prevalence over eight years, including high-risk ponds where *A. opacum* is present every year (incidence = 1.0), intermediate risk (incidence >0.65 but <1.0), and low risk ponds (<0.65). These thresholds created groups with similar numbers of members and corresponded to our general understanding of predation risk in these study ponds. DAPC was performed on these predation risk categories, and the segregation of the three groups in discriminant function space was assessed using the Wilks' λ metric from multivariate analysis of variance (MANOVA) within the *vegan* package of R (Oksanen et al. 2012). To ensure that the MANOVA

results were not an artifact of forced clustering of groups by the DAPC, we also randomized the dataset by shuffling individuals among predation risk classes and performing MANOVA on the randomized data. We repeated this 10,000 times to obtain a permutational MANOVA distribution of Wilks' λ . Significance was assessed using the permutational *P*-value, representing the proportion of the 10,000 randomizations where the empirical Wilks' λ was greater than the λ calculated from randomized data.

STATISTICAL ANALYSES OF TRAIT VARIATION

To assess the relative influence of selection, genetic differentiation, and spatial orientation of ponds on phenotypic trait differences among populations, we performed a series of multivariate redundancy analyses (RDA) followed by permutational analyses of variance (ANOVAs) within the *vegan* R package. RDA is an extension of multiple regression allowing several response variables to be assessed together. We started with a full model incorporating all explanatory variables (spatial, genetic, and predator selection data—see below). To reduce the full model to only those predictor variables significantly associated with phenotypic trait variation, we iteratively removed nonsignificant terms one at a time and retested the model at each step (Crawley 2007). Mean trait values for each population were used in analyses.

Before the RDA analyses, Moran's Eigenvector Mapping (MEM) was performed on the spatial data (i.e., position of ponds on the landscape) to determine the spatial relationship and levels of autocorrelation among ponds. MEM uses Moran's *I* and eigenfunction decomposition of spatial distance matrices to evaluate spatial relationships on multiple scales (Dray et al. 2006; Borcard et al. 2011). Moran's *I* is a direct analogue of Malecot's estimator of spatial correlations of gene frequencies (Malecot 1955; Epperson 2005). Multiple studies demonstrate that Moran's *I* describes accurately how gene flow influences neutral gene frequencies across space (Sokal and Oden 1978; Epperson and Li 1996; Hardy and Vekemans 1999; Epperson 2005). However, Moran's *I* can only be calculated for one spatial class at a time, which makes it difficult to use in a predictive statistical framework. MEM solves this problem by producing orthogonal eigenvectors that maximize Moran's *I* across multiple spatial scales at once which can then be incorporated directly into models of interest as spatial predictor variables. These new variables are used to estimate the variation in the response variable due entirely to spatial autocorrelation (Munoz 2009; Peres-Neto and Legendre 2010). MEM was also performed on the genetic data (pairwise *F_{ST}*, *G'_{ST}*, and Jost's *D*). Ultimately, MEM summarizes the matrix-based distance data in several new vector-based variables that can be directly incorporated into subsequent analyses (RDA in this study). In choosing which MEM variables to retain for the full model, we used the iterative procedure described above (Crawley 2007).

Once we established the most parsimonious full multivariate model, we then conducted a series of univariate multiple regression analyses using *glm* in R to determine to what degree each phenotypic trait (foraging rate, body size, and tail fin area) was associated with each explanatory variable. We then used variation partitioning within *vegan* to estimate the degree to which each explanatory variable influenced the phenotypic trait variation among populations (Peres-Neto et al. 2006). Similar to partial canonical ordination (e.g., partial RDA), variation partitioning quantifies the variance explained by each predictor variable while controlling for the effect of all other variables (Borcard et al. 1992; Peres-Neto et al. 2006).

Results

GENETIC DIFFERENCES AMONG POPULATIONS

We found no evidence of linkage disequilibrium among loci. Only one locus exhibited any level of departure from Hardy–Weinberg equilibrium, however, this deviation was limited to a single population. A single locus also exceeded the threshold null allele frequency ($v = 0.15$), but only for three of 13 populations. Additionally, none of our microsatellite loci showed any evidence of being under selection, so all 14 loci were used in subsequent analyses. F_{ST} , G'_{ST} , and Jost's D all produced similar results, and so only results using F_{ST} are presented below (see also Table S2).

At the regional scale, we found a high degree of genetic admixture among the Northford Ridge focal populations, which were clustered together relative to the two outlying populations (Fig. 2A). However, when the Northford populations were divided into three groups based on *A. opacum* risk, we found significant neutral genetic differentiation among these selective environments ($\lambda = 0.689$, $P < 0.001$; Fig. 2B). Results from the permutational MANOVA showed a significant difference between the empirical and 10,000 randomized datasets ($P = 0.0001$), further indicating that this pattern is not an artifact of the DAPC clustering algorithm. *k*-means clustering based on BIC also suggested three distinct groups, indicating that our choice of three predation categories had support using both genetic and natural history data. We also investigated if alternative categorizations of ponds by predation risk affected our conclusions (e.g., four or two categories rather than three) and found that the pattern of significant genetic differentiation by predation risk did not depend on how we defined these categories.

TRAIT DIFFERENCES AMONG POPULATIONS

Phenotypic divergence among Northford populations was strongly linked with selection pressure, genetic variation, and space. A single spatial MEM vector explained the trait data and was retained for the full model development. Four genetic MEM vectors were significantly linked with the trait data and retained.

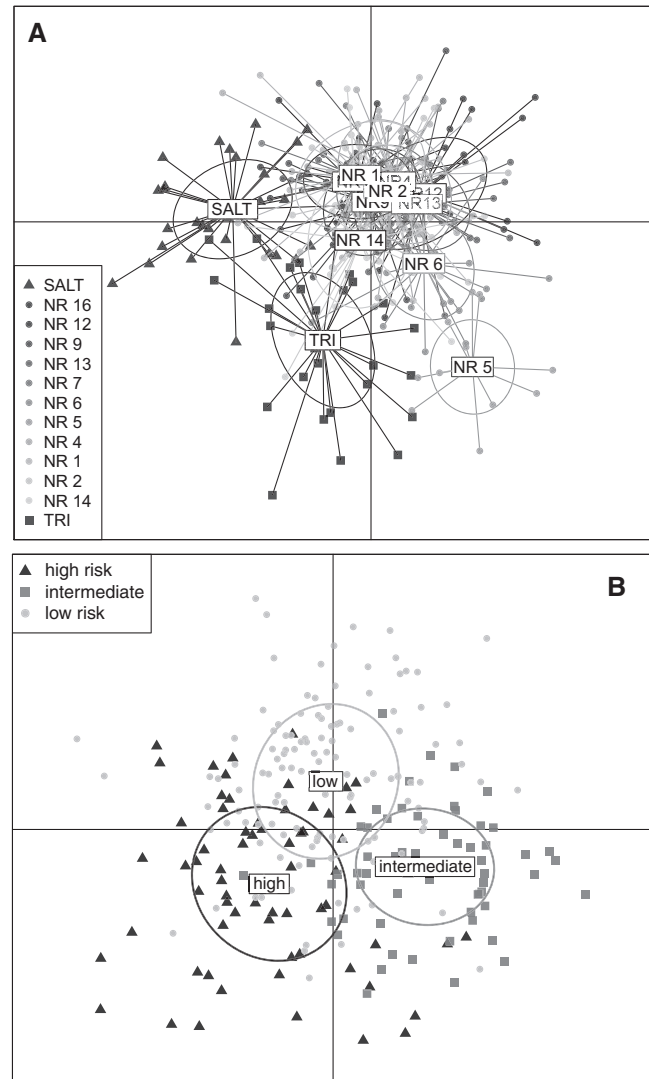


Figure 2. Genetic relatedness among *Ambystoma maculatum* individuals obtained using discriminant analysis of principal components (DAPC). Arranged on the first two principal component axes, points represent individuals and unique shades and inertia ellipses show genetic groups. Populations in the legend are ordered from south to north. In (A) there are three distinct genetic groupings, one for each of the outlying populations (SALT and TRI) and another for the Northford Ridge experimental populations. NR5 had the fewest samples ($n = 11$), likely leading to a spurious separation from the Northford group. (B) DAPC clustering of only the Northford populations, each categorized according to low, intermediate, or high *Ambystoma opacum* prevalence. Individuals collected from each pond type are represented by a symbol and color specific to that selection category. Figure S1 provides a color version of Figure 2A to allow easier population identification.

Forward model selection indicated that *A. opacum* prevalence was linked with the phenotypic trait data, whereas pond hydroperiod was not. Hydroperiod was subsequently removed from the model. In the combined RDA model, only one of the four genetic MEM variables was retained following model reduction. *A. opacum*

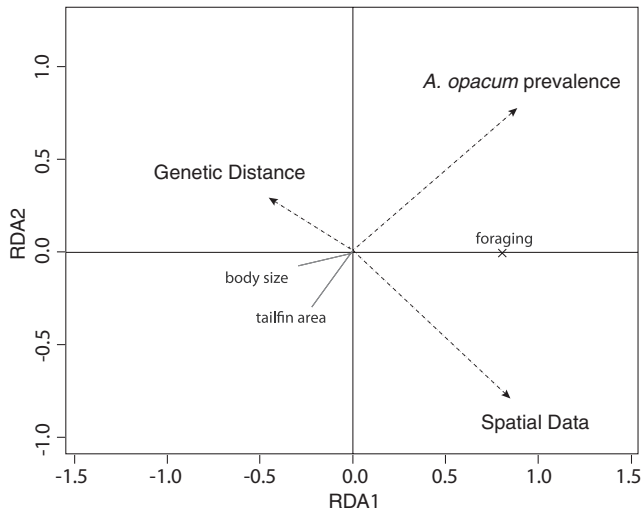


Figure 3. Biplot of the redundancy analysis (RDA) regressing *Ambystoma maculatum* response variables (foraging rate, body size, and tail fin area) on the three predictor variables retained in the full model (*Ambystoma opacum* predator prevalence, genetic distance among populations, and the spatial arrangement of ponds on the landscape). The gray text represents the position of each response variable in RDA space, with body size and tail fin area both located near the origin. *A. maculatum* foraging rate is the only variable significantly associated with the predictor variables. The dashed arrows represent the magnitude and directionality of the predictor variable loadings in RDA space. Permutational analysis of variance (ANOVA) indicates that *A. opacum* prevalence ($P = 0.0039$), genetic distance ($P = 0.0054$), and the spatial relationship among ponds ($P = 0.003$) were all significantly associated with *A. maculatum* foraging rates.

prevalence ($P = 0.0039$), neutral genetic divergence ($P = 0.0054$), and spatial position of ponds on the landscape ($P = 0.003$) were all significantly associated with the phenotypic trait data and were retained in the full model (Table S3, Fig. 3). Variance inflation scores were low (<1.2) for each predictor variable, confirming that the selection, genetic, and spatial variables were not strongly correlated.

Multiple regression models for each response variable indicated that foraging rate is driving the observed patterns; body size and tail shape were not associated with any of the three predictor variables (Table S4). The RDA biplot coordinates of each response variable vector confirm the role of foraging rate (Fig. 3). Increased foraging was related to higher *A. opacum* prevalence within spatially clustered ponds, and for more genetically similar populations (Fig. 3). Partitioning the variance among predictor variables (while isolating the effects of each variable) suggests that *A. opacum* prevalence (adjusted $r^2 = 0.500$), neutral genetic divergence (adjusted $r^2 = 0.309$), and space (adjusted $r^2 = 0.299$) each account for a substantial amount of the foraging rate variance among populations (Table S5). We also detected joint effects of *A.*

opacum prevalence and space (12%) and *A. opacum* prevalence and neutral genetics (–25%), suggesting that the effects of these factors are related and cannot be disentangled statistically (Table S5). Overall, the model explained 89% of the total trait variance.

Discussion

A key question in evolutionary biology centers on the degree to which populations can become adaptively differentiated in the presence of gene flow (Mayr 1963; Ehrlich and Raven 1969; Garant et al. 2007). Microgeographic adaptation is of particular interest because this adaptive evolution occurs at distances where gene flow is usually expected to overwhelm selection and prevent the evolutionary divergence of populations (Jain and Bradshaw 1966; Selander 1970; De Meester et al. 2002; Lenormand 2002; Hargeby et al. 2004; Skelly 2004; Eroukhanoff et al. 2009). Therefore, microgeographic adaptation must originate from some combination of strong selection, lower gene flow than expected based on dispersal, habitat selection, or spatially autocorrelated selection regimes (Ehrlich and Raven 1969; Nosil et al. 2005; Edelaar et al. 2008; Urban 2011). Discerning among these competing hypotheses requires data on adaptive trait variation among many populations, measurements of natural selection in the field, and neutral genetic variation. Few datasets meet these criteria. Here, we evaluated the relative contributions of natural selection, spatial arrangement of selection, and gene flow to microgeographic adaptation in natural populations of pond-breeding salamanders.

Despite occurring over short distances on the landscape, spotted salamander populations at Northford exhibited strong evidence of adaptive divergence in foraging traits. This divergence was associated with larval marbled salamander prevalence in ponds and levels of genetic divergence among ponds. The genetic signal of phenotypic divergence indicated strong clustering of populations based on *A. opacum* predation risk within ponds (Fig. 2B). This pattern held even after accounting for the spatial distribution of *A. opacum* risk across the landscape, which was also linked to phenotypic trait variation among populations. These results highlight the multifaceted components of adaptive divergence among populations experiencing heterogeneous environments, even at small spatial scales.

GENETIC SIGNAL OF ADAPTATION

We found strong evidence of adaptive divergence of foraging rates at microgeographic spatial scales. The average distance among the 11 focal ponds is 116 m, which is within the mean dispersal distance observed for *A. maculatum* adults (118 m; Semlitsch and Bodie 2003). It is important to note, however, that estimates of actual offspring dispersal (emigration) distances, as opposed to seasonal migration distances, are not well characterized for most

pond-breeding amphibians (Semlitsch 2008). Given the microgeographic adaptive divergence among populations, we expected to find limited gene flow as determined by neutral microsatellite markers. The breeding behavior of this species also lends itself to low gene flow. *Ambystoma maculatum* exhibit high site fidelity, such that most adults return to their natal pond to breed (Vasconcelos and Calhoun 2004). In addition, adults exhibit outbreeding avoidance, indicating potential for the reproductive isolation of immigrants (Chandler and Zamudio 2008). In contrast to these expectations, neutral microsatellite markers did not reveal low levels of gene flow among all Northford Ridge populations. Only the outlying populations were clearly differentiated from the Northford populations (Fig. 2A). Substantial admixture among spotted salamander populations has been observed previously (Zamudio and Wiczorek 2007; Purrenhage et al. 2009), including across our study area (Richardson 2012). A lack of neutral genetic structure might not be unexpected across these very fine spatial scales for microsatellite markers, which evolve rapidly but not rapidly enough to differentiate among local populations connected by moderate gene flow. Our analysis suggests that microgeographic adaptation is not occurring solely on the basis of limited gene flow.

Even with migration, adaptation can still occur if selection is strong. Consistent with this hypothesis, we found strong neutral genetic structure when we classified individuals based on the *A. opacum* risk they encounter within ponds (Fig. 2B), providing a rare example of neutral genotypic sorting by selection regime (see also Aguilera et al. 2007). This pattern was not explained by our microsatellite loci being linked to selected traits, as none of our 14 loci showed any signs of nonneutrality. This pattern suggests that gene flow is infrequent among populations facing divergent selection, likely due to strong natural selection or habitat selection based on genotype. Given the strong selection indicated by our regression analyses, we suggest that selective barriers might explain these neutral genetic patterns. This result indicates that natural selection induced by *A. opacum* predation acts as a stronger barrier to gene flow than space alone. Short-term laboratory selection experiments additionally suggest strong antagonistic selection between predation by *A. opacum* and predation by predators more commonly found in non-*A. opacum* ponds like *Dytiscus* beetle larvae (Urban 2010). Long-term field estimates of selection intensity are needed to substantiate this conclusion. Nonetheless, our results are most consistent with a signal of strong selective barriers. Selective barriers have long been thought to operate in a way that diminishes the role that gene flow plays in linking gene pools together (Ehrlich and Raven 1969; De Meester et al. 2002). Many of the other iconic examples of microgeographic adaptation also involve selective barriers. For instance, adaptive divergence in peppered moths, *Cepaea* land snails, and *Timema* stick insects

all occur because visual predators quickly remove prey that are not camouflaged in local environments (Cain and Sheppard 1954; Clarke and Sheppard 1966; Nosil 2004).

Even with the significant genetic divergence among ponds experiencing different predation risk, substantial admixture still occurs across predation environments (Fig. 2B). This is not surprising given the microgeographic scale of our study, but does raise the possibility of several mechanisms being responsible for the observed patterns of phenotypic and genetic divergence. Relatively high levels of gene flow among our populations may constrain adaptive divergence to some degree, leading to less pronounced adaptive differences between populations than would be expected in the absence of gene flow. For this reason, even though significant phenotypic differences have arisen among our focal populations in response to variable predation at this microgeographic scale, we do not anticipate the optimal levels of adaptive divergence expected when gene flow is very low (Lenormand 2002; Garant et al. 2007). Our sampling strategy may also contribute to the genetic admixture observed between phenotypically diverged populations. We collected genetic samples from developing embryos within each pond to ensure adequate sample sizes. Selection from *A. opacum* occurs during the larval stage for *A. maculatum*, meaning that offspring of first-generation immigrants may be overrepresented in our samples relative to larvae sampled after strong *A. opacum* selection had removed maladapted offspring. Future work that examines neutral genetic divergence before and after selection, and that experimentally manipulates selection and gene flow in natural populations and monitors adaptive divergence (Rasanen and Hendry 2008), would be useful to confirm observed patterns.

Neutral genetic markers have been used extensively to estimate the correspondence between phenotypic and genetic divergence between populations experiencing different environments. Although neutral markers evolve rapidly relative to many other markers available, they also have important limitations related to their delayed signal of selection events (Goldstein and Schlotterer 1999; Labonne and Hendry 2010). A previous study has shown that the inferences from neutral markers can be limited depending on the values of several important parameters, including selection, migration, recombination rates, and population size (Thibert-Plante and Hendry 2010). Simulations suggest that accurate detection of adaptive divergence with neutral markers can be achieved most consistently with strong selection and intermediate levels of gene flow (Thibert-Plante and Hendry 2010). This prediction occurs because high levels of gene flow will overwhelm natural selection, whereas low levels of gene flow promote divergence among all populations as a result of drift rather than adaptive differences. In our study system, we observe intermediate levels of gene flow, the signal of strong selection and

population sizes within the range tested, which enhance our ability to use neutral genes to detect selection (Thibert-Plante and Hendry 2010). However, other factors such as recombination rates not estimated as part of this study may also shape the observed patterns (Thibert-Plante and Hendry 2010). Additionally, the correlational analyses used in most studies of ecological divergence are limited in their ability to infer the direction of causality between gene flow inhibiting adaptive divergence and the inverse (Rasanen and Hendry 2008). However, our use of replicated populations, controlled common environment experiments and long-term data on natural selection provides strong evidence that *A. opacum* predation imparts such strong selection that maladapted genotypes dispersing across the landscape are efficiently removed from the population.

PARTITIONING THE CONTRIBUTIONS TO ADAPTIVE TRAIT DIVERGENCE

Selection from *A. opacum* was clearly the most important factor determining trait variation, explaining 50% of the variation. Neutral genetic structure and space contributed equally to trait variation at 30% each. The association of neutral genetic structure with trait variation suggests that barriers exist that are correlated with trait divergence. Because these are neutral markers, the most parsimonious explanation is that selection barriers operate to allow differentiation among subgroups exposed to dissimilar selection, as corroborated by discriminant analyses. The significant role of space indicates a contribution to trait divergence independent from selection or neutral gene flow and suggests a role for spatial autocorrelation in selection (discussed below).

Although we examined three traits under selection by *A. opacum*, only foraging rate was strongly linked with *A. opacum* prevalence and neutral genetic divergence, even after controlling for admixture and space. Previously, we found that six pond characteristics including two other predators (*D. verticalis* and *N. viridescens*), total predator density, pond temperature, canopy cover, and pond area were not significantly associated with foraging variation (Urban 2007a). This suggests that strong selection within ponds with high *A. opacum* prevalence has led, at least in part, to genetic divergence. Models suggest that increased foraging rates allow *A. maculatum* larvae to reach a size refuge sooner, reducing predation pressure earlier in development (Urban 2007b). Neither body size nor relative tail fin area were linked to selection regime or neutral genetic variation. Given equivalent food rations in the experiment, body size differences would require changes in assimilation efficiency, which do not appear to have evolved in high-risk populations. Although strongly selected, relative tail fin area also was not correlated with predation risk in the wild. The lack of adaptive divergence in this trait might originate from low additive genetic variation, substan-

tial pleiotropy, or a plastic response that occurs without a genetic component.

MICROGEOGRAPHIC CONTEXT

In this study, we found strong evidence of adaptive divergence of foraging rates at spatial scales within the mean dispersal distance of *A. maculatum*. This suggests that gene flow is lower than expected based on dispersal ability alone, and is not high enough to homogenize the gene pools of *A. maculatum* populations at Northford Ridge. Even if individuals are able to traverse the focal landscape, gene flow is limited to those dispersers that can successfully breed and produce fit offspring. The genetic data indicate that intense natural selection imparted by *A. opacum* predation contributes to reduced gene flow (Fig. 2B). Additionally, high site fidelity to the natal pond probably contributes to population insularity in *A. maculatum*, further reducing gene flow and facilitating divergence (Vasconcelos and Calhoun 2004). Microgeographic adaptation has been documented in an increasing number of systems, and is likely to be more common than previously realized (De Meester et al. 2002; Hargeby et al. 2004; Ellis and Weis 2006; Eroukmanoff et al. 2009).

The moderate spatial autocorrelation of *A. opacum* predation risk on the landscape also likely facilitates microgeographic adaptation. This outcome was supported by the significant contribution of space in determining interpopulation trait variation. On Northford Ridge, ponds with low and intermediate *A. opacum* risk are evenly distributed across the landscape (Fig. 1). However, all three of the highest risk ponds are located in the southwestern area of the ridge, where springs create conditions conducive to *A. opacum* overwinter survival. The spatial autocorrelation of selection environments ensures that similar selection regimes located close together share adapted migrants. Gene flow in these cases occurs more frequently from similar selective regimes and thus is not as disruptive to local adaptation as genes flowing from populations facing antagonistic selection. This spatial autocorrelation is one reason that microgeographic adaptation can occur with substantial gene flow (Urban 2011), and this process could explain why many studies find both elevated levels of gene flow and local adaptive divergence (Sambatti and Rice 2006; Berner et al. 2009; Zheng and Song 2010; Andrew et al. 2012; Galligan et al. 2012). In addition to spatial environmental autocorrelation, habitat choice by dispersing individuals may contribute to patterns of phenotypic and genetic divergence among populations. Dispersal and gene flow are often considered spatially random across the landscape, however, individuals may be able to assess habitat attributes and select the habitat that best matches their phenotype, providing an important performance advantage (Edelaar et al. 2008). More research will be needed to determine if dispersing spotted salamander adults choose breeding habitats based on their phenotype.

Conclusions

Even though spotted salamander populations at Northford often occur within 100 m of each other, they strongly diverge in foraging intensity. This microgeographic adaptive divergence is most closely associated with selection from marbled salamander prevalence in ponds, followed by space and neutral genetic divergence among populations. We found little evidence of neutral differentiation among all ponds, but strong differentiation among populations sharing the same selection regime, providing one of the few examples of genotypic sorting by natural selection across the landscape. Moreover, selection was the most important factor determining foraging variation. Hence, strong selective barriers likely operate in this system and promote microgeographic adaptation. These selective barriers eliminate the maladapted individuals that arrive from nearby ponds, limiting their contribution to local gene pools. Our results indicate that correlations between neutral genetic structure and adaptive trait variation can reveal the operation of selective barriers. Neutral genetic divergence has been shown to accompany rapid adaptive divergence among populations when selection is strong in other systems as well (Senar et al. 2006; Thibert-Plante and Hendry 2010; De Luna et al. 2012). Selective barriers likely play an important role in maintaining microgeographic adaptation more generally. Additional examples of microgeographic adaptation are likely to be uncovered in populations under strong selection as we explore genetic differentiation across finer and finer scales.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Primer information and polymerase chain reaction (PCR) conditions used to amplify the 14 *Ambystoma maculatum* microsatellite loci.

Table S2. Pairwise genetic distances between each pair of ponds at Northford Ridge.

Table S3. Test statistics from the redundancy analysis (RDA) and permutational analysis of variance (ANOVA) of predation risk, genetic divergence, and spatial landscape effects on phenotypic variation in spotted salamanders (*Ambystoma maculatum*).

Table S4. Detailed results from the multiple regression analyses of each phenotypic trait assessed for *Ambystoma maculatum*.

Table S5. Variation partitioning results.

Figure S1. A color reproduction of Figure 2A for population identification.