

Spatial dependence of phenotype-environment associations for tadpoles in natural ponds

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Abstract Within natural habitats, phenotypes are shaped by many environmental factors. Consequently, heterogeneity of these factors can promote phenotypic divergence. However, because environments exhibit heterogeneity at different spatial scales, phenotypic divergence should also exhibit such scale-dependence. Using hierarchical linear models, I determined how multiple environmental factors at two spatial scales affected the morphology of wood frog (*Rana (Lithobates) sylvatica*) tadpoles collected from natural ponds. Among ponds, predation risk intensity and tadpole density were strong predictors of tadpole morphology, while within ponds, other environmental variables such as water depth and leaf litter were more important. Spatial analyses revealed that water depth and leaf litter, but not predation risk intensity or tadpole density, exhibited heterogeneous spatial distributions within ponds, suggesting that spatial properties of environmental variables influenced the scale at which they shaped phenotypes. Furthermore, patterns of phenotypic variation with respect to predation risk intensity and tadpole density among ponds largely matched observations from previous laboratory studies. These results emphasize the importance of considering phenotype-environment associations across multiple spatial scales.

Keywords Adaptive divergence · Environmental heterogeneity · GIS · Hierarchical linear model · Phenotypic plasticity · Spatial autocorrelation

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Introduction

Environments influence the phenotypes of organisms by inducing phenotypic change (i.e. phenotypic plasticity) and selecting phenotypes that confer the highest fitness within that environment (i.e. natural selection; Langerhans et al. 2007). Consequently, phenotypes of organisms collected in natural habitats are often associated with environmental characteristics exerting strong inductive or selective pressure (e.g. Losos 1990; Keeley et al. 2005; Kerfoot and Schaefer 2006; Langerhans et al. 2007; Van Buskirk 2009a). Generally, this relationship is termed a ‘phenotype-environment association’ (Langerhans et al. 2007). Because such phenotypic changes induced or selected for by environmental variables are usually adaptive (e.g. Dudley and Schmitt 1996; Van Buskirk and Relyea 1998), examining phenotype-environment associations can reveal important historical and contemporary factors influencing phenotypic variation among natural populations, as well as enable predictions of how future changes in the environment may potentially affect phenotypic variation.

Because of the association between phenotype and environment, the spatial variance (i.e. heterogeneity) of an environmental variable often becomes a strong promoter of phenotypic divergence. Indeed, theoretical models predict that spatially heterogeneous environments favor the development of adaptively plastic traits (e.g. Via and Lande 1985; Moran 1992; Scheiner 1998; Sultan and Spencer 2002; Ernande and Dieckmann 2004; Leimar et al. 2006) and the divergence of phenotypes into local polymorphisms (Scheiner 1998; Sultan and Spencer 2002). Additionally, several empirical studies confirm these predictions (e.g. Juenger and Bergelson 2002; Svensson and Sinervo 2004; Lind and Johansson 2007). Subsequently, this phenotypic differentiation may either directly promote mechanisms of reproductive isolation or correlate with other such phenotypes (e.g. Kirschel et al. 2009), leading to the divergence of populations, and possibly speciation (see reviews by Robinson and Parsons 2002; West-Eberhard 2003; Schlichting 2004).

Environmental variables often exhibit heterogeneity at different spatial scales (Wiens 1989; Conover et al. 2006). Accordingly, several studies have examined how the influence of different environmental factors on patterns of species abundance, richness, and diversity changes across multiple spatial scales (e.g. Cushman and McGarigal 2002; Fridley et al. 2007; Talley 2007). However, few studies have extended this concept of scale-dependence to the effects of environments on phenotypes. Such studies are of great interest for evolutionary biology, as they enable predictions on the spatial scale at which populations may be expected to exhibit phenotypic divergence. For instance, because environmental heterogeneity promotes phenotypic divergence, and such heterogeneity is scale-dependent, phenotypes should differentiate at the spatial scale in which a strong inductive or selective environmental factor exhibits heterogeneity.

In this study, I examine the influence of five environmental variables—predation risk, intraspecific competition, water depth, structural complexity, and leaf litter—on the morphology of wild-caught wood frog (*Rana (Lithobates) sylvatica*) tadpoles at multiple spatial scales. *R. sylvatica* tadpoles inhabit temporary ponds that can span several environmental gradients, such as hydroperiod, overhead canopy cover, and predator and competitor composition (Wellborn et al. 1996; Van Buskirk 2005). Additionally, environmental factors can demonstrate spatial variability within ponds, creating local microhabitats (Alford 1986). Therefore, temporary pond habitats present environmental factors that can exhibit heterogeneity on a regional scale (e.g. comparing ponds with different predator densities) or a local scale (e.g. comparing predator distribution among microhabitats within a pond).

Predation and competition are known to impose strong inductive and selective pressure on tadpole morphological phenotypes. Furthermore, inducible changes are highly reversible and can occur within days (Relyea 2003a), permitting tadpoles to rapidly adjust phenotypes to changing environments. Laboratory experiments demonstrate that predators induce and select for relatively deep tailfins and short bodies (Van Buskirk and Relyea 1998), and induce shorter intestines (Relyea and Auld 2004) and less-developed mouthparts (Relyea and Auld 2005). Increased intraspecific competition induces tadpoles to develop longer bodies, shallower tailfins (Relyea 2002), longer intestines (Relyea and Auld 2004), and larger mouthparts (Relyea and Auld 2005), all of which increase the competitive ability of tadpoles (Relyea 2002). With respect to external body morphology, these patterns of trait variation observed in laboratory studies are consistent with trait variation of tadpoles in natural ponds (i.e. tadpoles from high-predation risk ponds have deeper tailfins; Van Buskirk and McCollum 1999; Van Buskirk 2009a). Structural complexity, defined as the amount of vertical structure in a given area, does not appear to affect any tadpole morphological trait (M. J. Michel, unpublished), although a recent field survey concluded that tadpoles collected from densely vegetated ponds had deeper heads and tailfins than those collected from sparsely vegetated ponds (Van Buskirk 2009a). No experiments, to my knowledge, have tested the effects of water depth and leaf litter abundance on tadpole morphology.

I predicted that environmental variables would have the strongest association with tadpole phenotypes at the same scale in which they demonstrate heterogeneity. For example, if predators and competitors exhibited strong heterogeneity within ponds (i.e. ‘hotspots’, or ‘coldspots’; Thompson 1999; Brodie et al. 2002), then I expected tadpole phenotypes to be strongly correlated with estimates of predation risk and competitive intensity within ponds. Conversely, if predators and competitors exhibited mostly uniform distributions within ponds, then I expected little phenotypic differentiation at the local spatial scale. To analyze such a multiscale dataset, I used hierarchical linear models (Bryk and Raudenbush 1992; Gelman and Hill 2007; McMahan and Diez 2007), which permitted the partitioning of phenotypic variance, as well as its relationship with environmental factors, into within-pond and among-pond scales.

Materials and methods

Field sampling

I selected five open and five closed canopy ponds (Table 1) known to be *R. sylvatica* breeding sites at the University of Notre Dame Environmental Research Center (Gogebic county, MI). Over the entire surface area of each pond, I established a 2 m × 2 m sampling grid, which served as the sampling locations (hereafter termed “sites”, or “sampling sites”) for tadpoles and the five environmental variables. Thus, the size of the pond determined the number of sampling sites (Table 1). A systematic sampling scheme allowed for subsequent spatial analysis of all five environmental variables (Fortin and Dale 2005).

Tadpole predators were sampled at each sampling site using one 42 cm by 23 cm unbaited Gee minnow trap composed of 6.4 mm galvanized wire mesh and two 22 mm diameter entrance holes. Six pieces of Styrofoam were mounted inside each trap to prevent sinking and subsequently drowning air-breathing organisms. For approximately 3 weeks before tadpole sampling, traps were checked once daily and all potential tadpole predators were counted and released. Predators included large-sized diving water beetle larvae

Table 1 Information for the ten sampled ponds

Pond	Canopy designation	Area (m ²)	# Sampling sites	# Sites with Tadpoles	Sampling date
Pond 1	Open	191.0	49	24	June 2
Pond 2	Open	26.8	8	2	June 4
Pond 3	Open	168.9	44	30	June 5
Pond 4	Closed	86.7	20	3	June 11
Pond 5	Open	110.9	35	18	June 9
Pond 6	Open	143.2	48	26	June 8
Pond 7	Closed	33.9	11	0	June 11
Pond 8	Closed	56.7	13	11	June 3
Pond 9	Closed	59.2	31	7	June 10
Pond 10	Closed	47.1	18	10	June 11

(Dytiscidae, >4 cm length, 19.4% of all captures), medium-sized dytiscid larvae (>1.5 cm and <4 cm length, 15.5%), large-sized dytiscid adults (>4 cm length, 35.6%), medium-sized dytiscid adults (>1.5 cm and <4 cm, 18.0%), adult giant water bugs (Belostomatidae, 7.1%), adult newts (*Notophthalmus viridescens*, 2.0%), scavenger beetle larvae (Hydrophilidae, 1.4%), adult water scorpions (*Ranatra* spp. 0.6%), and dragonfly larvae (Aeshnidae, 0.3%). Because different predator species pose varying mortality risks for tadpoles, I multiplied a predation risk weight factor, based on Van Buskirk and Arioli (2005) and personal observations (i.e. predation trials involving adult dytiscid beetles), to the site-specific absolute abundance totals for each predator. Large dytiscid larvae, belostomatids, and aeshnids received a weight of 3, medium dytiscid larvae received a weight of 2, and all other predators received weights of 1. Larger weights denoted predators that present higher predation risk to tadpoles. I then standardized by the number of nights each site was trapped to obtain an estimate of predation risk intensity per trap-night.

As a proxy for intraspecific competition, I obtained an estimate of *R. sylvatica* tadpole density. Density was quantified using two methods: (1) tadpoles freely entered minnow traps, and a count was recorded on the last day of trapping, and (2) 2 days after minnow traps were removed from a pond, each sampling site within a pond was sampled for tadpoles by dipnetting within a 30 cm diameter PVC pipe that was thrust vertically through the water column to the pond substrate. This sampling method does not disturb tadpole captures at sites that are at least 1 m apart (Mullins et al. 2004). Both methods were performed at each sampling site once per pond. I assumed that these two methods provided reasonably equal capture probabilities, and obtained an average tadpole count for each site. All tadpoles that were captured using these two methods were taken back to the laboratory, euthanized, and preserved in 10% formalin for later morphological measurement. *R. sylvatica* was the only anuran species present among the closed canopy ponds. Spring peeper (*Pseudacris crucifer*) tadpoles were present in most open canopy ponds, but had recently hatched and were rarely caught in dipnets. Although *R. sylvatica* tadpoles can alter their morphology in response to interspecific competitors (Relyea 2002), the size disparity and low abundance of *P. crucifer* likely limited their influence on *R. sylvatica* phenotypes.

Structural complexity and water depth were recorded at the time of pipe sampling. Before I dipnetted for tadpoles within each pipe, I removed and counted all macrophyte stems including submerged and emergent plants. The majority of aquatic plants were sedges and bulrushes (Cyperaceae), pondweeds (*Potamogeton* spp.) and buttercups

(*Ranunculus* spp.). Woody debris consisted mostly of logs on the bottom of the ponds, and, consequently did not contribute to vertical structure. Water depth was obtained using a ruler drawn on the inside of each pipe. Leaf litter abundance was recorded at the end of June after all 10 ponds had dried. I placed a 30 cm diameter PVC ring over each sampling site and counted the number leaf litter pieces greater than 30 mm diameter.

Morphological measurements

I was interested in four tadpole morphological traits: size of the labial toothrows (Mouth), gut length (Gut), body length (BL), and maximum depth of the tailfin (TFD; See Fig. 1 in Relyea and Auld 2005 and Fig. 1 in Relyea 2000). For each tadpole, I first obtained a wet mass (to the nearest 0.000 g) and then a photograph of the lateral view, after propping the tail on two microscope slides to present a more natural representation (Relyea 2000). Each photograph was viewed using the program ImageJ, scaled to size using a millimeter gradient present in the photograph, and measured for BL (tip of snout to body terminus) and maximum TFD (to the nearest 0.00 cm). After whole-body photography, the mouth of each tadpole was photographed using a 3.34 megapixel Nikon Coolpix 990 digital camera affixed to a dissecting scope (Relyea and Auld 2005). Using ImageJ, mouthpart photographs were scaled to size and the length of each labial tooththrow and the perimeter of the upper jaw sheath were measured. Wood frog tadpoles typically have a 3/4 tooththrow formula, and only lengths of tooththrows that had fully-formed teeth were measured. After mouth photography, each tadpole was dissected and the length of the intestine from the midgut to rectum was unfolded and then measured (Relyea and Auld 2004).

Before statistical analyses were conducted on morphological traits, they were standardized by tadpole body size. For TFD, BL, and Gut, I log-transformed each variable and then conducted a univariate analysis of covariance (ANCOVA) with pond-of-origin as the factor and log-transformed tadpole mass as the covariate. First, I confirmed that there were no interactions between pond-of-origin and tadpole mass (i.e. homogenous slopes). Then, I added the resulting residuals for each individual to the marginal trait means estimated by the ANCOVA of the pond-of-origin of that individual, thus producing size-independent measures of all four traits for each individual. This method has been used successfully in previous studies (see Schoeppner and Relyea 2008). For mouthpart morphology, I first conducted a principal components analysis (PCA) using the length of each tooththrow and of the upper jaw sheath as the variables. All variables loaded positively into the first factor score, which explained 62.3% of the variation in the original data (eigenvalue = 4.99). I identified this factor score as a representation of total mouthpart size and saved the score for each individual. I then removed variation due to body size in the same manner described above. Using the standardized traits, I obtained a mean value of each morphological trait for each sampling site within a pond.

Spatial analyses

Each environmental variable and tadpole morphological trait was analyzed for evidence of spatial autocorrelation at a distance class of 2 m using the global Moran's I statistic tool in ArcMap 9.2 (ArcGIS Desktop 9.2, ESRI Corporation, Redlands, CA). A value of Moran's I near 1 represents a clustered spatial pattern, a value near -1 represents a uniform pattern and a value near 0 represents a random pattern (Fortin and Dale 2005). The spatial autocorrelation tool in ArcGIS 9.2 provides a test of the null hypothesis of no spatial pattern (i.e. $I = 0$) using a permutation test. Directional patterns were not anticipated for

any of the variables, thus all measures of autocorrelation were omnidirectional. A distance class of 2 m was chosen as a compromise between selecting a large value to eliminate effects of tadpole movement within ponds and an adequate sample size within the smaller ponds. Previous field studies using tagged tadpoles suggest that the majority of daily tadpole movements are between 1 and 4 m within open-canopy ponds (M. J. Michel and C.V. Pilar, unpublished). Based on this observation, an environmental variable demonstrating significantly clustered spatial distributions above the specified distance class of 2 m was perceived as exhibiting heterogeneity.

Statistical analysis

The objective of this study was to determine how environmental variables at the within-pond scale and at the among-pond scale affect morphology of wild-caught tadpoles. For this, I used hierarchical linear models, which are commonly used for such multiscale datasets. The response variables were the four morphological measurements of tadpoles (mouthpart size, gut length, body length, and tailfin depth). The predictor variables were site-specific measures of each environmental variable (water depth, leaf litter abundance, structural complexity, tadpole density, predation risk intensity) at the within-pond scale and pond-mean values of these same five environmental variables at the among-pond scale. Because these models can quickly become complicated with the inclusion of explanatory variables and their interactions at any level, I initially conducted an exploratory data analysis (EDA) to determine which independent variables explained the most variation in tadpole morphology at both scales. First, I excluded Ponds 2, 4, and 7 from analyses due to a low number of sites in which tadpoles were captured (Table 1). Then, for within-pond phenotypic variation, I conducted a multiple linear regression using measurements of all five site-specific environmental variables. To account for any spatial autocorrelation, I included a spatial autoregressive coefficient with the error term (i.e. a spatial error model; Wang 2006). Using a backwards stepwise procedure, I then identified which environmental variables were significant ($\alpha = 0.05$) for each particular morphological variable (for inclusion in subsequent hierarchical linear modeling) and repeated this process for every pond. Within-pond regressions were conducted using GeoDa 0.9.5 (Anselin 2003). To assess any potential multicollinearity between environmental variables, I ran correlations of all within-pond variables for each pond. For Pond 3, water depth was negatively correlated with leaf litter (Pearson's $r = -0.466$, $df = 42$, Bonferroni adjusted $P = 0.026$). For Pond 6, water depth was negatively correlated with structural complexity (Pearson's $r = -0.567$, $df = 46$, adjusted $P = 0.002$), and positively correlated with tadpole density (Pearson's $r = 0.487$, $df = 24$, adjusted $P = 0.019$). None of the other possible correlations were statistically significant.

For among-pond phenotypic variance, I used a backwards stepwise multiple linear regression for each morphological trait. However, the small sample size ($n = 7$ ponds) limited this analysis to the three environmental variables that exhibited the strongest relationship (based on values of Pearson's r) with each morphological trait (Table S1 in supplementary material). All remaining environmental variables in the final regression model (at $\alpha = 0.05$) were included in subsequent hierarchical linear modeling. Among-pond multiple linear regressions were conducted using R 2.7.0. Additionally, I tested for correlations between environmental variables among ponds; no correlations were significant (Table S2). Although a significant negative correlation between predation risk intensity and tadpole density may have been expected, such a correlation was not

Table 2 Significant ($\alpha = 0.05$) within-pond and among-pond predictors obtained from exploratory data analysis for each tadpole morphological trait

Phenotype	Within-pond predictors	Among-pond predictors
Mouthpart size	Leaf litter	Tadpole density Predation risk intensity
Gut length	Water depth Leaf litter	Tadpole density Predation risk intensity
Body length	None	Tadpole density Predation risk intensity
Tailfin depth	Water depth Predation risk intensity	Structural complexity Predation risk intensity

Bold indicates predictors that were included in the chosen models

found among these ponds. This result is consistent with a previous pond survey study (Van Buskirk 2009a).

After obtaining potentially important within-pond and among-pond predictors (Table 2), I began building hierarchical linear models separately for each morphological trait, following the procedures outlined in Gelman and Hill (2007) and Bryk and Raudenbush (1992) (see Appendix S1 in Supplementary Material for detailed model-building steps). For each model, I obtained estimates of deviance information criterion (DIC) to evaluate model fit. However, model selection was ultimately based on the percentage of total within-pond and among-pond phenotypic variance explained by the model (Gelman and Hill 2007). These values were calculated as the percent difference in the residual error term (σ^2 ; for within-pond variance) or the random error term (τ_{00} ; for among-pond variance) between the null model (i.e. no predictors) and the evaluated model (Appendix S1). DIC estimates were only used to ensure that each model provided a better fit than the null model. Models were fit using the linear mixed effects (lmer) function (Gelman and Hill 2007) in R.2.7.0 (R Core Development Team 2007).

Results

Spatial autocorrelation estimates

Tadpole morphological traits did not exhibit evidence of significant spatial patterning in any pond (all P -values > 0.05). Values of Moran's I for Mouth ranged from -0.10 to 0.04 ; for Gut, -0.15 to 0.12 ; for BL, -0.59 to -0.01 ; and for TFD, -0.38 to 0.10 . For the environmental variables, water depth, structural complexity, and leaf litter abundance showed significantly clustered spatial patterns in most ponds, while tadpole density and predation risk intensity showed random spatial patterns in the majority of ponds (Table 3).

Hierarchical linear models

Mouthpart size

The null model estimated that 44.2% of the unexplained variation in mouthpart size was among ponds, while 55.8% existed within ponds (Table 4A). EDA identified predation risk intensity and tadpole density as important among-pond predictors, and leaf litter as an

Table 3 Spatial patterns of the five environmental variables assessed with Moran's I (C = Clustered, R = Random); significance determined by a permutation test of the null hypothesis $I = 0$

Pond	Water depth		Stems		Leaf litter		Tadpole density		Predation risk intensity	
	I	Pattern	I	Pattern	I	Pattern	I	Pattern	I	Pattern
Pond 1	0.440	C***	0.230	C*	0.180	C**	0.202	C*	0.320	C*
Pond 3	0.470	C***	-0.020	R	0.190	C*	0.113	R	0.149	R
Pond 5	0.650	C***	0.190	C*	0.140	R	-0.070	R	0.187	R
Pond 6	0.700	C***	0.300	C***	0.280	C*	0.123	R	-0.025	R
Pond 8	-0.004	R	-	-	0.110	R	-0.090	R	0.170	R
Pond 9	-	-	-	-	-	-	0.142	C*	0.286	R
Pond 10	0.480	C*	-	-	-0.090	R	0.234	R	0.014	R

Dashes indicate no value Moran's I was calculated due to a low sample size

* $0.05 \leq P < 0.01$; ** $0.01 \leq P < 0.001$; *** $P \leq 0.001$

important within-pond predictor. The inclusion of tadpole density and predation risk intensity each separately reduced among-pond variation by 20%, and a combined model was only able to reduce among-pond variation by an additional 1% (Table S3 in supplementary material). Site-specific leaf litter abundance reduced within-pond variation by 4.5%. A model combining both among-pond predictors and site-specific leaf litter abundance, but no interactions among them, reduced among-pond variation by 33.1% and within-pond variation by 6.3% (Table 4A). Among ponds, mouthpart size was negatively correlated with predation risk intensity (estimate \pm 1 SE: -0.649 ± 0.609 ; Fig. 1a) and positively correlated with tadpole density (0.054 ± 0.042), although this relationship was strongly driven by an outlier pond (Pond 8; Fig. 1b). After the removal of this pond, a model including tadpole density did not explain any among-pond variance in mouthpart size (Table S3); thus Pond 8 seems to have had a major influence on the relationship between mouthpart size and tadpole density among ponds. Within ponds, tadpoles collected at sites with a high abundance of leaf litter had smaller mouthparts (-0.007 ± 0.005 ; Fig. 1c).

Gut length

The null model estimated that 26.7% of the unexplained variation in gut length existed among ponds while 73.3% of this variation existed within ponds (Table 4B). EDA identified predation risk intensity and tadpole density as important among-pond predictors, and water depth and leaf litter as important within-pond predictors. The inclusion of tadpole density and predation risk intensity reduced among-pond variance by 3.3 and 17.8% respectively (Table S3). A model combining these two predictors reduced among-pond variation by 52.8% and the inclusion of an interaction term reduced this variation by 69.2% (Table 4B). Within-pond predictor variables failed to explain >1% of the within-pond phenotypic variance. Generally, gut length decreased as predation risk intensity increased, except for the pond with the highest tadpole density, at which tadpoles had the shortest gut lengths (-0.367 ± 0.251 ; Fig. 2).

Body length

The null model suggested that 25.3% of unexplained BL variation existed among ponds while 74.7% was within ponds (Table 4C). EDA identified predation risk intensity and

Table 4 Factors affecting within-pond and among-pond variance of the four phenotypes (chosen model) compared to a model in which no factors were included (null model)

Model	Parameter(s) (Estimate ± 1 SE)	σ^2 ($\times 10^{-4}$)	τ_{00} ($\times 10^{-4}$)	DIC
(A) Mouthpart size				
Null model	Intercept (−0.131 ± 0.116)	110	87.0	94
Chosen model	Intercept (−0.047 ± 0.278)	103	58.0	75
	Within-pond leaf litter (−0.007 ± 0.005)			
	Among-pond tadpole density (0.054 ± 0.042)			
	Among-pond predation risk intensity (−0.649 ± 0.609)			
(B) Gut length				
Null model	Intercept (1.076 ± 0.011)	1.91	0.695	−426
Chosen model	Intercept (1.039 ± 0.064)	1.92	0.214	−451
	Among-pond tadpole density (0.078 ± 0.059)			
	Among-pond predation risk intensity (0.237 ± 0.242)			
	Tadpole density × Predation risk intensity (−0.367 ± 0.251)			
(C) Body length				
Null model	Intercept (0.132 ± 0.003)	0.192	0.065	−723
Chosen model	Intercept (0.136 ± 0.005)	0.189	0.006	−760
	Among-pond tadpole density (0.002 ± 8.66E−04)			
	Among-pond predation risk intensity (−0.026 ± 0.010)			
(D) Tailfin depth				
Null model (among)	Intercept (0.009 ± 0.003)	–	0.327 ^a	−46 ^b
Chosen model	Intercept (0.022 ± 0.004)	–	0.106 ^a	−52 ^b
	Among-pond predation risk intensity (0.010 ± 0.003)			
Null model (within)	Intercept (0.011 ± 0.002)	0.440	0.000	−623
Chosen model	Intercept (0.009 ± 0.002)	0.410	0.000	−635
	Within-pond water depth (6.00E−04 ± 2.63E−04)			

Variance estimates are σ^2 (within-pond scale) and τ_{00} (among-pond scale)

^a Residual sums of squares from linear model

^b Akaike’s Information Criterion (AIC); used to evaluate fit of linear models

tadpole density as important among-pond predictors, but did not identify any within-pond predictors as significant. The inclusion of tadpole density reduced among-pond variance from 6.50×10^{-5} to 2.66×10^{-5} , a 59.1% reduction, while the inclusion of predation risk intensity reduced among-pond variance to 2.70×10^{-5} , a 58.5% reduction (Table S3). A combined model reduced among-pond variance by 91.3% (Table 4C). Inclusion of an interaction term did not substantially reduce among-pond variance. The models confirmed

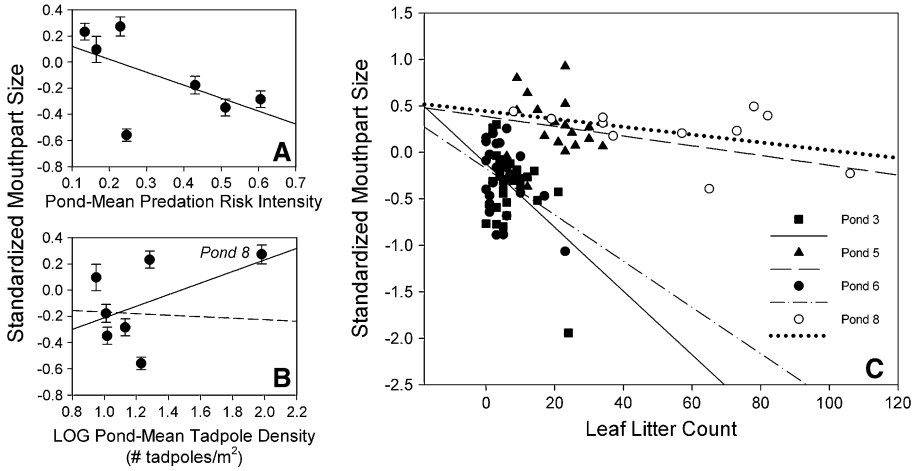


Fig. 1 **a** Among-pond spatial scale relationship between standardized tadpole mouthpart size and predation risk intensity and **b** tadpole density. *Dotted line* in **b** represents best-fitting trendline after omission of Pond 8. *Symbols* are pond-means \pm 1 SE. **c** Within-pond spatial scale relationship between standardized tadpole mouthpart size and leaf litter abundance for four ponds. For clarity, only ponds with significant negative slopes are shown

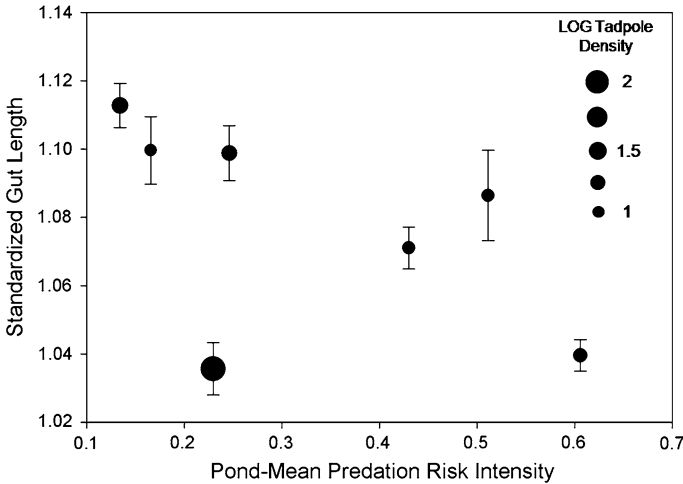


Fig. 2 Interactive effects of predation risk intensity and tadpole density on standardized tadpole gut length at the among-pond spatial scale. *Symbols* are pond means \pm 1 SE. Size of symbol corresponds to the logarithm of tadpole density of a pond

the lack of important site-specific predictors, in that no predictor explained more than 1.5% of within-pond variation. The final model suggests that ponds with high predation risk intensity produced tadpoles with short bodies (-0.026 ± 0.010 ; Fig. 3a), while ponds with high tadpole density produced tadpoles with long bodies ($0.002 \pm 8.66 \times 10^{-4}$; Fig. 3b), although this relationship may have been driven by the outlier pond (Pond 8). After the removal of this pond from the analyses, tadpole density reduced among-pond variance by

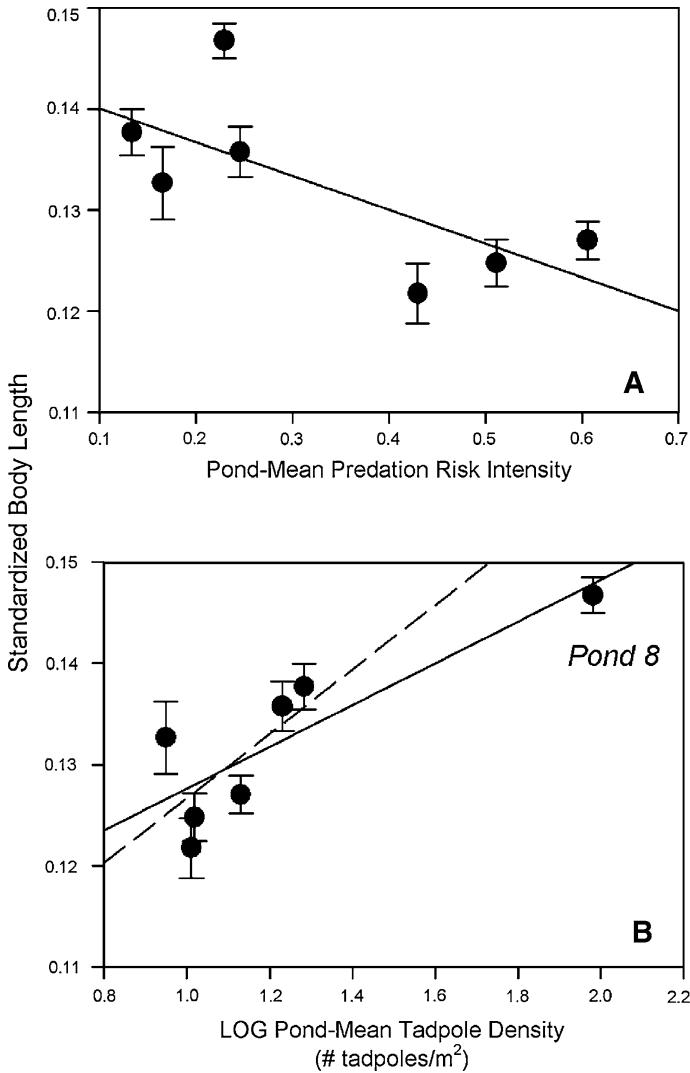


Fig. 3 Among-pond spatial scale relationships between standardized body length and **a** predation risk intensity and **b** tadpole density. *Dotted line in b* represents best-fitting trendline after omission of Pond 8. *Symbols* are pond-means ± 1 SE

72.5%, and a combined model with predation risk reduced among-pond variance by 99.9% (Table S3); thus this pond does not appear to have influenced the among-pond relationship between body length and tadpole density.

Tailfin depth

The null model estimated unexplained within-pond variance in tailfin depth as 4.4×10^{-4} and unexplained among-pond variance as 1.39×10^{-11} , suggesting that more than 99.9% of the total variation in TFD was within ponds (Table 4D). Because <0.01% of the

variation in TFD remained at the among-pond scale, among-pond TFD variance was pooled and analyzed separately using a backwards stepwise multiple linear regression. EDA identified predation risk intensity and structural complexity as important among-pond predictors. No predictors were significant (P -value > 0.05) from a combined model with or without an interaction term (Table S3). Only predation risk intensity remained significant in the final model ($P = 0.024$, Table 4D). EDA identified water depth and predation risk as important within-pond predictors. Site-specific water depth and predation risk intensity reduced within-pond variation by 6.8 and 2.3% respectively, and, combined, reduced within-pond variation by 10.5%. In this model, however, only water depth was a significant predictor, as judged by the standard error (Tables 4D, S3). The separate among- and within-pond models suggest that tadpoles collected from ponds with high levels of predation risk intensity had deeper tailfins (0.025 ± 0.011 ; Fig. 4a), while, at a within-pond scale, tadpoles collected in deeper water had deeper tailfins ($5.96 \times 10^{-4} \pm 2.35 \times 10^{-4}$; Fig. 4b).

Discussion

Correlations between the morphological phenotypes of tadpoles and environmental factors exhibited a strong signature of scale-dependence. At the within-pond scale, environmental variables that demonstrated spatial heterogeneity explained the most phenotypic variance. Within ponds, water depth and leaf litter were the strongest predictors of two of the four morphological phenotypes (Table 2). These environmental variables also showed clustered spatial distributions within most ponds (Table 3). Conversely, predation risk intensity and tadpole density exhibited random spatial distributions in the majority of ponds. Thus, at the within-pond scale, the absence of ‘hotspots’ or ‘coldspots’ of these two environmental factors precluded their importance in explaining tadpole phenotypic variance. However, among ponds, predation risk intensity and tadpole density were the most important predictors of tadpole phenotypic variance, suggesting that tadpoles adjust their phenotypes in response to pond-mean levels of these two variables. Therefore, at a fine spatial scale, tadpole phenotypes might exhibit differentiation with respect to local differences in water depth or leaf litter abundance, while, on a broader scale, tadpole populations may exhibit phenotypic differentiation with respect to pond-level differences in predation risk and tadpole density.

Theoretical models show that, for a given scale, the ability of an organism to accurately predict environmental change is a significant prerequisite for plastic trait responses (e.g. Moran 1992; Scheiner 1998; Leimar et al. 2006). At the within-pond scale, tadpole perceptions of water depth and leaf litter microhabitat differences may have been more accurate than perceptions of local predator and tadpole density. First, the spatial properties of water depth and leaf litter exhibited clustered spatial distributions in most ponds, but predation risk intensity and tadpole density exhibited mostly random spatial distributions. Second, because predators and tadpoles are mobile and can emit chemical cues that disperse throughout a pond, their presence is likely less predictable than static environmental variables such as water depth and leaf litter. Third, there may be a temporal difference between the important within- and among-pond predictors. Water depth and leaf litter are likely more temporally invariable than predation risk intensity and tadpole density, although temporal heterogeneity was not examined in this study. The scale of temporal variability is important in the context of the pace of tadpole plasticity: at the within-pond scale, tadpoles may adjust phenotypes more readily to environments that temporally vary

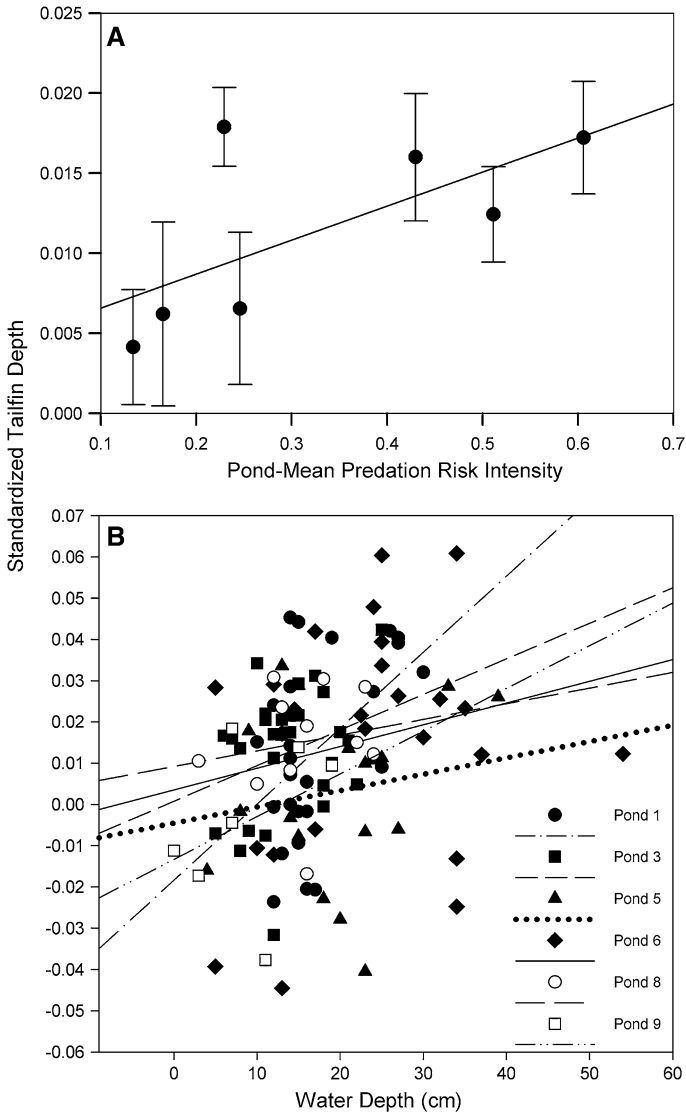


Fig. 4 **a** Among-pond spatial scale relationship between standardized tadpole tailfin depth and predation risk intensity. *Symbols* are pond-means \pm 1 SE. **b** Within-pond relationship between standardized tadpole tailfin depth and water depth for six ponds. For clarity, only ponds with significant positive slopes are shown

at a scale greater than the few days required for tadpole morphological plasticity (Relyea 2003a). However, these conclusions assume that the within-pond phenotype-environment correlations observed here arose mostly through phenotypic plasticity and not nonrandom habitat selection, yet this study was unable to distinguish between these two mechanisms.

At which scale might populations of *R. sylvatica* exhibit phenotypic divergence? The presence of environmental heterogeneity at the scale of microhabitats is thought to have driven the population divergence of various taxa, including sticklebacks (*Gasterosteus*

spp.) responding to differences between limnetic and benthic habitats (Day et al. 1994), various teleost species of northern postglacial lakes responding to differences between littoral and pelagic habitats (reviewed in Robinson and Parsons 2002), and anoles (*Anolis* spp.) responding to differences between ground, bush, trunk and other microhabitats (Losos 1990; Losos et al. 2000). In these studies, the environmental variables exerted strong selective and inductive pressure on phenotypes. However, the important within-pond predictors identified in this study, water depth and leaf litter, are not known to exhibit such strong influence on tadpole phenotypes. Furthermore, within-pond phenotypic divergence would require some degree of reproductive isolation among the differing phenotypes. Although phenotypic changes during the tadpole stage can affect the phenotypes of metamorphs (Relyea 2001; Van Buskirk and Saxer 2001), it is unknown if these changes affect any amphibian isolation mechanisms, such as mate choice. Conversely, at the among-pond scale, much morphological variation was explained by predation risk intensity and tadpole density, which are two environmental variables known to impose strong selection and induction on tadpole traits (Van Buskirk et al. 1997; Van Buskirk and Relyea 1998; Relyea 2002; Teplitsky et al. 2005). Reproductive isolation is also more likely at the among-pond scale because of strong philopatry exhibited by *R. sylvatica* adults (Berven and Grudzien 1990), although there seems to be little evidence for finescale population genetic structure (Berven and Grudzien 1990; Crosby et al. 2009). Thus, phenotypic divergence among amphibian populations is more likely to occur on a regional scale.

Several mechanisms may explain why only a small portion of within-pond trait variation was explained. First, environmental variables that I did not quantify, such as the level of tadpole food resources, could have been significant within-pond predictors. Second, tadpoles simply may not respond phenotypically to local-scale environmental variables. The environmental variables known to impose strong selective and inductive pressure on tadpole phenotypes—predation risk intensity and tadpole density—were mostly randomly distributed within a pond. Thus, the unpredictability of these environments may have precluded strong phenotypic responses (Scheiner 1998; Leimar et al. 2006). Finally, substantial phenotypic variation may be explained by variation in genotypes and genotype-by-environment interactions. Keeley et al. (2007) estimated that heritable differences explained 52.7% of variation in rainbow trout (*Oncorhynchus mykiss*) morphology, as opposed to environmental differences, which explained 7.3%. Proulx and Magnan (2004) also found that genetic factors explained approximately 2% more variation than environmental factors in brook charr (*Salvelinus fontinalis*) morphology. Because I was only able to observe phenotypic differences at the population level, future studies should examine individual-level differences in genotypes and genotype-by-environment interactions.

The results of this field survey also suggest that tadpole phenotypic responses to predation risk and competitor density observed in laboratory experiments are accurate predictors of tadpole phenotypic change among natural populations. Many tadpole predators both select for (Van Buskirk et al. 1997; Van Buskirk and Relyea 1998; Teplitsky et al. 2005) and induce deep tailfins and long bodies (Lardner 1998; Relyea 2003b; McIntyre et al. 2004; Schoepner and Relyea 2008), which matched the patterns observed in this field study. Similarly, Van Buskirk (2009a) found the same relationships between predation risk and tadpole body length and tailfin depth for *R. temporaria*, a closely related species. Tadpoles from high predation risk ponds also had shorter guts and smaller mouthparts. Decreases in gut length and mouthpart size are thought to be costs incurred by tadpoles in order to produce deep tailfins and short bodies (Relyea and Auld 2004, 2005).

Ponds with high tadpole densities produced tadpoles with longer bodies, which is also likely due to a combination of selection and plasticity. For *R. sylvatica*, laboratory experiments demonstrate that intraspecific competition induces longer bodies (Relyea 2002, 2004) and that these changes enhance growth and developmental rates in highly competitive environments (Relyea 2002). Again, Van Buskirk (2009a) also observed this relationship between competitor density of a pond and body length for *R. temporaria* tadpoles. Competitors also induce longer gut lengths (Relyea and Auld 2004) and shallower tailfins (Relyea 2002) in laboratory experiments, but these patterns were not observed here. Compared to body size, tailfin depth may be under stronger selective and inductive pressure from predators such that its response to increased competition in natural ponds may be constrained. Changes to gut length may be similarly affected by other environmental variables in ponds, such as food quality and quantity.

At the within-pond scale, there were two unexpected relationships. In 6 of the 7 ponds, tadpoles with deep tailfins were found in deeper water, and in 4 of the 7 ponds, tadpoles with smaller mouthparts were found in microhabitats with high amounts of leaf litter. A deep tailfin is thought to increase swimming speed (Dayton et al. 2005, but see Van Buskirk and McCollum 2000), thus a deep tailfin may improve the ability of tadpoles to move throughout deeper waters. Alternatively, both dissolved oxygen and temperature decrease with water depth and a large tailfin may provide more surface area for cutaneous respiration (sensu Van Buskirk 2009a) and thermoregulation. Interestingly, a comparative study of 82 species of anuran tadpoles found a positive relationship between tailfin depth and preference for pelagic habitats (Van Buskirk 2009b). *R. sylvatica* tadpoles are known to consume leaf litter, and a narrow mouth is sometimes associated with the consumption of detritus (Bonacci et al. 2008), thus the relationship between mouthpart size and leaf litter abundance observed here may have been adaptive. Future laboratory experiments should be conducted to examine possible plastic responses of tadpoles to water depth and leaf litter abundance, and the fitness consequences of these responses.

In this paper, I have demonstrated that phenotype-environment associations between tadpoles and environmental variables of ponds were scale-dependent and stronger at an among-pond scale. Consequently, it is more likely that phenotypic divergence would occur at broader spatial scales, provided other prerequisites for divergence (e.g. reduced gene flow) are met. Importantly, spatial properties of environmental factors were critical in determining their importance in the differentiation of phenotypes at both scales. Variables that are well-defined and spatially clustered are predicted to correlate with phenotypes at a finer spatial scale, as opposed to variables that are mobile and randomly distributed. Hierarchical linear models and measures of spatial autocorrelation can facilitate the recognition of these patterns and have the potential to be valuable statistical tools to investigate these types of patterns in other systems (Fortin and Dale 2005; McMahon and Diez 2007). Future studies should consider such scale effects as field studies conducted at fine spatial scales may obscure potentially important environmental variables, while those conducted over broader scales may overlook potentially important fine-scale environmental factors.

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