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FORAGING TRAIT (CO)VARIANCES IN STICKLEBACK EVOLVE DETERMINISTICALLY AND DO NOT PREDICT TRAJECTORIES OF ADAPTIVE DIVERSIFICATION

Daniel Berner,[1,2] William E. Stutz,[3] and Daniel I. Bolnick[3,4]

[1]*Zoological Institute, University of Basel, CH-4051 Basel, Switzerland*

[2]*E-mail: daniel.berner@unibas.ch*

[3]*Section of Integrative Biology, University of Texas at Austin, Austin, Texas 78712*

[4]*Howard Hughes Medical Institute*

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How does natural selection shape the structure of variance and covariance among multiple traits, and how do (co)variances influence trajectories of adaptive diversification? We investigate these pivotal but open questions by comparing phenotypic (co)variances among multiple morphological traits across 18 derived lake-dwelling populations of threespine stickleback, and their marine ancestor. Divergence in (co)variance structure among populations is striking and primarily attributable to shifts in the variance of a single key foraging trait (gill raker length). We then relate this divergence to an ecological selection proxy, to population divergence in trait means, and to the magnitude of sexual dimorphism within populations. This allows us to infer that evolution in (co)variances is linked to variation among habitats in the strength of resource-mediated disruptive selection. We further find that adaptive diversification in trait means among populations has primarily involved shifts in gill raker length. The direction of evolutionary trajectories is unrelated to the major axes of ancestral trait (co)variance. Our study demonstrates that natural selection drives both means and (co)variances deterministically in stickleback, and strongly challenges the view that the (co)variance structure biases the direction of adaptive diversification predictably even over moderate time spans.

KEY WORDS: Adaptive landscape, disruptive selection, evolutionary divergence, P matrix, sexual dimorphism, trophic morphology.

A major goal of evolutionary biology is to understand natural selection's role in driving patterns of biological diversification. Progress toward this goal has certainly been impressive as regard the evolution of population means (e.g., Endler 1986; Mousseau et al. 2000; Schluter 2000). In contrast, our understanding of the relationship between natural selection and the structure of variance and covariance (hereafter simply "(co)variance") among multiple traits remains thin. Two aspects of this relationship are of central interest.

The first major question concerns the importance of natural selection in shaping the multivariate genetic (co)variance structure relative to other evolutionary processes such as mutation, drift, or gene flow (Roff 2000; Arnold et al. 2001; Steppan et al. 2002; Jones et al. 2003; McGuigan 2006; Guillaume and Whitlock 2007; Jones et al. 2007; Arnold et al. 2008). In particular, stabilizing selection is predicted to reduce trait variance whereas disruptive selection should increase variance. Correlational selection, which favors specific combinations of trait values, should drive the evolution of positive or negative genetic covariance between jointly selected traits (Lande and Arnold 1983; Phillips and Arnold 1989; Sinervo and Svensson 2002; Jones et al. 2003; Arnold et al. 2008).

Unfortunately, it has proved difficult to connect these general theoretical expectations with specific empirical tests. This

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is due in part to the lack of powerful statistical tools permitting the comparison of (co)variance matrices across multiple populations and selective environments (Houle et al. 2002; Steppan et al. 2002; Blows 2007; Hine et al. 2009). More importantly, however, few organismal systems provide the depth of biological detail (such as the shape of the adaptive landscape, ancestral character states, or the genetic architecture of phenotypes) needed to propose and test specific predictions on the adaptive evolution of trait (co)variances (Turelli 1988; Arnold 1992; Roff 2000; Steppan et al. 2002; McGuigan 2006; Arnold et al. 2008). For instance, even though some studies have documented environment-related changes in phenotypic or genetic (co)variances (Badyaev and Hill 2000; Roff and Mousseau 2005; Berner et al. 2008; Doroszuk et al. 2008), the specific selective mechanisms driving these changes remain unknown or unconfirmed (but see Blows and Higgie 2003).

The second major question concerns the extent to which the genetic (co)variance structure influences trajectories of adaptive diversification. Quantitative genetic theory predicts that a population's response to multivariate selection can be slowed down, accelerated, and/or biased directionally by patterns of genetic trait (co)variance (Lande 1979; Arnold 1992; Björklund 1996; Arnold et al. 2001; Blows and Hoffmann 2005; Agrawal and Stinchcombe 2009; Kirkpatrick 2009). The actual evolutionary trajectory will depend, on the one hand, on the shape of the adaptive landscape, and on the other hand on diverse features of the (co)variance matrix such as its eccentricity and temporal stability, and the strength of trait covariance. Despite a solid theoretical framework for multivariate evolution, it thus remains elusive to which extent the (co)variance structure constrains or facilitates adaptive diversification in nature (Agrawal and Stinchcombe 2009).

Empirical studies have not clarified the issue; some studies support the notion that (co)variances strongly influence the rate or direction of evolution (e.g., Schluter 1996; Blows and Higgie 2003; Bégin and Roff 2004; Hunt 2007) whereas others argue against it (e.g., Merilä and Björklund 1999; Badyaev and Hill 2000; McGuigan et al. 2005; Berner and Blanckenhorn 2006; Berner et al. 2008). A major difficulty here is that information on the adaptive landscape has rarely been incorporated and hence it often remains uncertain whether diversification has been driven by selection.

OUR STUDY

The objective of the present investigation is to use multiple natural populations of threespine stickleback fish (*Gasterosteus aculeatus*) to explore the relationship between natural selection and the multivariate phenotypic (co)variance structure. Stickleback are particularly well suited for this investigation. A first reason is that a key selective force acting within populations has been identified. Lacustrine (lake-dwelling) populations frequently experience intraspecific frequency-dependent competition for food resources (Bolnick 2004; Bolnick and Lau 2008). Within any given lake, individuals specialize on a subset of the available prey resources (Svanbäck and Bolnick 2007; Araujo et al. 2008; Bolnick and Paull 2009). Some individuals consume preferentially limnetic prey (zooplankton occurring in the open water) whereas others specialize on benthic prey (mainly macro-invertebrates occurring on the bottom substrate). This divergent resource specialization is most clearly seen in the sympatric limnetic and benthic species pairs (Schluter and McPhail 1992), where reproductive isolation allows each species to approach its respective multivariate phenotypic optimum.

In the majority of lakes, however, there is only a single panmictic population and the limnetic–benthic prey distribution generates disruptive selection. That is, individuals specializing on either limnetic or benthic resources have higher fitness than generalist individuals exploiting both resource types. Because prey use is determined in part by foraging morphology (Bentzen and McPhail 1984; Lavin and McPhail 1986; Ibrahim and Huntingford 1988; Schluter 1993, 1995; Robinson 2000; Araujo et al. 2008), frequency-dependent competition for prey should lead to persistent disruptive selection on foraging traits. This has been confirmed directly for gill raker morphology (Bolnick 2004; Bolnick and Lau 2008): selection favors individuals with long and numerous or few short gill rakers over individuals that are intermediate in these traits. The strength of this selection varies among populations, presumably due to variation among lakes in the opportunity for individual specialization on limnetic versus benthic prey. Indeed, lakes that offer the two foraging habitats in roughly balanced proportion appear to exhibit the strongest disruptive selection (Bolnick and Lau 2008).

Despite these extensive data on selection acting within lacustrine stickleback, it is not known whether trait (co)variances exhibit adaptive divergence among stickleback populations. Suggestive evidence derives from the finding of relatively consistent differences in multivariate phenotypic (co)variance structure within multiple independent pairs of parapatric lake and stream stickleback (Berner et al. 2008). Another study found that phenotypic variances of individual traits were related to lake size, perhaps reflecting differences in trophic niche diversity among populations (Nosil and Reimchen 2005). Finally, lacustrine populations tend to exhibit strongest sexual dimorphism in lakes in which disruptive selection is also expected to be strongest (Bolnick and Lau 2008).

A first goal of our study is to test the hypothesis that differences among lakes in the strength of disruptive selection have driven predictable divergence in the magnitude of trait (co)variance among populations. This divergence should be strongest in traits known to be targeted by selection (gill raker morphology). Alternatively, trait (co)variances in stickleback may

not easily be shaped by the adaptive landscape. In this case, the (co)variance structure of (derived) lacustrine populations should closely resemble the ancestral state. We examine these alternative hypotheses by combining phenotypic (co)variance matrix comparison among lacustrine stickleback with indirect information on selective conditions within habitats, and with information on ancestral patterns of trait (co)variance.

In addition to the within-population niche variation mentioned above, substantial adaptive divergence is also evident among lacustrine stickleback populations. Specifically, larger lakes contain proportionally more open-water habitat and hence limnetic resources, whereas smaller lakes tend to provide more littoral habitat with benthic resources. Consequently, foraging traits such as gill raker length and number differ predictably among lacustrine populations (Lavin and McPhail 1985; Ibrahim and Huntingford 1988; Schluter and McPhail 1992; Berner et al. 2008). It is unclear whether (or to which extent) this among-lake divergence is biased by within-population (co)variances. A previous analysis showed that the major axis of divergence among lacustrine populations aligned with the major axis of the phenotypic and genetic (co)variance matrix (Schluter 1996). The study concluded that the (co)variance structure can strongly bias patterns of adaptive diversification. However, the (co)variance matrix was estimated from a (derived) lacustrine population rather than from the common ancestor of the lacustrine populations. If the (co)variance structure itself evolves rapidly, comparing axes of diversification and derived (co)variance may be misleading. The second goal of our study is therefore to test the hypothesis that morphological adaptation to lake environments has been biased by ancestral patterns of trait (co)variance.

Material and Methods

STICKLEBACK SAMPLES AND MORPHOLOGICAL TRAITS

Our investigation is based on data from 18 lacustrine stickleback populations (including the 14 studied in Bolnick and Lau 2008) and a single marine population, sampled in the spring of 2005, 2006, and 2009 on central Vancouver Island, British Columbia, Canada (Table 1). Stickleback were caught using unbaited minnow traps as described in Bolnick and Lau (2008). Sample size per site ranged from 196 to 495 (mean: 376), with a total of 6979 fish.

The lakes group to several different watersheds that were almost certainly colonized independently by marine stickleback, as this has been inferred for numerous stickleback populations based on genetic markers (Thompson et al. 1997; Hendry and Taylor 2004; Berner et al. 2009). Even within watersheds, genetic data indicate that gene flow is usually very low between adjacent lakes (Caldera and Bolnick 2008). It is therefore unlikely that the morphological patterns investigated here are influenced materially by gene flow, so that we treat the 18 lacustrine populations as independent datapoints. Conveniently, it is also known that colonization of the lakes has occurred after the last glacial retreat. The lacustrine populations are therefore not older than roughly 12,000 years (generations) (Clague and James 2002). In fact, molecular data suggest that some populations might be substantially younger (Caldera and Bolnick 2008; Berner et al. 2009). This places our investigation in a robust temporal framework.

Table 1. Geographic location, sample year, and sample size for the 18 lacustrine stickleback populations, and for the marine population (last row).

Lake	Latitude (*N*)	Longitude (*W*)	Sample year	Sample size
Big Mud	50°12′01″	125°33′59″	2006	397
Blackwater	50°10′40″	125°35′20″	2005	485
Cecil	50°14′13″	125°32′35″	2006	399
Cedar	50°12′09″	125°33′58″	2005	243
Dugout	50°10′57″	125°31′26″	2006	196
Farewell	50°12′01″	125°35′14″	2005	300
First	50°03′07″	125°47′09″	2005	493
Gosling	50°02′43″	125°30′41″	2006	399
Gray	50°03′27″	125°35′40″	2006	398
Little Mud	50°12′23″	125°33′00″	2006	395
Little Woss	50°10′51″	126°36′39″	2005	299
McCreight	50°17′08 ″	125°38′46″	2005	495
McNair	50°13′40″	125°34′31″	2006	399
Mohun	50°09′47″	125°29′18″	2006	396
Ormond	50°10′49″	125°31′30″	2006	201
Roberts	50°12′45″	125°32′03″	2006	397
Second	50°03′28″	125°47′03″	2006	400
Snow	50°18′44″	125°35′41″	2005	475
Sayward Estuary	50°22′38″	125°57′05″	2009	212

Only a single marine sample (Sayward Estuary) was used as representative of the ancestral stickleback type. This limited sampling is justified because the morphology of marine fish is generally uniform over wide geographic regions, and because the watersheds of all lakes sampled drain into the sea within a few kilometers from Sayward Estuary, or into that estuary itself (First Lake, Second Lake). Furthermore, fossils indicate that marine stickleback morphology has remained highly constant over millions of years (Bell and Foster 1994). We therefore assume that our present-day marine sample closely resembles the common ancestor of the derived lacustrine stickleback populations studied.

On each individual, we determined body mass (formalin-preserved) and measured or counted five morphological traits by following the methods described in Bolnick (2004): total length, body depth, gape width, gill raker number, and gill raker length.

The specific trait combination was chosen for consistency with previous work on divergence among lacustrine stickleback populations (same traits as in Schluter and McPhail 1992; Schluter 1996), and because the link between these traits and foraging performance is relatively well established. Specifically, body length and depth together influence overall body shape and hence swimming and maneuvering performance (Webb 1984; Walker 1997; Blake 2004), whereas gape width, gill raker number, and gill raker length influence prey capture and handling efficiency (Bentzen and McPhail 1984; Lavin and McPhail 1986; Ibrahim and Huntingford 1988; Schluter 1993; Gerking 1994; Robinson 2000). For most of these traits, substantial additive genetic variance has been reported from several stickleback populations (Hagen 1973; Lavin and McPhail 1987; Schluter 1996; Hatfield 1997; Hermida et al. 2002; Aguirre et al. 2004; for instance, heritability estimates range from 0.23 to 0.81 for gill raker length).

Stickleback often exhibit sexual dimorphism in foraging morphology (e.g., Reimchen and Nosil 2004; Bolnick and Lau 2008). We therefore performed all analyses below for males and females separately, and for the sexes pooled. All three sets of analyses produced similar results supporting identical conclusions. We therefore present only results for the sexes pooled.

CHARACTERIZATION AND COMPARISON OF PHENOTYPIC (CO)VARIANCE STRUCTURE

Throughout this study, our focus lies on phenotypic rather than genetic patterns of trait (co)variance. Obtaining statistically robust estimates of genetic (co)variances for all 19 study populations would have been prohibitively labor-intensive. Furthermore, such estimates typically suffer low precision (Lynch and Walsh 1998), so that estimates of phenotypic (co)variances may often provide meaningful surrogates for their genetic counterpart (Cheverud 1988; Roff 1996; Schluter 1996; Roff et al. 1999; Badyaev and Hill 2000; Bégin and Roff 2004; but see Willis et al. 1991). This is expected to hold in particular for traits with substantial heritability (Lande 1979), which is that case for the traits studied here (see above).

Prior to any analysis, it was necessary to decouple trait (co)variances from means and measurement scales. We did so by mean-scaling the traits within each population separately (i.e., dividing raw trait measurements by the corresponding population mean). Even though mean-scaling is recommended (Houle 1992; Hansen and Houle 2008; Kirkpatrick 2009), all analyses were repeated with ln-transformed traits, which produced similar results (not presented). In a second preliminary step, the traits were body size-adjusted because all except for gill raker number are correlated with overall body size. For this, we regressed each of the five mean-scaled traits against linearized (cube-root transformed) body mass, again for each population separately. The residuals were treated as scale- and size-independent morphology.

These data were used to estimate the (co)variance matrix for each population. After spectral decomposition of the matrices, we calculated confidence intervals for both the eigenvalues and the trait loadings on the eigenvectors across the lake populations to explore the consistency of lacustrine (co)variance matrices, and differences between lake populations and the ancestor. (We here corrected for the fact that the eigenvectors showed unstable sign structure [i.e., sign of the trait loadings] among populations, and that eigenvectors 2 and 3 sometimes swapped rank order because their eigenvalues were similar. The corresponding adjustments were straightforward because all eigenvectors were easily identified owing to strong loading by a single trait.)

For more formal matrix comparison, we combined three complementary approaches. First, we tested whether populations differed significantly in phenotypic (co)variance structure by using the jackknife multivariate analysis of variance (MANOVA) method (Roff 2002; Bégin et al. 2004). Briefly, this approach converts population-level (co)variances to individual (co)variance pseudovalues by sequentially deleting single individuals from a population and recalculating (co)variances according to the standard jackknife procedure (Manly 2007). The resulting pseudovalues represent approximate random variables that can be analyzed in factorial designs. We subjected the pseudovalues to MANOVA with population as factor. This was done both for the 18 lacustrine populations only, and then repeated for the full dataset including the marine population.

Second, we tested whether the lacustrine populations had diverged from their marine ancestor in specific attributes of the (co)variance matrix. Following Jones et al. (2003), we tested for differences in (1) matrix eccentricity (expressed as the ratio of the first eigenvalue to the sum of the remaining eigenvalues), (2) overall matrix size (sum of all eigenvalues), and (3) matrix orientation (angle between the first eigenvectors of the lacustrine vs. marine (co)variance matrices). Variance in gill raker length was included as an additional key attribute because this trait has been shown to be the main target of disruptive selection (Bolnick 2004; Bolnick and Lau 2008). Significance of lake versus marine shifts in these four attributes was assessed by using bootstrap (resampling with replacement) tests following the logic described in Manly (2007, p. 73). In short, we tested the null hypothesis that the marine value for each of the matrix attributes was a random sample drawn from the distribution based on the lacustrine populations. The test distribution was generated by bootstrapping the lakes at the population level, and the marine population at the specimen level. To test the significance of the angle (matrix orientation), we proceeded analogously by applying the bootstrap vector comparison methodology described in Berner (2009). The strength of these bootstrap tests is that they took into account error in the estimation of both the lacustrine and marine population means. However, testing the above hypothesis with the

corresponding two-tailed one-sample *t*-test for each (co)variance matrix attribute produced consistent results (not presented). All bootstrap (and permutation) tests in this article are based on 999 iterations.

In the third approach, we used (co)variance tensor analysis (Hine et al. 2009) to describe among-population differences in (co)variance structure. In brief, a (co)variance tensor summarizes (co)variances among (co)variance matrix elements across multiple populations. Spectral decomposition of the (co)variance tensor produces independent components of divergence in (co)variance structure, the eigentensors. Eigentensors themselves can then be decomposed to obtain eigenvectors (independent linear combinations of the original traits) that drive matrix divergence along an eigentensor (fuller detail on the procedure is given in Hine et al. 2009). We used (co)variance tensor analysis to characterize the major axes of matrix divergence among the lacustrine stickleback populations only, and among the full set of populations.

ANCESTRAL TRAIT (CO)VARIANCE AND DIVERGENCE IN POPULATION MEANS

We next examined whether the ancestral (co)variance structure predicts the orientation of the trajectory of marine-lake divergence, or the major axes of diversification among lacustrine populations. Before these questions could be addressed, we had to rescale body mass for the marine sample. The reason is that marine populations (but not the lacustrine populations studied) generally display a series of bony armor plates along their body (Bell and Foster 1994). As a consequence, marine specimens had strikingly higher body mass for given length measurements compared to the lacustrine samples. This did not affect (within-population) estimation of the (co)variance matrix, but made it impossible to compare all 19 population means in a single body size-free morphological space. We therefore computed individual scores along the first eigenvector extracted from the correlation matrix between body length and body depth (raw measurements; all 6979 specimens pooled). These scores ("PC1-size") were regarded as a robust alternative size metric. We then regressed linearized body mass against PC1-size separately for all lake individuals pooled, and for the marine individuals (the pooled analysis of the lake fish was justified because regression parameters were nearly identical across populations; details not shown). Finally, estimates for the two sets of slope and intercept parameters could be used to statistically remove the extra mass due to armor plates for the marine specimens.

We next performed mean-scaling with all individuals pooled (i.e., individual trait values were divided by the grand mean), followed by regression against linearized body mass (adjusted in the marine fish). The resulting residuals represented scale- and size-independent morphology that still retained population-level differences. These data were used to compute the means for the 19 populations.

The hypothesis that divergence among multivariate means has been biased by the (co)variance structure was examined in two ways. First, we tested whether ancestral (marine) (co)variances predict vectors of morphological change from marine to freshwater mean phenotypes. We here calculated for each population separately the vector connecting its mean to the ancestral marine population mean, and then tested whether the resulting 18 divergence vectors aligned with the first eigenvector of the ancestral (co)variance matrix. Second, we tested whether ancestral (co)variances predict vectors of among-lake divergence in trait means. For this, we extracted the three major eigenvectors from the (co)variance matrix calculated on the lake means, and tested whether these axes aligned with their counterparts extracted from the ancestral (co)variance matrix. (We here compared eigenvector 2 with eigenvector 3 and vice versa because they displayed reversed rank order.) Vector alignment was tested for significance by using the bootstrap vector comparison protocol described in Berner (2009). Bootstrapping was performed at the population level for the lake means, and at the specimen level for the marine population.

SELECTION AS A DRIVER OF (CO)VARIANCE MATRIX EVOLUTION

We explored whether (co)variance matrix evolution was driven by selection by taking three different correlative approaches. First, we hypothesized that if (co)variances evolve adaptively, they should be related to habitat features that likely reflect the shape of the adaptive landscape. To examine this hypothesis, we extracted each lacustrine population's score along the major axis of matrix divergence (the first eigentensor). We then tested whether these scores were correlated with the surface area/perimeter (SA:P) ratio estimated for each lake. The SA:P ratio provides a crude estimate of the relative availability of open-water (limnetic) versus littoral (benthic) habitat, and thus the likely strength of disruptive selection arising from habitat heterogeneity. We predicted a correlation between eigentensor scores and habitat heterogeneity, and considered the possibility for both linear and quadratic relationships. No attempt was made to relate (co)variance matrix divergence directly to estimates of quadratic selection gradients (Bolnick and Lau 2008), as these data were available only for a subset of the populations studied here.

The availability of multivariate population means allowed us to carry out a second indirect test for the role of selection in (co)variance matrix evolution. Differential availability of benthic versus limnetic prey among lakes leads to among-population divergence in phenotypic means (Lavin and McPhail 1985; Ibrahim and Huntingford 1988; Schluter and McPhail 1992; Berner et al.

Table 2. Description of the three leading eigenvectors of the phenotypic (co)variance matrix. The first data row indicates the importance (percentage of total variance explained) of each eigenvector (EVec), the subsequent rows give the trait loadings on the eigenvectors. The left-hand columns give the summary statistics over the 18 lake populations (mean values, with associated 95% confidence intervals in parentheses); the right-hand columns show the estimates for the ancestral (marine) population.

	Lake populations			Marine population		
	EVec1	EVec2	EVec3	EVec1	EVec2	EVec3
Eigenvalue (%)	69.3 (±0.032)	13.5 (±0.016)	12.7 (±0.020)	57.5	25.5	11.1
Body length	0.016 (±0.011)	0.007 (±0.016)	0.002 (±0.031)	0.069	−0.058	0.052
Body depth	0.000 (±0.007)	0.002 (±0.016)	−0.021 (±0.016)	−0.038	−0.058	−0.002
Gape width	0.122 (±0.044)	0.057 (±0.153)	0.933 (±0.046)	0.532	0.842	−0.053
Gill raker number	0.001 (±0.022)	0.945 (±0.046)	−0.059 (±0.155)	−0.017	0.074	0.996
Gill raker length	0.987 (±0.008)	−0.007 (±0.028)	−0.117 (±0.044)	0.843	−0.528	0.049

2008). If resource-based selection is driving deterministically both (co)variances and means, an association between the two should be detectable. For instance, large lakes may drive shifts toward a predominantly limnetic phenotype with long gill rakers *and* reduced variance in that trait. We tested this by regressing the populations' first eigentensor scores against their scores on the major axis of divergence in mean morphology (first eigenvector of the (co)variance matrix of population means).

The third approach, finally, was based on the empirical finding that in lacustrine stickleback, sexual dimorphism represents an adaptive response to disruptive selection (Bolnick and Lau 2008). Lakes with a resource distribution promoting strong disruptive selection should thus exhibit the stickleback populations with the strongest sexual dimorphism and hence highest (co)variances. To test this, we expressed the magnitude of sexual dimorphism for each population as the length of the vector connecting the male and female multivariate mean (data were first made scale- and body size-independent within each population, see above). We then regressed population scores along the first eigentensor against the magnitude of sexual dimorphism.

These three tests were performed by using random permutation, with the eigentensor scores (response) permuted over the lakes, and the linear model's *F*-value used as test statistic (Manly 2007). We report all analyses based on the lacustrine populations only. Including the marine population in the latter two tests (the SA:P ratio used in the first test could not be calculated for the marine population) produced very similar results. Similar results were also obtained by performing univariate tests with gill raker length data only (i.e., substituting variance in gill raker length for the eigentensor scores, population mean gill raker length for population eigenvector scores, and sexual dimorphism in gill raker length for multivariate dimorphism). All analyses and graphics were carried out with the R statistical language (R Development Core Team 2009).

Results

(CO)VARIANCE MATRIX CHARACTERIZATION AND COMPARISON

Several aspects of the phenotypic (co)variance structure were shared across the 18 lacustrine populations. The first eigenvector (EVec1) generally accounted for a very substantial proportion (70% on average; range: 56–80%) of the total variation and essentially reflected variance in gill raker length (Table 2, Fig. 1). EVec2 and EVec3 together captured nearly all of the remaining variation (around 13% each) and were driven largely independently by gill raker number and gape width. Hence, there was no indication of

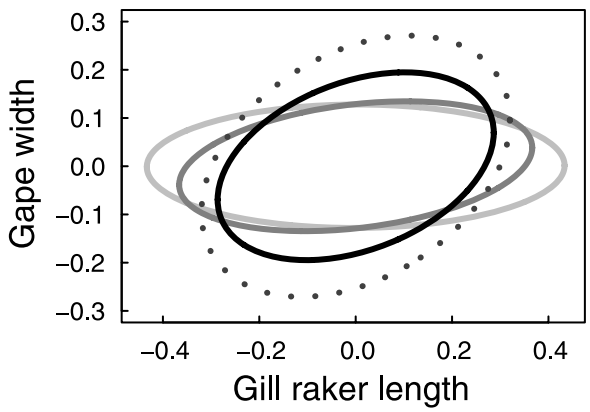

Figure 1. Differences in phenotypic (co)variance structure among stickleback populations visualized in the gill raker length/gape width plane. Shown are 95% confidence ellipses fitted to body size-adjusted and mean-scaled data from three lacustrine stickleback populations (solid ellipses), and the marine population (dotted ellipse). The lake populations were chosen to represent the minimum (black; McCreight) and maximum (light gray; Little Mud) scores along the major axis (first eigentensor) of (co)variance matrix divergence, as well as an intermediate population (dark gray; Dugout) (see Fig. 2).

substantial covariance among body size-adjusted morphological traits in the lacustrine fish; phenotypic variation was mainly in the diagonal elements of the (co)variance matrix. The marine stickleback population clearly differed in (co)variance structure from the average lacustrine population (Table 2, Fig. 1). Here, EVec1 accounted for a relatively smaller proportion of the total variation, and both EVec1 and EVec2 showed substantial (positive and negative) covariance between gape width and gill raker length. (For more detail on (co)variance and correlation matrices for the lake and marine populations see Appendix 1 and Table S1.)

Jackknife MANOVA indicated highly significant overall (co)variance matrix divergence in stickleback, both when comparing across lacustrine populations only ($F_{270,80445} = 4.17$, $P < 0.0001$, F approximated by Wilks' lambda), and with the marine population included ($F_{270,80445} = 5.29$, $P < 0.0001$). In both analyses, most of the univariate tests for shifts in individual elements of the (co)variance matrix remained significant after controlling the false discovery rate (Benjamini and Hochberg 1995) at the 0.01 level (details not presented). This made it particularly valuable to explore matrix divergence by using the specific bootstrap tests, and in an integrated way by using covariance tensor analysis.

The bootstrap tests confirmed a strikingly (43%) lower eccentricity (ratio of first eigenvalue to the sum of the remaining eigenvalues) of the marine (co)variance matrix as compared to the lacustrine populations ($P = 0.001$). Overall matrix size (sum of all eigenvalues) was 31% greater in the ancestral population ($P = 0.004$), but gill raker length variance was 18% lower ($P = 0.042$). Moreover, the orientation of EVec1 of the marine population was clearly different ($P = 0.001$) from the lacustrine populations: the average marine-lake angle was 25°, as compared to an average of 6.3° within 30 randomly chosen lacustrine population pairs.

Covariance tensor analysis revealed that most of the divergence in phenotypic (co)variance structure was captured by a single major eigentensor (Fig. 2, Table 3), irrespective of whether the lacustrine populations were analyzed alone or together with the marine population. Nearly the entire variation along this eigentensor was contributed by a single eigenvector, which itself essentially reflected variance in gill raker length. Consistent with the bootstrap test above that indicated lower gill raker length variance for the ancestor, the marine population was positioned at the lower end along this eigentensor (Fig. 2). The second eigentensor captured a relatively minor proportion of the matrix divergence among populations and was characterized by a single eigenvector driven by positive covariance between gape width and gill raker length. This axis strongly separated the marine from the lacustrine populations. None of the subsequent eigentensors captured more than 3.3% of the divergence in the (co)variance matrix.

To summarize the salient patterns: all stickleback populations shared the common feature that variance in gill raker length was dominating the phenotypic (co)variance structure. Despite this similarity, (co)variance matrices had clearly evolved. The most striking features of matrix evolution concerned extensions and contractions along the gill raker length axis, and reduced covariance between gill raker length and gape width during transition from the ocean to freshwater.

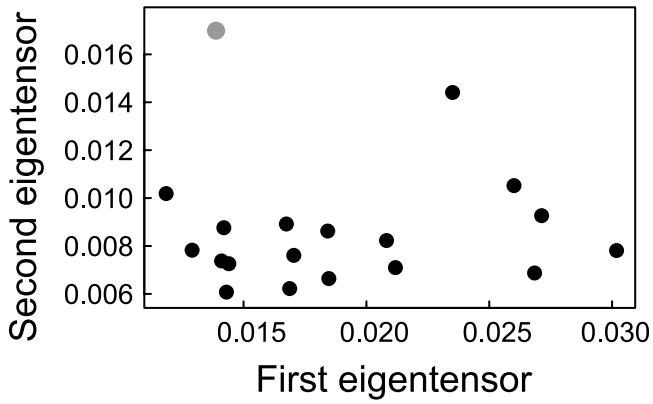

Figure 2. Divergence in the phenotypic (co)variance structure among the 19 stickleback populations. The dots (gray for the marine population) indicate the scores along the two major axes (eigentensors) of matrix evolution. The first eigentensor essentially reflects shifts in gill raker length variance, whereas the second eigentensor captures mainly covariance between gill raker length and gape width. For further details on the eigentensors see Table 3.

Table 3. Characterization of the two major independent axes of (co)variance matrix evolution (eigentensors) among the 19 stickleback populations (subsequent eigentensors captured an immaterial proportion of the variation among (co)variance matrices and are not described). The first data row gives the proportion of total variation among (co)variance matrices accounted for by each eigentensor. The second data row shows the proportion of variation along each eigentensor that is explained by its dominant eigenvector. The following rows specify the trait loadings along the dominant eigentensor-eigenvectors. Note that the first eigentensor is driven almost entirely by variance in gill raker length. The analysis with the marine population excluded produced similar results that are not described.

	First eigentensor	Second eigentensor
Eigenvalue (%)	74.9	18.7
	First eigentensor-eigenvector	First eigentensor-eigenvector
Eigenvalue (%)	98.1	98.6
Body length	−0.035	0.069
Body depth	0.027	−0.053
Gape width	−0.083	0.884
Gill raker number	0.012	−0.045
Gill raker length	0.996	0.458

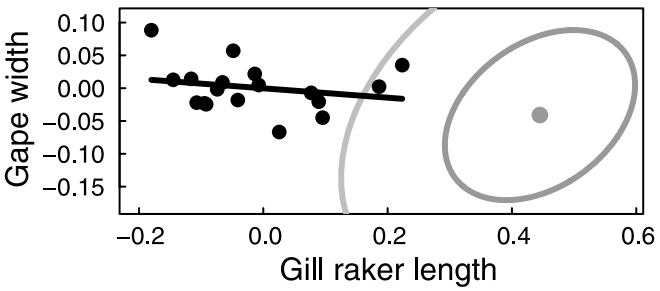

Figure 3. **Relationship between diversification among multivariate population means (dots; ancestor in gray) and the ancestral structure of trait (co)variance (ellipses; 95% confidence in light gray, 50% confidence in dark gray), visualized in the gill raker length/gape width plane. The major axis of divergence among populations (black line) matches the pattern of ancestral (co)variance poorly. Note that the black line here represents the average marine-lake trajectory as well as the major axis of among-lake diversification, as in this trait plane the two vectors are nearly identical.**

ANCESTRAL (CO)VARIANCES AND DIVERGENCE AMONG POPULATION MEANS

The divergence of lacustrine stickleback from the marine population was characterized by a dramatic reduction in gill raker length, with very minor shifts in the other traits (Fig. 3, Table 4; see Table S2 for trait means for each population). The 18 marine-lake divergence vectors deviated from the major axis of trait (co)variance estimated for the ancestor by 40° on average (range: 32–55°), and vector comparison consistently indicated noncollinearity between these axes (all 18 tests: $P = 0.001$; the divergence vectors and angles are described in detail in Table S3). The covariance structure of the ancestor also predicted poorly the major axes of divergence among the lake population means (described in Table 4): the first two eigenvectors clearly differed in orientation (angle for EVec1: 36.7°, $P = 0.001$; EVec2: 43°, $P = 0.009$); vector collinearity was indicated for EVec3 only (angle: 16.4°, $P = 0.3$). Our analysis of divergence in multivariate population means thus yielded no evidence for a strong directional association between axes of diversification and ancestral trait (co)variance.

Table 4. **The major axes of multivariate diversification among stickleback populations. The left-hand column describes the average trajectory of marine-lake divergence (mean trait loadings across the 18 vectors, with associated 95% confidence intervals in parentheses). The right-hand columns describe the three dominant eigenvectors of the (co)variance matrix calculated from the lake population means.**

	Marine-lake divergence	Divergence among lake means		
		EVec1	EVec2	EVec3
Eigenvalue (%)		73.7	14.6	7.9
Body length	−0.095 (±0.028)	0.049	0.058	0.075
Body depth	0.120 (±0.040)	0.038	0.196	0.248
Gape width	−0.092 (±0.042)	−0.001	−0.509	0.852
Gill raker number	−0.047 (±0.051)	0.179	0.821	0.445
Gill raker length	0.969 (±0.014)	0.982	−0.160	−0.094

SELECTION AS A DRIVER OF (CO)VARIANCE MATRIX EVOLUTION

The position of the lacustrine populations along the first eigentensor showed a significant linear association ($r = -0.47$, $P = 0.049$) with estimated lake habitat heterogeneity (SA:P ratio; Fig. 4A) (a quadratic model did not improve the fit). Stickleback in lakes with a high SA:P ratio tended toward low scores on the first eigentensor (i.e., low gill raker length variance) as compared to conspecifics from lakes with a low ratio. Divergence in (co)variance structure was also related negatively to divergence in population means (Fig. 4B; $r = -0.48$, $P = 0.041$). Populations with low variance in gill raker length tended toward long gill rakers on average (high scores on the major axis of divergence in means). Finally, eigentensor scores were correlated positively with the magnitude of sexual dimorphism (Fig. 4C; $r = 0.56$, $P = 0.015$). Lacustrine populations exhibiting relatively high gill raker length variance also displayed the most pronounced dimorphism in that character. (Note that sexual dimorphism was lowest—near-zero for gill raker length—in the marine population; Table S4.) We emphasize, however, that divergence in (co)variances was attributable only partly to sexual dimorphism, as sex-specific analyses produced results consistent with the pooled analysis.

Discussion

The role of multivariate (co)variances in evolution remains poorly understood. Key questions include the temporal stability of the (co)variance structure, whether natural selection is effective at shaping (co)variances, and to which extent (co)variances bias trajectories of diversification in multivariate means (Roff 2000; Steppan et al. 2002; Blows and Hoffmann 2005; McGuigan 2006; Arnold et al. 2008; Agrawal and Stinchcombe 2009). These questions have proved hard to resolve theoretically, so that empirical information is needed. We here set out to obtain such information by an investigation across multiple lacustrine stickleback populations and their ancestor. Our main findings are that the colonization of freshwater by marine stickleback has coincided with substantial shifts in phenotypic (co)variance structure. These changes appear to be adaptive, as they are correlated with lake

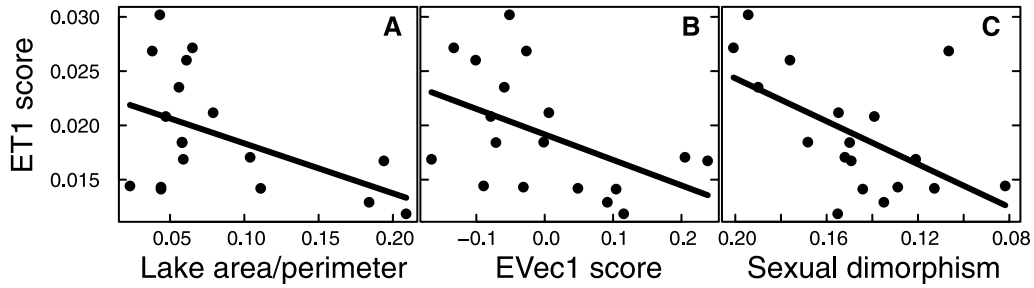

Figure 4. Divergence in (co)variance structure among stickleback populations is related to an ecological proxy for the strength of disruptive selection acting within populations (A), to divergence in morphological means (B), and to the magnitude of sexual dimorphism within populations (C). Populations with low gill raker length variance (low scores along the first eigentensor, ET1) tend to occur in lakes with presumably more stabilizing selection (high area/perimeter ratio), display long gill rakers on average [high scores on the first eigenvector (EVec1) of the (co)variance matrix of multivariate means], and low sexual dimorphism. Note that for consistency among the plots, the abscissa is inverted for (C). Only data for the lacustrine populations are shown.

ecology, population mean morphology, and sexual dimorphism. Finally, trajectories of marine-freshwater divergence in population means are not well predicted by ancestral (co)variances, suggesting that trait covariance has not appreciably biased evolutionary trajectories over the medium-term (thousands of generations).

IS (CO)VARIANCE MATRIX DIVERGENCE IN STICKLEBACK DRIVEN BY SELECTION?

Previous work has shown that lacustrine stickleback populations frequently experience disruptive selection on foraging traits owing to resource-mediated intraspecific competition (Bolnick 2004; Bolnick and Lau 2008). Further, the strength of disruptive selection acting in a given lake is predicted to some extent by the estimated opportunity for individual specialization on limnetic versus benthic resources. The consequences of disruptive selection and associated individual specialization on the multivariate (co)variance structure, however, have not been investigated previously.

In the present study, we found that the major axis of (co)variance matrix evolution among populations reflected shifts in gill raker length variance. This trait has been identified as the key trait in stickleback resource specialization (Schluter 1993, 1995; Robinson 2000; Bolnick and Paull 2009), and as the primary target of disruptive selection (Bolnick 2004; Bolnick and Lau 2008). We further found that divergence in (co)variance structure was related negatively to a crude proxy for the resource distribution and hence the expected strength of disruptive selection within a habitat. As predicted, lakes with a high surface area/perimeter ratio (likely corresponding to a primarily limnetic habitat imposing more stabilizing selection) tended to be occupied by stickleback exhibiting low variance in gill raker length relative to lakes with a low ratio. Very low variance was also observed for the strictly limnetic (Bell and Foster 1994) marine population that should experience stabilizing selection too, but for which the SA:P ratio could not be calculated.

Divergence in (co)variances was also correlated negatively to population divergence in multivariate means, which is known to reflect adaptation to local prey resources (Lavin and McPhail 1985; Ibrahim and Huntingford 1988; Schluter and McPhail 1992; Berner et al. 2008). Stickleback populations with long gill rakers on average tended toward low variance in that trait. Finally, we found that the magnitude of trait (co)variance was correlated positively with the magnitude of sexual dimorphism. This dimorphism has been shown to represents an adaptive response to disruptive selection (Bolnick and Lau 2008).

Combined, all these findings argue very strongly for resource-mediated disruptive selection as a key driver of (co)variances in stickleback. Molecular data further rule out drift as a major cause for evolution in (co)variances. The population with the highest observed score on the first eigentensor (i.e., lowest gill raker variance) inhabits the second largest lake in our dataset (McCreight) and has recently been shown to exhibit nearly twice as much neutral genetic variation compared to the population with the lowest eigentensor score (Little Mud, the second smallest lake sampled) (Caldera and Bolnick 2008). Low (co)variance in foraging traits is thus certainly not simply due to loss of variation by drift in small populations.

At this point it is important to evaluate to which extent the observed patterns of trait (co)variance observed within stickleback populations are genetically based or reflect phenotypic plasticity (e.g., foraging-induced). Stickleback from several populations have been raised on limnetic versus benthic food treatments in the laboratory to quantify plasticity in foraging traits. Although adaptive plasticity has been reported for gape width and the number and length of gill rakers by Day et al. (1994), the shifts were modest when compared to genetic differences between the focal limnetic and benthic stickleback morphs. For gill raker length, the key trait in our article, subsequent investigations found no significant diet-induced plasticity (Day and McPhail 1996; Wund et al. 2008; D. Berner and A. Hendry, unpubl. data). Furthermore,

stickleback foraging traits typically exhibit substantial levels of additive genetic variance (Hagen 1973; Lavin and McPhail 1987; Schluter 1996; Hatfield 1997; Hermida et al. 2002; Aguirre et al. 2004). Not denying a contribution by phenotypic plasticity, on balance the available evidence points to a substantial genetic basis to the observed phenotypic patterns, as demonstrated directly for various other organisms as well (Cheverud 1988; Roff 1996; Roff et al. 1999; Badyaev and Hill 2000; Bégin and Roff 2004). Nevertheless, it would be desirable to assess environmental effects on the (co)variance structure directly through reciprocal transplant experiments across distinct foraging habitats in nature.

Taken together, our study strongly indicates that the phenotypic (and probably also genetic) (co)variance structure among stickleback foraging traits has responded deterministically to the curvature of the adaptive landscape. These responses have occurred on a relatively short timescale of a few thousand generations, maybe substantially less (Caldera and Bolnick 2008; Berner et al. 2009). Our study provides rare evidence for evolution in multivariate (co)variances in relation to an identified selective mechanism in nature (see also Blows and Higgie 2003).

VARIATIONAL BIAS TO ADAPTIVE DIVERSIFICATION?

Another key finding of our study was the striking divergence among populations in mean gill raker length, both between lacustrine populations and the ancestor, and among lacustrine populations. This divergence certainly has a strong genetic basis, as this has been found in common garden experiments using several divergent population pairs (Day et al. 1994; Hatfield 1997; Wund et al. 2008; D. Berner and W. Salzburger, unpubl. data). Moreover, differences in gill raker length among lacustrine populations are linked to variation among lakes in the relative availability of limnetic and benthic prey resources (Lavin and McPhail 1985, 1986; Schluter and McPhail 1992; Berner et al. 2008). For the traits studied here, the gill raker length axis thus clearly represents the major line of repeated adaptive divergence between marine and lacustrine stickleback, and among lacustrine populations themselves. A dominant role of gill raker length in population divergence has also been inferred in previous comparisons among lacustrine stickleback populations (Schluter and McPhail 1992), and of lake-stream stickleback population pairs (Berner et al. 2008). Note also that the relatively extreme mean gill raker length exhibited by the marine population is consistent with the ancestral stickleback's highly limnetic lifestyle (Bell and Foster 1994).

Surprisingly, both the trajectories of marine-lake divergence and the major axes of diversification among lake means were poorly predicted by the major axes of ancestral trait (co)variance. Provided that the phenotypic (co)variances estimated for our marine sample reflect the true ancestral genetic (co)variance structure at least approximately, our work argues against directional genetic bias to adaptive diversification even on a relatively short timescale. It appears that traits relevant to stickleback adaptive diversification simply exhibit enough genetic variation segregating independently in the ancestor. This conclusion clearly disagrees with the one drawn in a previous analysis also based on lacustrine stickleback populations from Vancouver Island, and the same suite of morphological traits. Specifically, Schluter (1996) documented alignment between the major axis of among-population diversification and the major axis of both phenotypic and genetic (co)variance estimated for a single lacustrine population. An obvious explanation for the disagreement between the two studies is that here we were able to infer the ancestral (co)variance matrix. Indeed, when comparing ad hoc the divergence among lake population means with the average *lacustrine* (co)variance structure, collinearity was supported for all three leading eigenvectors (EVec1: angle 12.4°, $P = 0.092$; EVec2: 21.4°, $P = 0.119$; EVec3: 32.6°, $P = 0.075$). This finding highlights that collinearity between vectors of trait (co)variance within and among populations can arise if (co)variances within populations evolve to align with the adaptive landscape (Arnold 1992; Arnold et al. 2001; McGuigan 2006). Collinearity thus need not necessarily reflect variational constraints on the direction of adaptation, as commonly assumed.

CONCLUSIONS

We have shown that the structure of multivariate (co)variance among stickleback foraging traits has evolved rapidly and, to a great extent, in response to variation among environments in a key feature of the adaptive landscape. We have also shown that ancestral patterns of trait (co)variance do not predict trajectories of adaptive population divergence over a moderately short time scale (thousands of generations). Our study implicates natural selection as a highly deterministic driver of both means and (co)variances in stickleback. Both the observed (co)variance matrix instability and the poor match between axes of (co)variance within and among populations seriously challenge the hope of quantitative genetics that trajectories of adaptive diversification may be predicted by the structure of trait (co)variance. We caution, however, against an overly strong generalization of our findings. The role of (co)variances in diversification may vary greatly among traits and organisms due to differences in genetic detail and patterns of selection. The identification of general trends, if they exist, therefore awaits a more extensive empirical base. As our stickleback study has shown, comparative investigations performed with populations of known evolutionary relationship and within a strong ecological context are likely to be most informative. We anticipate that even deeper insights into the role of (co)variances in diversification will arise from further progress in developmental genetics and from more direct quantifications of the shape of the adaptive landscape across multiple habitats.

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Appendix 1. **Phenotypic variances and covariances (multiplied by 1000, lower semimatrix) and correlations (upper semimatrix, in bold) among the five foraging traits (body size-adjusted). The upper matrix presents averages across the 18 lacustrine populations, with associated 95% confidence intervals in parentheses. The lower matrix shows values for the marine population, with 95% confidence intervals calculated from jackknife standard errors. Full (co)variance and correlation matrices for all lacustrine populations separately are given in the Table S1.**

Trait	Body length	Body depth	Gape width	Gill raker number	Gill raker length
Lake populations					
Body length	0.656 (±0.069)	**−0.184 (±0.055)**	**0.020 (±0.068)**	**0.029 (±0.017)**	**0.075 (±0.049)**
Body depth	−0.119 (±0.037)	0.693 (±0.095)	**−0.042 (±0.033)**	**0.002 (±0.032)**	**0.012 (±0.033)**
Gape width	0.058 (±0.112)	−0.075 (±0.054)	3.971 (±0.684)	**−0.028 (±0.040)**	**0.195 (±0.052)**
Gill raker number	0.047 (±0.028)	0.004 (±0.056)	−0.117 (±0.156)	3.906 (±0.359)	**0.003 (±0.038)**
Gill raker length	0.259 (±0.185)	0.053 (±0.130)	1.735 (±0.547)	0.029 (±0.333)	20.437 (±2.740)
Marine population					
Body length	1.213 (±0.272)	**−0.099 (±0.151)**	**0.079 (±0.159)**	**0.040 (±0.139)**	**0.300 (±0.132)**
Body depth	−0.110 (±0.165)	1.012 (±0.237)	**−0.223 (±0.171)**	**−0.012 (±0.136)**	**−0.087 (±0.164)**
Gape width	0.302 (±0.611)	−0.779 (±0.574)	12.068 (±2.534)	**0.025 (±0.131)**	**0.354 (±0.121)**
Gill raker number	0.087 (±0.299)	−0.025 (±0.267)	0.170 (±0.895)	3.916 (±0.645)	**−0.055 (±0.144)**
Gill raker length	1.356 (±0.679)	−0.358 (±0.662)	5.051 (±2.139)	−0.443 (±1.156)	16.840 (±3.161)

Supporting Information

The following supporting information is available for this article:

Table S1. Trait (co)variances (×1000) and correlations (shaded gray) for the 18 lacustrine stickleback populations.
Table S2. Trait means for the 18 lacustrine stickleback populations and the marine population (last row).
Table S3. Trajectories of divergence in mean morphology between each lacustrine stickleback population and the ancestor.
Table S4. Magnitude of sexual dimorphism in gill raker length (male minus female mean), and in all traits combined ('multivariate'; length of vector connecting male and female centroids), within the lacustrine and marine stickleback populations.

Supporting Information may be found in the online version of this article.

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Please note: [Correction added after online publication April 7, 2010: last line of abstract corrected to read: **"biases the direction of adaptive diversification predictably"**]