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Decoupling of genetic and phenotypic divergence in a headwater landscape

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Abstract

In stream organisms, the landscape affecting intraspecific genetic and phenotypic divergence is comprised of two fundamental components: the stream network and terrestrial matrix. These components are known to differentially influence genetic structure in stream species, but to our knowledge, no study has compared their effects on genetic and phenotypic divergence. We examined how the stream network and terrestrial matrix affect genetic and phenotypic divergence in two stream salamanders, *Gyrinophilus porphyriticus* and *Eurycea bislineata*, in the Hubbard Brook Watershed, New Hampshire, USA. On the basis of previous findings and differences in adult terrestriality, we predicted that genetic divergence and phenotypic divergence in body morphology would be correlated in both species, but structured primarily by distance along the stream network in *G. porphyriticus*, and by overland distance in *E. bislineata*. Surprisingly, spatial patterns of genetic and phenotypic divergence were not strongly correlated. Genetic divergence, based on amplified DNA fragment length polymorphisms, increased with absolute geographic distance between sites. Phenotypic divergence was unrelated to absolute geographic distance, but related to relative stream vs. overland distances. In *G. porphyriticus*, phenotypic divergence was low when sites were close by stream distance alone and high when sites were close by overland distance alone. The opposite was true for *E. bislineata*. These results show that small differences in life history can produce large differences in patterns of intraspecific divergence, and the limitations of landscape genetic data for inferring phenotypic divergence. Our results also underscore the importance of explicitly comparing how terrestrial and aquatic conditions affect spatial patterns of divergence in species with biphasic life cycles.

Keywords: amphibian, dendritic network, evolution, gene flow, morphology, salamander

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Introduction

Landscape attributes can play a central role in maintaining genetic and phenotypic diversity by mediating the effects of drift on divergence, and by setting the template for adaptive and plastic divergence (Wright 1951; Endler 1977; Manel *et al.* 2003). When phenotypic divergence is controlled primarily by drift and gene flow, neutral genetic divergence and phenotypic divergence should be correlated, and the landscape attributes predicting these two measures of divergence should be similar (Merila & Crnokrak 2001; McKay & Latta 2002; Ramstad *et al.* 2010). Alternatively, patterns of genetic and phenotypic divergence that are uncorrelated and predicted by different landscape attributes suggest that phenotype is plastic, or controlled by local selection (Storz 2002; Leinonen *et al.* 2006; Lehtonen *et al.* 2009). Access to highly variable molecular markers has led to a surge of landscape genetic data in recent years, but complementary phenotypic data are often lacking. By comparing genetic and phenotypic divergence at the landscape scale, we can expand understanding of how these two components of intraspecific biodiversity are

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maintained, and of the mechanisms causing them to become decoupled.

Streams and rivers occur in hierarchical networks where smaller stream channels join to form larger ones in a dendritic pattern that resembles branches on a tree (Horton 1945; Strahler 1952). This consistent network architecture constrains evolutionary, demographic and ecological processes in aquatic organisms (Grant *et al.* 2007; Muneepeerakul *et al.* 2008; Hughes *et al.* 2009). More specifically, in species linked to streams during all or part of their life cycle, the landscape affecting genetic and phenotypic divergence is comprised of two fundamental components: the stream network and terrestrial matrix. This division has been useful for exploring the landscape genetics of stream species, where differences in aquatic vs. terrestrial gene flow cause genetic population structure to align with the stream network or terrestrial matrix (Meffe & Vrijenhoek 1988; Finn *et al.* 2007; Hughes *et al.* 2009; Mullen *et al.* 2010). Stream organisms also exhibit phenotypic structure at the landscape scale (Reznick *et al.* 2001; Chaput-Bardy *et al.* 2007). However, to our knowledge, no study has compared effects of the stream network and terrestrial matrix on both genetic and phenotypic divergence in stream species.

A community of salamanders in the family Plethodontidae, the lungless salamanders, is associated with small, headwater streams in eastern North America (Petranka 1998). Species diversity in headwater salamander communities declines from south to north along the Appalachian Uplift, with up to six species in western Virginia (Resetarits 1997), but only two or three species in New Hampshire (Taylor 1993). The larvae of stream plethodontids are strictly aquatic, but adults occupy a range of habitat zones extending from the channel into the riparian corridor (Organ 1961; Hairston 1987). We know that these lateral habitat associations are correlated with morphological and behavioural traits (Wilder & Dunn 1920; Dunn 1926) and maintained by interspecific interactions (e.g. Wilder & Dunn 1920; Hairston 1987; Rissler *et al.* 2004). Much less is known about how these habitat associations and, more broadly, variation in terrestriality among adults are related to genetic and phenotypic divergence in headwater landscapes.

The two predominant stream salamanders in the Hubbard Brook Experimental Forest (HBEF), New Hampshire, where this work was conducted, are *Gyrinophilus porphyriticus* and *Eurycea bislineata* (Burton & Likens 1975). Postmetamorphic adults of these species differ in their use of terrestrial habitat. Adults of *G. porphyriticus* are associated with aquatic habitat within the stream channel and have strongly keeled and laterally compressed tails, short limbs and flattened posture suited to life in flowing water (Brandon 1966; Petranka 1998). *G. porphyriticus* adults can move into riparian forests at night, but return to the stream during the day, where they are found under cover in the channel and along the wetted edge (Greene *et al.* 2008). Adults of *E. bislineata* return to streams to breed, but are primarily associated with terrestrial habitat (MacCulloch & Bider 1975; Petranka 1998). *E. bislineata* adults are slender, with weakly laterally compressed tails, and are found under rocks and wood in riparian zones and on the forest floor, far from stream channels (Bishop 1941; Perkins & Hunter 2006).

In a previous study, we examined genetic and phenotypic divergence in *G. porphyriticus* and *E. bislineata* at paired sites in six HBEF streams, where each pair of sites was separated by a standardized distance of 1 km along the stream (Lowe *et al.* 2008). That analysis showed that within-stream measures of genetic divergence and phenotypic divergence were correlated, suggesting that phenotypic divergence along individual streams was driven, in part, by gene flow and drift. However, we only examined divergence within streams, and did not quantify divergence between sites in different streams. Therefore, that study did not provide insight on broader patterns of genetic and phenotypic structure across the Hubbard Brook Watershed, or on the relative influence of the stream network and terrestrial matrix on divergence. Additionally, in that study, phenotypic divergence was assessed based on only one measure of body morphology (trunk length).

Our goal here is to build on this earlier study by examining effects of the stream network and terrestrial matrix on genetic and phenotypic divergence in *G. porphyriticus* and *E. bislineata* throughout the Hubbard Brook Watershed, using more measures of body morphology to quantify phenotypic divergence. On the basis of our previous results and differences in terrestriality of *G. porphyriticus* and *E. bislineata* adults, we hypothesize that genetic and phenotypic divergence are correlated across the Hubbard Brook Watershed in both species, but affected differently by the stream network and terrestrial matrix. We predict that genetic and phenotypic divergence in *G. porphyriticus* is structured primarily by the stream network, such that divergence between sampling sites increases with distance along stream channels, independent of distance by overland pathways. In *E. bislineata*, we predict that genetic and phenotypic divergence is structured primarily by the terrestrial matrix, such that divergence between sampling sites increases with overland, Euclidean distance, independent of distance along the stream network.

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Materials and methods

Study sites and sampling protocol

The Hubbard Brook Watershed (Fig. 1) is in the White Mountains of central New Hampshire, USA (43°56′N, 71°45′W). The majority of the watershed is within the 31.6 km^2 HBEF. We sampled six hydrologically independent streams throughout the Hubbard Brook Watershed. These streams have low conductivity (12.0–15.0 μS), slight acidity (pH of 5.0–6.0), high dissolved oxygen content (80–90% saturation) and moderate midday summer temperatures (13.0–17.0 °C) (Likens & Buso 2006). Dominant tree species in the watershed are *Acer saccharum, Fagus grandifolia, Betula alleghaniensis, Picea rubens, Abies balsamea, B. papyrifera*.

We sampled downstream and upstream sites along each stream (Fig. 1). Downstream sites were reaches between 100 and 200 m from the confluence with a higher-order (i.e. larger) stream; upstream sites were reaches between 1200 and 1300 m from this confluence. To avoid sampling individuals from the same family group, morphological data and tissue samples were collected from randomly sampled individuals distributed throughout the 100-m study sites.

For *G. porphyriticus*, tissue samples were collected in June, July and August of 2003 (n = 10 individuals per site); morphological data were collected during the same period in 2005 (n = 9–15 individuals per site). For *E. bislineata*, tissue samples were collected in June, July and August of 2005 (n = 9–12 individuals per site); morphological data were collected during the same period in 2006 (n = 8–11 individuals per site). Because sampling was random within sites—to characterize the genetic and phenotypic attributes of all *G. porphyriticus* and *E. bislineata* individuals at a site—it is unlikely that these differences in sampling date biased our results.

Larval and adult *G. porphyriticus* were photographed for morphological analysis. Ratios of larvae to adults sampled did not differ among or within streams [analysis of variance (ANOVA): stream effect: F = 2.46, d.f. = 5, 5, P = 0.17; site effect: F = 0.03, d.f. = 1, 5, P = 0.88]. All *E. bislineata* sampled for morphology were postmetamorphic adults. A small section of tail tissue was clipped from captured salamanders and stored in 90% ethanol at −80 °C.

We photographed animals to collect morphological data. Animals were placed on a level, lighted stage. The camera was held approximately 20 cm above the stage, which allowed us to capture the entire dorsal surface of the animal in the photograph, along with a ruler. The ruler was used to calibrate morphological measurements in mm. We used photographs to measure head, trunk and leg morphology. We also measured snout-vent length (SVL)—the standard measure of body size in amphibians (Heyer *et al.* 1994).

AFLP amplification, scoring and analyses

We used standard phenol methods to extract total DNA from tissue samples. Amplified DNA fragment length polymorphism (AFLP) loci were developed using the AFLP Plant Mapping Kit [Applied Biosystems (ABI), Foster City, CA, USA]. We used the Eco RI-ACA primer labelled with the FAM fluorochrome paired with the Mse I-CAC primer to selectively amplify loci. Amplified products were run on an ABI 3100 sequencer; peaks were categorized and scored using ABI Genotyper v3.0 software.

Hardy–Weinberg equilibrium is often assumed in analyses using dominant AFLP markers, raising concerns about their suitability for population genetic studies relative to codominant markers (Holsinger *et al.* 2002; Holsinger & Wallace 2004). Our analytical tech-

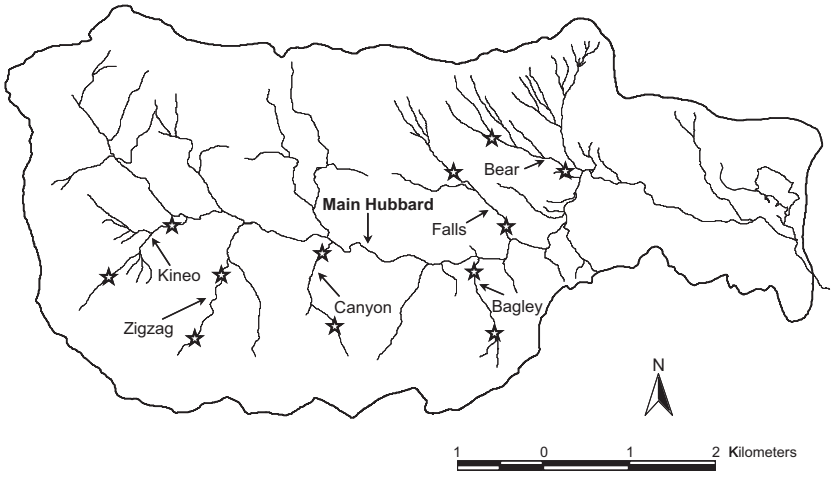

Fig. 1 Map of the Hubbard Brook Experimental Forest, New Hampshire, USA, which comprises the majority of the Hubbard Brook Watershed. Study streams and the main Hubbard Brook are labelled. Stars indicate the locations of sites where *Gyrinophilus porphyriticus* and *Eurycea bislineata* tissue samples and morphological data were collected. Sampling sites within each stream were 1 km apart, measured along the stream.

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niques do not rely on this assumption, but do assume deviations from Hardy–Weinberg equilibrium and linkage equilibrium are similar across sites. We used Φ_{ST} to quantify genetic divergence between sites (Excoffier *et al.* 1992); Φ_{ST} is analogous to F_{ST} (Wright 1951), describing the level of genetic divergence between sites. We chose AFLPs because of their high variability and genome-wide coverage, which we expected to be necessary for resolving population structure within and among streams (Freeland 2005). Genome-wide coverage may produce loci that are under selection, but the majority of AFLP loci are expected to be neutral (e.g. Colbeck *et al.* 2011).

We estimated pairwise Φ_{ST} values using the analysis of molecular variation (AMOVA) framework in WIN AMOVA v1.55 (Excoffier *et al.* 1992). This method uses pairwise Euclidian distances among AFLP marker profiles and does not require indirect estimates of allele frequencies. Input files were produced with AMOVA-PREP v1.1 (Miller 1997).

In previous studies, we found no difference among streams or between downstream and upstream sites in genetic diversity, quantified as average heterozygosity and percent polymorphic loci (Lowe *et al.* 2006, 2008). This suggests that demographic histories for processes that could influence genetic diversity at these sites (e.g. population bottlenecks and demographic expansions) have been similar over the recent past (Hartl & Clark 1997; Marko & Hart 2011).

Morphological analyses

To quantify morphological variation in the head, trunk and legs, we generated size-adjusted shape variables using principal component analysis (PCA). We extracted seven principal components from the covariance matrix representing head, trunk and leg morphology. The covariance matrix was comprised of log-transformed SVL and the two, log-transformed measurements of each body element [head (max. length and width), trunk (max. length and width) and legs (humerus length and femur length)]. The first principal component (MORPH PC1) was expected to represent generalized size because SVL was positively correlated with all morphological measurements. MORPH PC2–PC7 were expected to be size-adjusted shape variables representing different aspects of morphology (Bookstein 1989; Jungers *et al.* 1995; Adams & Beachy 2001).

We restricted our analyses of morphological divergence to the second principal component (MORPH PC2) because it accounted for the highest proportion of morphological variation after size correction and, therefore, was most likely to represent ecologically meaningful variation. In a previous study of morphological divergence in *G. porphyriticus* in the southern Appalachians, Adams & Beachy (2001) found that results using this PCA-based method of size correction were similar to results using two other methods: residuals from regressions of morphological characters on SVL, and Burnaby's (1966) size-independent shape variables.

It is difficult to determine sex in the two study species using external traits, so we could not include sex as a variable in morphological analyses. However, for an earlier study (Lowe & McPeek 2012), we sexed 35 *G. porphyriticus* individuals (15 males, 20 females) in a population in northern New Hampshire and found no morphological differences between the sexes ($F < 2.88$, d.f. = 1, 33, $P > 0.09$).

We used pairwise phenotypic distances (P_{ST}) to quantify divergence in MORPH PC2 between study sites. Phenotypic variance components were quantified for all pairs of sites using the one-way ANOVA framework, and P_{ST} was calculated as

$$P_{ST} = \frac{\sigma^2_{GB}}{(\sigma^2_{GB} + 2\sigma^2_{GW})}$$

as in Raeymaekers *et al.* (2007) and Saint-Laurent *et al.* (2003).

Analysis of genetic and phenotypic divergence

We wanted to compare how the stream network vs. terrestrial matrix influenced spatial patterns of genetic and phenotypic divergence in *G. porphyriticus* and *E. bislineata*. Specifically, we tested the prediction that pairwise genetic and phenotypic distances (Φ_{ST} and P_{ST}) between sites throughout the Hubbard Brook Watershed were influenced by geographic distances along the stream network (i.e. 'stream distance'), as opposed to straight-line distances through terrestrial habitat (i.e. 'overland distance'). Stream distance was the shortest pathway along streams between two sites, where distances were measured along the landscape surface to incorporate changes in elevation along the path. Overland distance was the Euclidean distance connecting two points, also incorporating changes in elevation along the path (Mullen *et al.* 2010). We quantified distances and elevations with a Global Positioning System receiver (Garmin Ltd., Olathe, KS, USA) and Terrain Navigator software (MAPTECH®; Amesbury, MA, USA) with an enhanced digital elevation model of the Hubbard Brook Watershed.

We expected stream and overland distances to be correlated, which precluded using these data directly to compare effects on genetic and phenotypic divergence (Graham 2003). To address this problem of multicollinearity, we used PCA to transform the distance data into two independent variables representing (i) absolute

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geographic distance between sites, whether along streams or overland, and (ii) relative stream vs. overland distance between sites, independent of absolute geographic distance. The second principal component differentiated sites that were closer by stream than overland distances from those that were closer by overland than stream distances. With these derived variables, we were able to assess the overall effect of geographic distance on genetic and phenotypic divergence throughout the Hubbard Brook Watershed, and the relative importance of stream and overland distances in mediating divergence.

To assess the role of genetic drift in driving patterns of phenotypic divergence throughout the watershed, we first tested for correlation between Φ_{ST} and P_{ST} values using a Mantel test that corrects for nonindependence of pairwise points (Mantel 1967). To characterize the spatial structure of genetic and phenotypic divergence, we then tested for effects of the two distance principal components (DISTANCE PC1 and PC2) on Φ_{ST} and P_{ST} with Mantel tests. All Mantel tests were performed with Isolation by Distance version 3.16 (Jensen *et al.* 2005), with 10,000 matrix randomizations.

Results

Genetic and phenotypic variation

We identified 92 polymorphic AFLP loci from the 120 *G. porphyriticus* tissue samples, and 90 polymorphic loci from the 124 *E. bislineata* tissue samples. Peak profiles were highly repeatable in estimated sizes and fluorescence intensity.

Gyrinophilus porphyriticus head, trunk and leg measurements were positively correlated with SVL (r = 0.91–0.99). MORPH PC1 was positively weighted by all six variables and accounted for 91.1% of total variation in body morphology. MORPH PC2 accounted for 3.0% of total variation in body morphology and was negatively weighted by SVL, head length, head width, trunk length and humerus length, and positively weighted by femur length and trunk width (Table 1). In *E. bislineata*, head, trunk and leg measurements were positively correlated with SVL (r = 0.67–0.99). MORPH PC1 was positively weighted by all six variables and accounted for 69% of total variation in body morphology. MORPH PC2 accounted for 13.6% of total variation in body morphology and was negatively weighted by SVL, head width and trunk length, and positively weighted by head length, trunk width, humerus length and femur length (Table 1).

Distance measures

As expected, pairwise stream and overland distances were strongly correlated (r = 0.88, n = 66, $P < 0.001$). DISTANCE PC1 accounted for 94.4% of the covariation in stream and overland distances and was positively weighted by both distance measures. DISTANCE PC2 accounted for the remaining covariation in stream and overland distances (5.6%) and was negatively weighted by overland distance and positively

Table 1 Percent of the total variance explained and factor loadings (eigenvectors) for principal components derived from measurements of body morphology of *Gyrinophilus porphyriticus* and *Eurycea bislineata* individuals sampled at sites throughout the Hubbard Brook Watershed, New Hampshire, USA (Fig. 1). All measurements were in mm, converted from photographs taken at capture

			Eigenvectors						
Species	Principal component	% variance explained	Snout-vent length	Head length	Head width	Trunk length	Trunk width	Humerus length	Femur length
Gyrinophilus porphyriticus	PC1	91.0	0.39	0.38	0.38	0.39	0.38	0.37	0.36
	PC2	2.9	−0.11	−0.19	−0.18	−0.08	0.01	−0.31	0.91
	PC3	2.4	−0.06	−0.33	−0.27	0.03	−0.32	0.83	0.15
	PC4	1.8	−0.04	0.76	−0.08	−0.29	−0.53	0.06	0.14
	PC5	1.2	−0.48	0.04	0.35	−0.63	0.42	0.27	0.05
	PC6	0.7	0.03	0.28	0.79	0.05	0.54	0.08	0.8
	PC7	0.0	−0.78	0.18	0	0.6	0	0	0
Eurycea bislineata	PC1	69.0	0.41	0.41	0.42	0.38	0.36	0.29	0.37
	PC2	13.6	−0.4	0.13	−0.1	−0.5	0.09	0.67	0.33
	PC3	9.2	0.15	0.33	0.1	0.11	−0.68	0.44	−0.44
	PC4	3.7	0.27	−0.45	−0.6	0.41	0.01	0.41	0.12
	PC5	2.9	−0.01	0.12	−0.11	−0.04	0.63	0.21	−0.73
	PC6	1.6	0.09	0.69	0.66	0.04	0	0.26	0.13
	PC7	0.0	−0.75	0.14	0	0.64	0.01	0	0

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weighted by stream distance (loadings = −0.23, 0.23, respectively).

Genetic and phenotypic divergence

In *G. porphyriticus*, there was no significant correlation between pairwise Φ_{ST} and P_{ST} values for sites throughout the Hubbard Brook Watershed (Mantel; $r = -0.22$, $n = 66$, $P = 0.10$). Genetic distance (Φ_{ST}) was positively related to DISTANCE PC1 ($r = 0.29$, $n = 66$, $P = 0.02$; Fig. 2A), and unrelated to DISTANCE PC2 ($r = -0.01$, $n = 66$, $P = 0.48$; Fig. 2B). Phenotypic distance (P_{ST}) was unrelated to DISTANCE PC1 ($r = -0.08$, $n = 66$, $P = 0.29$; Fig. 3A) and positively related to DISTANCE PC2 ($r = 0.30$, $n = 66$, $P = 0.04$; Fig. 3B).

In *E. bislineata*, there was a positive, but statistically nonsignificant correlation between pairwise Φ_{ST} and P_{ST} values (Mantel; $r = 0.27$, $n = 66$, $P = 0.06$). Genetic distance was positively related to DISTANCE PC1 ($r = 0.35$, $n = 66$, $P = 0.01$; Fig. 2C) and unrelated to DISTANCE PC2 ($r = 0.00$, $n = 66$, $P = 0.50$; Fig. 2D). Phenotypic distance showed a weak positive relationship to DISTANCE PC1 ($r = 0.21$, $n = 66$, $P = 0.07$; Fig. 3C), and a stronger, statistically significant negative

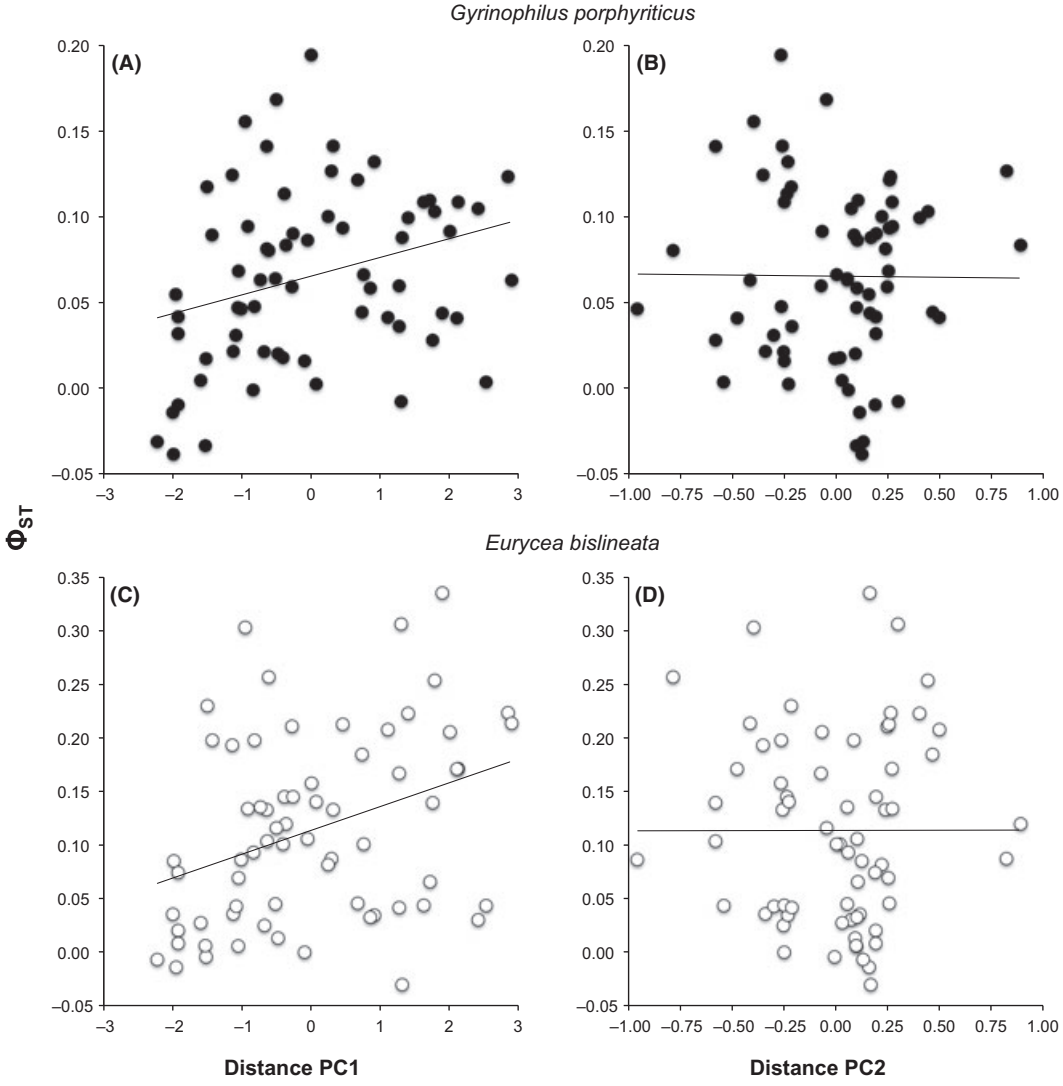

Fig. 2 Scatterplots of the relationships between distance principal components (Distance PC1 and PC2) and pairwise genetic distances (Φ_{ST}) in *Gyrinophilus porphyriticus* (A, B) and *Eurycea bislineata* (C, D). Least squares regression lines are plotted to show trends. Distance PC1 represents variation in absolute geographic distance between sites and is positively weighted by overland and stream distance. Distance PC2 represents variation in relative stream vs. overland distance between sites and is negatively weighted by overland distance and positively weighted by stream distance. Using Mantel tests, we found significant positive relationships between Distance PC1 and Φ_{ST} in both species, but no relationships between Distance PC2 and Φ_{ST}.

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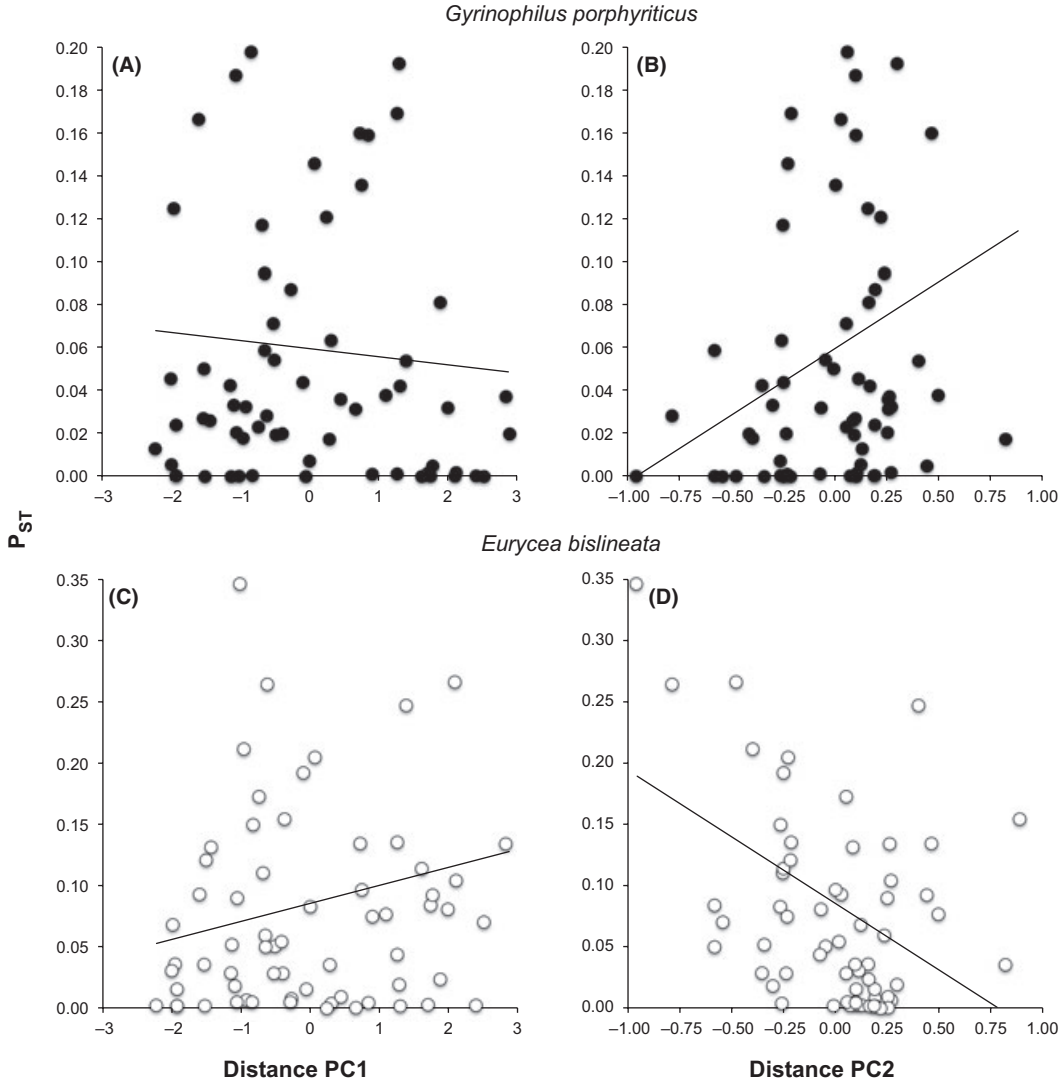

Fig. 3 Scatterplots of the relationships between distance principal components (Distance PC1 and PC2) and pairwise phenotypic distances (P_{ST}) in *Gyrinophilus porphyriticus* (A, B) and *Eurycea bislineata* (C, D). Least squares regression lines are plotted to show trends. Distance PC1 represents variation in absolute geographic distance between sites and is positively weighted by overland and stream distance. Distance PC2 represents variation in relative stream vs. overland distance between sites and is negatively weighted by overland distance and positively weighted by stream distance. Using Mantel tests, we found a significant positive relationship between Distance PC2 and P_{ST} in *G. porphyriticus*, and a significant negative relationship between Distance PC2 and P_{ST} in *E. bislineata*. There were no relationships between Distance PC1 and P_{ST}.

relationship to DISTANCE PC2 ($r = -0.37$, $n = 66$, $P = 0.02$; Fig. 3D).

Discussion

Across the Hubbard Brook Watershed, we found that genetic and phenotypic divergence were largely decoupled in *G. porphyriticus* and *E. bislineata*. In both species, pairwise genetic and phenotypic distances (Φ_{ST} and P_{ST}) were not strongly correlated, and these two measures of divergence responded differently to measures of intersite distance. Genetic distances in both species increased with DISTANCE PC1—an index of absolute geographic distance between sampling sites incorporating both stream and overland distances (Fig. 2). Phenotypic distances were not strongly related to DISTANCE PC1 in both species, but were strongly related to DISTANCE PC2—an index of relative stream vs. overland distance between sites. Phenotypic divergence of *G. porphyriticus* individuals was low when sites were close by stream distance alone and high when sites were close by overland distance alone

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(Fig. 3B). In contrast, phenotypic divergence of *E. bislineata* individuals was low when sites were close by overland distance alone and high when sites were close by stream distance alone (Fig. 3D).

At AFLP loci, *G. porphyriticus* and *E. bislineata* exhibited isolation by distance within the Hubbard Brook Watershed. Assuming that the majority of AFLP loci were neutral, this suggests that genetic divergence was driven primarily by effects of distance on gene flow (Kimura & Weiss 1964). The lack of relationship between DISTANCE PC2 and genetic distance in both species suggests that gene flow occurs both along the stream network and by overland routes. All life history stages of *G. porphyriticus* and *E. bislineata* are capable of dispersal along stream channels (Stoneburner 1978; Bruce 1986; Lowe 2009). Larvae and adults of *G. porphyriticus* show no difference in the frequency, distance or upstream-bias of dispersal, suggesting that both stages contribute to gene flow along streams (Lowe 2009, 2010). In contrast, larvae of *E. bislineata* are known to drift downstream (Johnson & Goldberg 1975; Stoneburner 1978; Bruce 1986), whereas adult dispersal along streams is rare, and short distance (Ashton & Ashton 1978; Bruce 1986). This characteristic suggests that *E. bislineata* larvae may contribute more to gene flow along streams than adults. The stage responsible for overland dispersal in these species is unknown, although the strictly aquatic larvae are excluded. In the only published study of overland dispersal in stream salamanders, Grant *et al.* (2010) found that newly metamorphosed juveniles of *Desmognathus fuscus* and *D. monticola* (both Plethodontidae) had the highest probability of overland dispersal between headwater streams. It is also important to note that patterns of phylogeographic divergence in *E. bislineata* and closely related species are associated with historic drainage connections (Kozak *et al.* 2006), suggesting that stream corridors are important pathways for dispersal and gene flow at larger spatial scales (e.g. between watersheds).

In *G. porphyriticus* and *E. bislineata*, phenotypic divergence was not strongly related to genetic divergence and geographic distance (DISTANCE PC1), suggesting that at the scale of the entire watershed, variation in body morphology is not controlled by drift and gene flow. These two species did, however, exhibit distinctly different patterns of phenotypic variation across the Hubbard Brook Watershed. In *G. porphyriticus*, phenotypic divergence was structured primarily by the stream network: sites that were relatively close by stream vs. overland distance showed the greatest phenotypic similarity. Phenotypic divergence in *E. bislineata* was structured primarily by the terrestrial matrix: sites that were relatively close by overland vs. stream distance showed the greatest phenotypic similarity. These results show that, in general, *E. bislineata* individuals at upstream sites in adjacent streams are more morphologically similar than individuals at sites separated by an equivalent distance along the same stream (Fig. 1). The opposite is true for *G. porphyriticus*—individuals at sites in the same stream are, in general, more morphologically similar than individuals at sites separated by an equivalent distance over land. This difference highlights the complexity of dendritic systems, like stream networks, where the linear, branching structure can decouple spatial processes affecting evolutionary and ecological dynamics from the surrounding, three-dimensional landscape (Grant *et al.* 2007).

We cannot say whether variation in body morphology in *G. porphyriticus* and *E. bislineata* is because of local adaptation or plasticity. However, regardless of the mechanism of control, we can conclude that diversity in body morphology in these two species is a function of different components of environmental variation. In *G. porphyriticus*, body morphology appears to be responding to environmental attributes specific to the stream environment, such that sites that are nearby along stream channels, and therefore likely to have similar aquatic conditions (Vannote *et al.* 1980; Ganio *et al.* 2005), produce individuals with similar body morphology. Overland proximity does not lead to similar body morphology in *G. porphyriticus*, suggesting that body morphology is not responding to conditions specific to the terrestrial environment. In contrast, *E. bislineata* body morphology appears to respond to terrestrial conditions, and not to in-stream conditions. Morphological measurements for *E. bislineata* were from adults only, whereas measurements for *G. porphyriticus* were from adults and larvae. However, previous analyses showed no differences between *G. porphyriticus* larvae and adults in these size-adjusted morphological variables (Lowe & McPeek 2012), so it is unlikely that the inclusion of *G. porphyriticus* larvae produced such a strong difference between species in patterns of phenotypic divergence (Fig. 3).

Stream and terrestrial environments are tightly connected in headwater systems (Likens & Bormann 1974). For example, chemical conditions in headwater streams (e.g. pH, conductivity, nutrient concentrations) are often regulated by processes acting in upslope, terrestrial areas (Likens & Bormann 1974; Johnson *et al.* 1981; Likens 1999). Physical conditions (e.g. temperature, light, precipitation) are also spatially autocorrelated across both environments, causing, for example, upslope and riparian forests to be similar in structure and composition (Schwarz *et al.* 2003). These strong connections between stream and terrestrial environments result in a limited number of conditions that might explain the

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different responses of *G. porphyriticus* and *E. bislineata* body morphology to stream and overland proximity.

Interactions with species that are restricted to either the stream or terrestrial environment may explain the very different patterns of phenotypic divergence in *G. porphyriticus* and *E. bislineata*. If *G. porphyriticus* body morphology is affected by interactions with strictly aquatic species that are patchily distributed in the watershed (e.g. predatory fish, aquatic invertebrates; Resetarits 1991; Greene *et al.* 2008), we would expect relative proximity by stream distance alone to have the strongest effect on morphological divergence. Likewise, *E. bislineata* body morphology may be affected by interactions with terrestrial species encountered by adults, such as small mammals, terrestrial invertebrates or terrestrial salamanders (e.g. *Plethodon cinereus*, the red back salamander, which is abundant at the HBEF; Burton & Likens 1975). This hypothesis is consistent with evidence of local adaptation and phenotypic plasticity in response to interspecific interactions in other amphibians (e.g. Relyea 2001; Richardson 2006; Urban 2007).

It is exciting to consider that where interspecific interactions occur—the stream vs. terrestrial environment—could account for such different patterns of phenotypic divergence in two closely related species, even in the same watershed. These results underscore the need for explicit comparison of how terrestrial and aquatic conditions, both biotic and abiotic, drive landscape-level patterns of adaptive and plastic divergence in species with biphasic life cycles (Wilbur 1980; Ward 1992). More broadly, our results show the limitations of genetic data for inferring phenotypic divergence in complex landscapes.

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Data accessibility

Population AFLP data: DRYAD entry doi:10.5061/dryad.kd741614.

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