Phenotypic Divergence along Lines of Genetic Variance

Katrina McGuigan,[*] Stephen F. Chenoweth,[†] and Mark W. Blows[‡]

Department of Zoology and Entomology, University of Queensland, Brisbane, Queensland, 4072 Australia

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ABSTRACT: Natural populations inhabiting the same environment often independently evolve the same phenotype. Is this replicated evolution a result of genetic constraints imposed by patterns of genetic covariation? We looked for associations between directions of morphological divergence and the orientation of the genetic variance-covariance matrix (**G**) by using an experimental system of morphological evolution in two allopatric nonsister species of rainbow fish. Replicate populations of both *Melanotaenia eachamensis* and *Melanotaenia duboulayi* have independently adapted to lake versus stream hydrodynamic environments. The major axis of divergence (**z**) among all eight study populations was closely associated with the direction of greatest genetic variance ($\mathbf{g}_{max}$), suggesting directional genetic constraint on evolution. However, the direction of hydrodynamic adaptation was strongly associated with vectors of **G** describing relatively small proportions of the total genetic variance, and was only weakly associated with $\mathbf{g}_{max}$. In contrast, divergence between replicate populations within each habitat was approximately proportional to the level of genetic variance, a result consistent with theoretical predictions for neutral phenotypic divergence. Divergence between the two species was also primarily along major eigenvectors of **G**. Our results therefore suggest that hydrodynamic adaptation in rainbow fish was not directionally constrained by the dominant eigenvector of **G**. Without partitioning divergence as a consequence of the adaptation of interest (here, hydrodynamic adaptation) from divergence due to other processes, empirical studies are likely to overestimate the potential for the major eigenvectors of **G** to directionally constrain adaptive evolution.

Keywords: adaptation, genetic drift, genetic covariance, $\mathbf{g}_{max}$, rainbow fish, *Melanotaenia*.

[*] Corresponding author. Present address: Center for Ecology and Evolutionary Biology, 5289 University of Oregon, Eugene, Oregon 97403-5289; e-mail: mcguigan@darkwing.uoregon.edu.

[†] E-mail: schenoweth@zen.uq.edu.au.

[‡] E-mail: mblows@zen.uq.edu.au.

Independent evolution of the same phenotype in natural populations occupying the same habitat is convincing evidence for the action of natural selection (Endler 1986; Schluter and Nagel 1995). Examples of replicated (convergent or parallel) evolution are increasingly common (Hendry and Kinnion 2001), especially in fish (e.g., Endler 1995; Bernatchez et al. 1996; Pigeon et al. 1997; Ruber et al. 1999; Rundle et al. 2000). Although such patterns represent strong evidence of adaptation, we do not fully understand why independently evolving populations arrive at the same solution to a particular environmental challenge. The response of a multivariate set of traits to selection depends jointly on the selection gradient (β) and the matrix of additive genetic variances and covariances (**G**; Lande 1979). Therefore, replicated independent evolution of the same phenotype could be the consequence of a single selective optimum, or, in the presence of multiple selective optima, the orientation of **G** may constrain populations to evolve toward a particular peak (Lande 1979, 1980; Cheverud 1984; Zeng 1988; Arnold 1992; Björklund 1996; Schluter 1996).

To test the hypothesis that replicated evolution is due to directional constraints imposed by **G**, it is necessary to statistically associate the vector describing the direction of divergence with the pattern of genetic covariation. In an influential article that has transformed how genetic constraints are studied by empiricists, Schluter (1996) developed an approach that compared the major axis of phenotypic divergence among populations (**z**) to the major axis of **G** ($\mathbf{g}_{max}$, the genetic line of least resistance). Blows and Higgie (2003) extended this approach by considering the entire space of phenotypic divergence and genetic variance, comparing **G** to the divergence variance-covariance matrix of population means, **D** (Lande 1979), of which Schluter's **z** was the first principal component (Schluter 1996). Vector and matrix comparisons have been used to investigate directional constraints imposed on evolution by the genetic basis of the traits under study (e.g., Schluter 1996; Arnold and Phillips 1999; Merila and Björklund 1999; Badyaev and Hill 2000; Blows and Higgie 2003). As yet, it is perhaps too early to draw any general conclusions

concerning the prevalence of genetic constraints on adaptive divergence and the timescale over which they may operate.

In addition to its potential influence on adaptation, **G** is likely to exert a directional influence on phenotypic evolution driven by random genetic drift (Lande 1979; Arnold et al. 2001; Phillips et al. 2001). However, previous studies of the directional influence of **G** on evolution have not distinguished divergence as a consequence of selection from that due to neutral processes. Comparing the orientation of **G** with directions of phenotypic divergence potentially confounds associations generated by natural selection with those generated by genetic drift (Phillips et al. 2001). Here, we show how naturally replicated adaptive evolution presents an opportunity to investigate this issue. We use just such a system to demonstrate statistical approaches for partitioning divergence due to different evolutionary processes and for associating those divergence vectors with the orientation of **G**.

Phenotypic Divergence in Rainbow Fish Morphology

Rainbow fish (Atheriniformes: Melanotaeniidae) are small, ubiquitous freshwater fish, endemic to Australia and New Guinea (Allen 1995). Replicate populations of two nonsister allopatric species, *Melanotaenia eachamensis* and *Melanotaenia duboulayi*, have morphologically adapted to the distinct hydrodynamic selection regimes of lakes and streams (McGuigan et al. 2003). We used this system of replicated hydrodynamic adaptation to partition phenotypic divergence into three levels: between habitats, between species, and between replicate populations within species and habitat. We then explore the relationship between **G** and the direction of hydrodynamic adaptation between habitats and contrast this to the association of **G** with the direction of divergence due to other processes.

Stream populations of both *M. eachamensis* and *M. duboulayi* have repeatedly colonized closed catchment lakes. Lake populations have evolved a distinct body shape, correlated with divergent locomotor performance and muscle morphology (McGuigan et al. 2003). Habitat-specific morphology persisted in fish bred from lake or stream parents and raised in a common laboratory environment, indicating a genetic basis to the habitat divergence (McGuigan et al. 2003). Replication of a heritable, habitat-specific locomotor phenotype indicated that divergence between lake and stream fish was a consequence of natural selection on hydrodynamics (McGuigan et al. 2003).

There is substantial morphological variation among rainbow fish that is not associated with differences in hydrodynamic regime (Allen and Cross 1982; Allen 1995; McGuigan et al. 2000, 2003; McGuigan 2001). Rainbow fish species boundaries inferred using molecular markers are consistent with the distribution of morphological variation (Zhu et al. 1994, 1998; Jones 1999; McGuigan et al. 2000; McGuigan 2001). On the basis of current and historical biogeography and the distribution of molecular and morphological variation, the species group containing *M. eachamensis* and *M. duboulayi* was hypothesized to have diverged through random genetic drift following repeated range fragmentation of a widespread, stream-dwelling northern ancestor (McGuigan et al. 2000; McGuigan 2001). However, hypotheses of adaptive divergence have not been explicitly tested, and uncharacterized differences in selection regimes might also have contributed to phenotypic divergence among species. *Melanotaenia eachamensis* and *M. duboulayi* both occupy hydrodynamic habitats ranging from headwater streams to lakes. Therefore, phenotypic divergence between species is unlikely to be due to adaptation to different flow regimes. As we show below, species divergence can be statistically partitioned from divergence between hydrodynamic selection regimes, and we can then determine whether species divergence is associated with the eigenstructure of **G**.

Finally, replicate populations within a single environment could also diverge. Such divergence might arise either from genetic drift or uncharacterized differences among replicate habitats generating population-specific selective optima. Statistical analyses in evolutionary biology tend to treat variation among populations within selection regimes (replicates) as nuisance or error variation. Conspecific rainbow fish populations occupying the same hydrodynamic environment are not morphologically identical (McGuigan et al. 2003). Replicate intraspecific populations, diverging through drift or selection, provide another estimate of divergence that we can associate with the eigenstructure of **G** to explore the influence of **G** on the direction of evolution at this level.

In summary, we studied rainbow fish morphological evolution using a single experimental design in which we partitioned phenotypic divergence due to natural selection by hydrodynamic habitat from divergence due to other processes. We developed an approach in which we decomposed **D** to extract vectors describing divergence among hydrodynamic habitats, among species, and among replicate populations within species and hydrodynamic habitat. The orientation of each phenotypic divergence vector was then associated with the orientation of **G** using matrix projection methodology (Blows et al. 2004). We addressed two questions. First, was hydrodynamic adaptation constrained to occur along genetic lines of least resistance? Second, did **G** influence divergence in similar ways irrespective of the evolutionary process that may have driven the divergence?

Methods

In northeast Queensland, Australia, 10 male and 10 female *Melanotaenia eachamensis* were collected from each of two lakes and one stream, while 22 males and 22 females were collected from a second stream (South Johnstone River). Ten male and 10 female *Melanotaenia duboulayi* were collected from two lakes and two streams in southeast Queensland (refer to the study by McGuigan et al. [2003] for collection site information). We elected to estimate genetic parameters (**G**) using the South Johnstone River *M. eachamensis* because the molecular phylogeny indicated that a northern stream-dwelling taxon was the ancestor of this group of rainbow fish. We generated 44 families from natural matings. Rainbow fish have external fertilization and no parental care. Green acrylic mops provided a laboratory surrogate for egg attachment. Mops were checked daily, and if eggs were present, mops were removed to 2-L hatching containers. After 30 eggs were obtained, adults were removed from the breeding tank, and the eggs/fry were replaced in the tank. Each parent was bred a second time (following the same procedure) with a different partner. All tanks were in the same room, maintained at 25°C with 12L : 12D. Families were assigned to tanks randomly. Fry were initially fed twice daily with Serra Vipera fry food, replaced with flake food when they were large enough to consume it.

Morphology was characterized using a truss network (Strauss and Bookstein 1982) with 10 landmarks and 21 interlandmark distances (fig. 1; McGuigan et al. 2003). Data were natural log (ln) transformed to ensure covariances were scale invariant and mean independent (Bookstein et al. 1985). Parents from the genetic experiment were measured prior to mating and offspring at 6 months of age when sexually mature. All wild-caught fish (including parents) were measured within 2 weeks of capture. To maintain equal sample sizes across groups, only 10 males and 10 females (randomly selected) from South Johnstone were included in estimates of phenotypic divergence.

Genetic Basis of Locomotor Morphology

A total of 375 offspring were obtained from the 44 families. Differential mortality resulted in families ranging from two to 15 offspring. This variation was not considered substantial enough to impact genetic estimates (Bohren et al. 1961; Falconer and Mackay 1996). Laboratory-reared offspring were regressed on wild-caught parents to estimate **G** (Riska et al. 1989). Variances did not differ significantly between males and females, nor were there consistent differences in estimates of maternal versus paternal heritability. Therefore, heritabilities were estimated using family

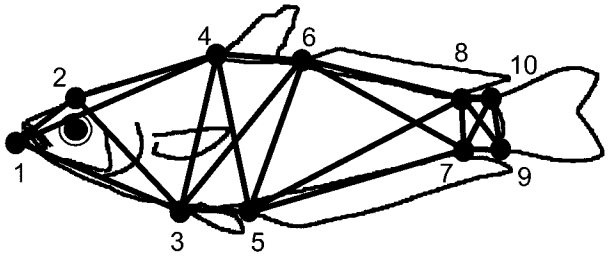

Figure 1: Truss network with 10 landmarks (Strauss and Bookstein 1982). Landmarks were most anterior point of snout (*1*), dip above front of eye (*2*), origin of pelvic fins (*3*), origin of first dorsal fin (*4*), origin of anal fin (*5*), origin of second dorsal fin (*6*), insertion of anal fin (*7*), insertion of second dorsal fin (*8*), posterior point of the caudal peduncle ventrally (*9*), and posterior point of the caudal peduncle dorsally (*10*). Interlandmark distances were identified with reference to the numbers of the two defining landmarks. Truss boxes are designated 1–4 anterior-posterior.

mean and parent midpoint regressions. Standard errors of heritability estimates were calculated according to Becker (1992, p. 103). Genetic correlations were estimated from the trait variances and covariances following Becker (1992, p. 134).

We utilized a principal components analysis (PCA) to formally explore the genetic covariation of the 21 morphological traits. Because all traits were measured on the same scale, eigenvectors were extracted from the covariance matrix. The first principal component, $\mathbf{g}_{max}$ (Schluter 1996), described the direction of greatest genetic variance, representing the direction of least resistance to evolution. Subsequent principal components described orthogonal, successively decreasing amounts of genetic variance and were designated $\mathbf{g}_2$, $\mathbf{g}_3$, $\mathbf{g}_4$, and so forth.

Phenotypic Divergence

Schluter (1996) introduced the vector **z**, the first principal component of variation among population means, to characterize the direction of phenotypic divergence. We estimated **z**, calculating the mean of each ln-transformed interlandmark distance in each of the eight populations, and extracted principal components from the covariance matrix. A general divergence vector, **z** represents the linear combination of traits explaining the greatest variation among populations from both species and from both habitats.

Schluter's (1996) approach was generalized by Blows and Higgie (2003) to accommodate multiple directions of divergence using the variance-covariance (**D**) matrix of population means (Lande 1979). Neither Schluter's (1996) nor Blows and Higgie's (2003) approach can differentiate

divergences at different levels of organization, although divergence at these different levels might be driven by different evolutionary processes. The major aim of this study was to exploit a system in which we could disentangle divergence due to hydrodynamic selection from that due to other processes and to thus determine whether the direction of locomotor adaptation was biased by **G**. To do this, we extended the approach of Blows and Higgie (2003) to generate vectors summarizing divergence in multivariate morphological space for each of the main effects of species, habitat, and replicate population.

Because of multicollinearity among traits, MANOVA could not be conducted on the raw data. Therefore, to estimate directions of phenotypic divergence, we first conducted a PCA on the 21 ln-transformed interlandmark distances from all 160 fish. This analysis generated 21 orthogonal principal components (extracted from the covariance matrix), from which we calculated principal component scores for each fish. The square matrix describing trait contributions (21 rows) to principal components (21 columns) was designated **C**.

To partition phenotypic divergence attributable to each source in our experimental design, McGuigan et al. (2003) employed the linear model:

$$Y_{ijklm} = \mu + H_i + Sp_j + S_k + P(HSp)_{l(ij)} + HSp_{ij} + HS_{ik} + SpS_{jk} + HSpS_{ijk} + \varepsilon_{m(ijkl)}, \quad (1)$$

where habitat (H), species (Sp), sex (S), the interactions between these main effects (habitat by species [HSp], habitat by sex [HS], species by sex [SpS], and habitat by species by sex [HSpS]), population nested within species and habitat (P[HSp]), and residual error (ε) represented all sources of variation in the experimental design. An ANOVA using equation (1) suggested that the principal components described biologically meaningful variation: the first principal component described body size, and several other principal components were significantly associated with the main effects of habitat, species, sex, and population (McGuigan et al. 2003).

A MANOVA (using eq. [1]) on the principal component scores for each of the 160 fish was used to obtain the sums of squares and cross-products (SSCP) matrices (here termed **H**) for each main effects of habitat, species, and replicate population. Each **H** was scaled by the appropriate error SSCP matrix (**E**) using (Rencher 1998)

$$\mathbf{D} = \mathbf{E}^{-1}\mathbf{H}, \quad (2)$$

where **D** matrices represented the divergence between habitats, species, or populations in 21-dimensional space.

Each **D** was subjected to a PCA (extraction on the covariance matrix) from which the first principal component was analogous to the first canonical variate of divergence ($\mathbf{c}_x$). At both the species and habitat levels, there was a single degree of freedom in the MANOVA, resulting in a single divergence vector that described morphological variation distributed between species or between habitats. Because there were four degrees of freedom at the population level, four canonical variates were extracted, each accounting for a decreasing proportion of the divergence between replicate populations within species and habitat.

This series of transformations resulted in the elements of each $\mathbf{c}_x$ being the contributions of principal components to divergence between groups. In contrast, the orientation of **G** was described by vectors (principal components), which had elements that were relative contributions of each interlandmark distance to genetic variance. Therefore, before comparing directions of phenotypic divergence with the orientation of **G**, each $\mathbf{c}_x$ was transformed back into the original ln-interlandmark distance trait space using

$$\mathbf{d}_x = \mathbf{C}\mathbf{c}_x, \quad (3)$$

where **C** was the 21-by-21 matrix of the contribution of each ln-transformed interlandmark distance (rows) to each principal component (columns).

Divergence vectors in the original trait space ($\mathbf{d}_x$) were designated $\mathbf{d}_{Sp}$, $\mathbf{d}_H$, or $\mathbf{d}_{P1}$ through $\mathbf{d}_{P4}$ for divergence between species, habitat, or replicate populations, respectively. The species divergence vector, $\mathbf{d}_{Sp}$, described only the trait covariation between *M. eachamensis* and *M. duboulayi* that was common to all fish, irrespective of sex, habitat, or population. Similarly, replicate population divergence vectors, $\mathbf{d}_{P1}$ through $\mathbf{d}_{P4}$, described only variation between populations that could not be attributed to habitat, species, sex, or any interactions. Finally, the habitat divergence vector, $\mathbf{d}_H$, described the covariation of traits between stream and lake fish that was common to both species of both sexes from all populations.

Comparison of Lines of Divergence and Genetic Variance

Although comparison with $\mathbf{g}_{max}$ has been used to test for evolution along lines of least selective resistance (Schluter 1996; Arnold and Phillips 1999; Merila and Björklund 1999; Badyaev and Hill 2000; Begin and Roff 2003), the approach has a number of limitations (Blows and Higgie 2003). In particular, eigenvectors other than $\mathbf{g}_{max}$ ($\mathbf{g}_2$, etc.), which can explain considerable proportions of the available genetic variance, might influence the direction of evolution. Therefore, to formally test whether divergence at any of our three levels of interest was associated with lines

of genetic variance, the divergence vectors were projected onto a subspace of **G** using (Blows et al. 2004)

$$\mathbf{p}_x = \mathbf{A}(\mathbf{A}^{\mathrm{T}}\mathbf{A})^{-1}\mathbf{A}^{\mathrm{T}}\mathbf{d}_x, \tag{4}$$

where the matrix **A** contained a subset of principal components of **G** ($\mathbf{A}^{\mathrm{T}}$ is the transpose of **A**) and $\mathbf{p}_x$ was the resultant projection of genetic variance that was closest to the direction of phenotypic divergence, $\mathbf{d}_x$. If all 21 principal components of **G** are considered simultaneously, the projection vector would be returned as the divergence vector ($\mathbf{d}_x = \mathbf{p}_x$) because the complete set of principal components spans the space. We projected $\mathbf{d}_x$ onto a progressive series of subspaces of **G**, starting with the subspace described by only the twentieth principal component and then adding one principal component at a time in ascending order of their eigenvalues until the subspace included all eigenvectors from 20 ($\mathbf{g}_{20}$) through 1 ($\mathbf{g}_{\max}$). In this fashion, we could explore the association of divergence vectors with subspaces of **G** describing increasing proportions of the total genetic variance. Importantly, this method does not require a priori assumptions about which eigenvectors of **G** might influence directions of divergence. Many of the eigenvectors with very small eigenvalues are likely to be unstable and have little biological meaning. By including these eigenvectors in the subspaces, our method effectively allows us to demonstrate their lack of effect on divergence rather than assuming a lack of effect as previous analyses have done. Our sequential projection analytical approach will reveal which, if any, eigenvectors of **G** are associated with each phenotypic divergence vector. For example, if only the direction of greatest genetic variance ($\mathbf{g}_{\max}$) is associated with phenotypic divergence, the analysis will reveal no association between divergence vectors and subspaces of **G** until $\mathbf{g}_{\max}$ is added to **A**.

The relationship between the direction of divergence ($\mathbf{d}_x$) and the projection of that divergence on the subspaces of **G** ($\mathbf{p}_x$) was assessed by calculating the correlation between the vectors,

$$r = \mathbf{p}_x^{\mathrm{T}}\mathbf{d}_x, \tag{5a}$$

and the angle between the vectors,

$$\theta = \cos r. \tag{5b}$$

Angles were naturally constrained to be between 0° (vectors describing the same direction) and 90° (unrelated vectors describing orthogonal directions).

To determine which eigenvectors of **G** were associated with morphological divergence, we estimated 95% confidence intervals for the angles between $\mathbf{d}_x$ and $\mathbf{p}_x$. Error in **G** was estimated by bootstrapping over families (i.e., randomly sampling families), generating 1,000 replicates of **G**. Error in divergence vectors was estimated by random reassignment of individuals. To estimate error in replicate population divergence vectors, fish retained the correct habitat, species, and sex but were randomly allocated to replicate populations and 1,000 randomized estimates of the four replicate population divergence vectors generated. To estimate error in species divergence, fish retained the correct habitat and sex but were randomized across species and population. Finally, to estimate error in habitat divergence, fish retained the correct species and sex but were randomized across habitat and population. Confidence intervals of an angle between the genetic projection and a direction of divergence were estimated by randomly pairing a bootstrapped replicate of **G** with a randomized divergence vector to generate 1,000 estimates of the particular angle under consideration. Ideally, population would not have been randomized in the species- and habitat-level tests, but there were too few populations to shuffle as entire nested units. Therefore, the species- and habitat-level confidence intervals included variation attributable to the population level, biasing them in a conservative manner. Because these procedures were computationally demanding, we calculated confidence intervals only for situations when adding a new principal component of **G** resulted in a large reduction (>45°) in the estimated angle between the projection and divergence vectors.

Results

Genetic Basis of Locomotor Morphology

Persistence of interhabitat divergence in *Melanotaenia eachamensis* raised in a common laboratory environment (McGuigan et al. 2003) led us to expect a heritable basis in at least some of the traits. Interlandmark distances ranged in heritability from 0.11 to 0.79, with 16 of the 21 estimates greater than two standard errors above zero, indicating significant genetic variance in these traits (table A1 in the online edition of the *American Naturalist*). Traits were, on average, strongly positively correlated (average $r_{\mathrm{A}} = 0.94$; table A1). This relationship was apparent in the PCA, where all traits contributed positively and relatively equally to $\mathbf{g}_{\max}$ (table 1). Equal contribution in the same direction from all traits is characteristic of a principal component describing size (Reist 1985; Jolliffe 1986), suggesting that most of the genetic variation in our rainbow fish was in body size (note lack of deformation in fig. 2*A*).

Caudal peduncle length traits (7.9, 7.10, 8.9, and 8.10) were more tightly correlated with one another than with other traits (table A1). This pattern was also apparent in the results of the PCA of **G**. The second principal component, $\mathbf{g}_2$, was dominated by caudal length traits, which

Table 1: First four eigenvectors of **G**

Trait	$\mathbf{g}_{max}$, 96.5%	$\mathbf{g}_2$, 2.3%	$\mathbf{g}_3$, .6%	$\mathbf{g}_4$, .3%
1.2	.18	.12	.13	−.17
1.3	.18	−.08	−.08	.04
1.4	.22	−.10	−.10	−.01
2.3	.20	−.10	−.10	−.08
2.4	.24	−.17	−.16	.09
3.4	.26	−.15	−.15	−.23
3.5	.23	−.08	−.07	.39
3.6	.26	−.12	−.12	.02
4.5	.24	−.17	−.16	−.20
4.6	.20	.07	.07	.45
5.6	.26	−.19	−.19	−.15
5.7	.24	−.15	−.15	.30
5.8	.21	−.10	−.10	.00
6.7	.23	−.11	−.11	.04
6.8	.12	−.14	−.14	.17
7.8	.22	.16	.16	−.44
7.9	.14	.50	.50	.02
7.10	.15	.36	.35	−.21
8.9	.26	.34	.35	.01
8.10	.24	.47	.47	.24
9.10	.21	−.10	−.11	−.24

Note: See figure 1 for trait definitions.

contributed in the opposite direction to most other traits (table 1). Fish with short caudal peduncles and large bodies are at one extreme of $\mathbf{g}_2$, and fish with long caudal peduncles and small bodies are at the other (fig. 2*B*).

Phenotypic Divergence

The major axis of divergence among the eight population means, **z**, also had characteristics of a vector describing body size, with strong positive contributions from all 21 traits (table 2; note lack of deformation in fig. 3*A* relative to other panels of fig. 3). Because **z** described almost all of the phenotypic variance among populations (>99.9%), this result suggested that most rainbow fish evolution involved changes in size. The first eigenvector describing divergence among replicate populations (nested within habitats and species; $\mathbf{d}_{P1}$) also had characteristics of a size vector, although it was dominated by variation in the anterior trunk (truss box 2) and the caudal peduncle (truss box 4; table 2; fig. 3*B*). The second replicate population divergence vector ($\mathbf{d}_{P2}$) was dominated by posterior trunk traits and caudal length, which contributed in the opposite direction to the other traits (table 2); fish with small bodies had long caudal peduncles and vice versa (fig. 3*C*). The species divergence vector ($\mathbf{d}_{Sp}$) also contrasted caudal peduncle length traits with the rest of the body but differed from $\mathbf{d}_{P2}$ in that it was dominated by traits describing anterior trunk depth (3.4, 3.6, 4.5, and 5.6) rather than traits describing the posterior trunk (table 2; fig. 3*D*). Habitat divergence ($\mathbf{d}_H$) described positive covariation of head length with caudal peduncle length and negative covariation of these with trunk length/depth (table 2). This result was consistent with a previous analysis of morphological divergence between lake and stream fish; lake fish had shorter median fins and longer heads than their stream counterparts (McGuigan et al. 2003; fig. 3*E*).

Comparison of Lines of Divergence and Genetic Variance

Rainbow fish have diverged (table 2, **z**) in a direction similar to that described by the first principal component of **G** (table 1, $\mathbf{g}_{max}$; $\theta = 12.9°$, $r = 0.97$; cf. figs. 2*A*, 3*A*). When investigating traits where divergence among some of the populations is known to have been driven by selection, a close association between $\mathbf{g}_{max}$ and **z** has been interpreted as adaptation biased in the direction of $\mathbf{g}_{max}$ (e.g., Schluter 1996). However, it is unlikely that adaptation to lake and stream habitats is the primary cause of the association between $\mathbf{g}_{max}$ and **z** in our system because both vectors describe variation in size, but evolution in body size was not implicated as important in the adaptation of rainbow fish to lakes (McGuigan et al. 2003).

When we associated the divergence vectors for habitat, species, and replicate population with subspaces of **G**, two general patterns emerged. First, projection of each divergence vector onto the largest subspace of **G** ($\mathbf{g}_{20}$ through $\mathbf{g}_{max}$ inclusive) resulted in small angles between the genetic projections and divergence vectors (fig. 4). Second, divergences were not associated with subspaces of **G** defined by principal components describing very small amounts of genetic variance; divergence vectors were orthogonal to subspaces of **G** defined by principal components with small eigenvalues, but angles approached zero as principal components with larger eigenvalues (up to $\mathbf{g}_{max}$) were included (fig. 4). These results indicate that all morphological divergence occurred in directions for which there is currently nonnegligible levels of genetic variance.

Aside from this generality, $\mathbf{d}_{P1}$–$\mathbf{d}_{P4}$, $\mathbf{d}_{Sp}$, and $\mathbf{d}_H$ differed in their patterns of association with **G** (fig. 4). Divergence among replicate populations appears to have occurred in proportion to the availability of genetic variance (fig. 4*A*). Inclusion of $\mathbf{g}_{max}$ in the subspace of **G** reduced the angle between the projection and $\mathbf{d}_{P1}$ from 57° ($r = 0.545$; 95% confidence interval [CI]: 11.7°–77.0°) to 0.36° ($r > 0.999$; 95% CI: 0.06°–4.9°). The significant effect on the association between $\mathbf{d}_{P1}$ and **G** of the addition of $\mathbf{g}_{max}$ to the subspace, as indicated by the nonoverlapping 95% confidence intervals, suggested that replicate populations mostly diverged in a direction close to that for which the maximum genetic variance was available (i.e., along $\mathbf{g}_{max}$).

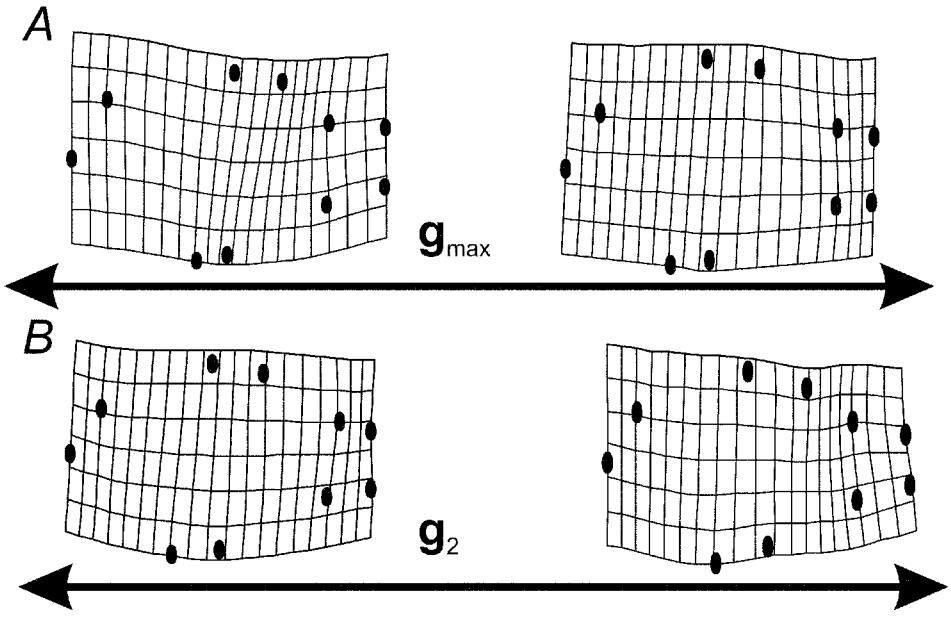

Figure 2: Shape variation along $\mathbf{g}_{max}$ (*A*) and $\mathbf{g}_2$ (*B*). Using the data in table 1, we calculated principal component scores for each fish (375 offspring and 44 parents) and identified fish representing the extremes described by each eigenvector. We also averaged each interlandmark distance to approximate the "average" form. The program Morpheus et al. (Slice 1998) was used to retrospectively infer landmark coordinates for the average and extreme fish. Thin-plate splines were generated using tspSpline (Rohlf 2004). The images on the left are the deformations of the mean fish to give the fish with the smallest principal component score for that eigenvector, while the images on the right are the deformations to give the fish with the highest score. Deformations were scaled by a factor of 1. Refer to figure 1 for landmark position information. Thin-plate splines were not used in hypothesis testing.

A similar pattern was observed for $\mathbf{d}_{P2}$ (fig. 2*A*), where the inclusion of both $\mathbf{g}_2$ and $\mathbf{g}_{max}$ reduced the angle from 54.3° ($r = 0.583$; 95% CI: 5.5°–60.6°) to 2.0° ($r = 0.994$; 95% CI: 0.10°–9.3°), although in this case the overlapping 95% confidence intervals indicate that this was not a significant reduction in angle. The third vector of population divergence ($\mathbf{d}_{P3}$) displayed an almost linear decline in angle as each successive principal component was added, while the angle between $\mathbf{d}_{P4}$ and the projection was strongly affected by the inclusion of $\mathbf{g}_3$, reducing the angle from 41.9° ($r = 0.744$) to 5.3° ($r = 0.995$; fig. 4*A*). Species divergence ($\mathbf{d}_{Sp}$) was not closely associated with any subspace of **G** until the inclusion of $\mathbf{g}_2$ and $\mathbf{g}_{max}$, whose combined effect significantly reduced the angle from 69.3° ($r = 0.351$; 95% CI: 13.5°–74.8°) to 2.4° ($r > 0.999$; 95% CI: 0.1°–7.5°; fig. 4*B*). Habitat divergence appeared to display a qualitatively different behavior to the other major divergence vectors ($\mathbf{d}_{P1}$, $\mathbf{d}_{Sp}$) because there was a marked decrease in angle starting with the addition of $\mathbf{g}_6$ and proceeding in a saltatory manner with the addition of $\mathbf{g}_4$, $\mathbf{g}_2$, and $\mathbf{g}_{max}$, resulting in reduction in the angle from 68.5° ($r = 0.366$) to a final value of 1.7° ($r > 0.999$; fig 4*B*).

It should be noted that **G** in table A1 is nonpositive definite, as with many estimated **G** matrices (Hill and Thompson 1978). Therefore, the estimate of the percent variance explained by $\mathbf{g}_{max}$ (96%) is likely to be an overestimate. To explore this issue, we applied a matrix "bending" procedure (Hayes and Hill 1981), as implemented by the program FLBEND (Henshall and Meyer 2002), which indicated that $\mathbf{g}_{max}$ might explain as little as 78% of the genetic variance. Bending leaves the eigenvectors of **G** unchanged (Hayes and Hill 1981), which are of most interest in our approach to investigating genetic constraints using matrix projection. Therefore, we do not consider the bent **G** further here, except to note that eigenvectors with very small eigenvalues may change rank during the bending procedure. In such cases, the subspaces containing these minor eigenvectors may change with the inclusion or exclusion of a particular eigenvector. Nevertheless, our conclusions concerning subspaces containing the major eigenvectors of **G** will be unaffected by the choice of bent or unbent **G** as their relative ranking remains unchanged by the bending procedure.

Table 2: Contribution of traits to the general divergence vector **z** and to the canonical variates describing divergence between habitats, species, and populations nested within species and habitat

Trait	**z**, 100%	$\mathbf{d}_{P1}$, 56.6%	$\mathbf{d}_{P2}$, 29.9%	$\mathbf{d}_{P3}$, 10.3%	$\mathbf{d}_{P4}$, 3.2%	$\mathbf{d}_{Sp}$, 100%	$\mathbf{d}_{H}$, 100%
1.2	.17	.04	−.02	.16	.09	−.07	.16
1.3	.26	.05	−.11	.34	.23	−.13	.23
1.4	.28	.09	−.10	.20	.19	−.14	.33
2.3	.25	.16	−.03	.05	.10	−.19	.05
2.4	.25	.15	−.10	.10	.31	−.15	.31
3.4	.23	.24	−.14	−.03	−.19	−.38	−.09
3.5	.17	.37	.08	.31	.28	−.18	−.31
3.6	.25	.23	−.17	−.16	−.01	−.31	.02
4.5	.23	.22	−.19	.21	−.25	−.37	−.15
4.6	.18	.16	−.27	−.32	.35	−.18	.06
5.6	.23	.26	−.23	−.36	−.24	−.35	−.02
5.7	.25	.02	−.34	−.26	.04	−.16	.16
5.8	.26	.11	−.32	−.20	−.10	−.24	.05
6.7	.23	.03	−.31	.26	.12	−.16	−.12
6.8	.21	−.03	−.45	.08	.12	−.20	−.30
7.8	.16	.31	−.06	.28	−.23	−.17	.01
7.9	.18	.39	.26	−.17	.09	.17	.31
7.10	.20	.31	.08	.06	.07	.01	.16
8.9	.20	.26	.16	−.16	.06	.12	.30
8.10	.17	.34	.35	−.11	−.07	.27	.48
9.10	.14	.09	−.11	.28	−.57	−.22	.09

Note: See figure 1 for trait definitions.

Discussion

Lines of Genetic Variance and the Direction of Evolution

Genetic covariance among traits might impose directional constraints on adaptive (Lande 1979, 1980; Arnold 1992; Björklund 1996; Schluter 1996) and neutral (Lande 1979; Phillips et al. 2001) evolution. In this study, we explored the relationship between **G** and the direction of adaptation to hydrodynamic regime versus divergence due to other processes. Our results suggest that adaptation to a hydrodynamic environment was not strongly influenced by eigenvectors of **G** that described large amounts of the genetic variance. This result was intuitive in so much as the dominant eigenvector of **G** describes size and morphological divergence among hydrodynamic regimes is only in shape (McGuigan et al. 2003). Evidence from rainbow fish therefore suggests that phenotypic divergence is not constrained to occur only along the genetic line of least resistance, consistent with a single selective optimum as the cause of replicated evolution.

The age of colonized lakes (700,000 years to 1 million years (myr); Jardine 1925; Longmore 1997) and the generation time in rainbow fish (one to two per year; Pusey et al. 2001) place an upper time limit on adaptation of lake rainbow fish of two million generations. Thus, our results support the idea that with sufficient time and some genetic variance, adaptation will proceed toward a fitness optimum, irrespective of the orientation of **G** (Lande 1979; Arnold 1992; Björklund 1996). Greater similarity between the direction of adaptive divergence and major eigenvectors of **G** might be expected when divergence has proceeded for a shorter period of time (Schluter 1996), although proportionality of **G** and **D** matrices may not be apparent even in the earliest stages of divergence (Blows and Higgie 2003).

Divergence at different levels appears to have been influenced differently by the pattern of genetic covariance among traits. In contrast to the weak association of the direction of hydrodynamic divergence with major eigenvectors **G**, divergence between species and replicate populations appears to have been strongly influenced by the orientation of **G**. We have experimentally inferred that hydrodynamic adaptation drove divergence between lakes and streams (McGuigan et al. 2003), but we do not know whether species or replicate populations adapted to local optima or diverged through drift. Species divergence was almost completely associated with the subspace defined by the first two eigenvectors of **G**. If species are diverging through selection, the selective optimum must lie along the major eigenvectors of **G**, in contrast to the position of the hydrodynamic selective optima. Alternatively, if species diverged through drift, our results suggest that **G** continues to exert a directional influence on divergence over relatively long time periods; *Melanotaenia eachamensis* and

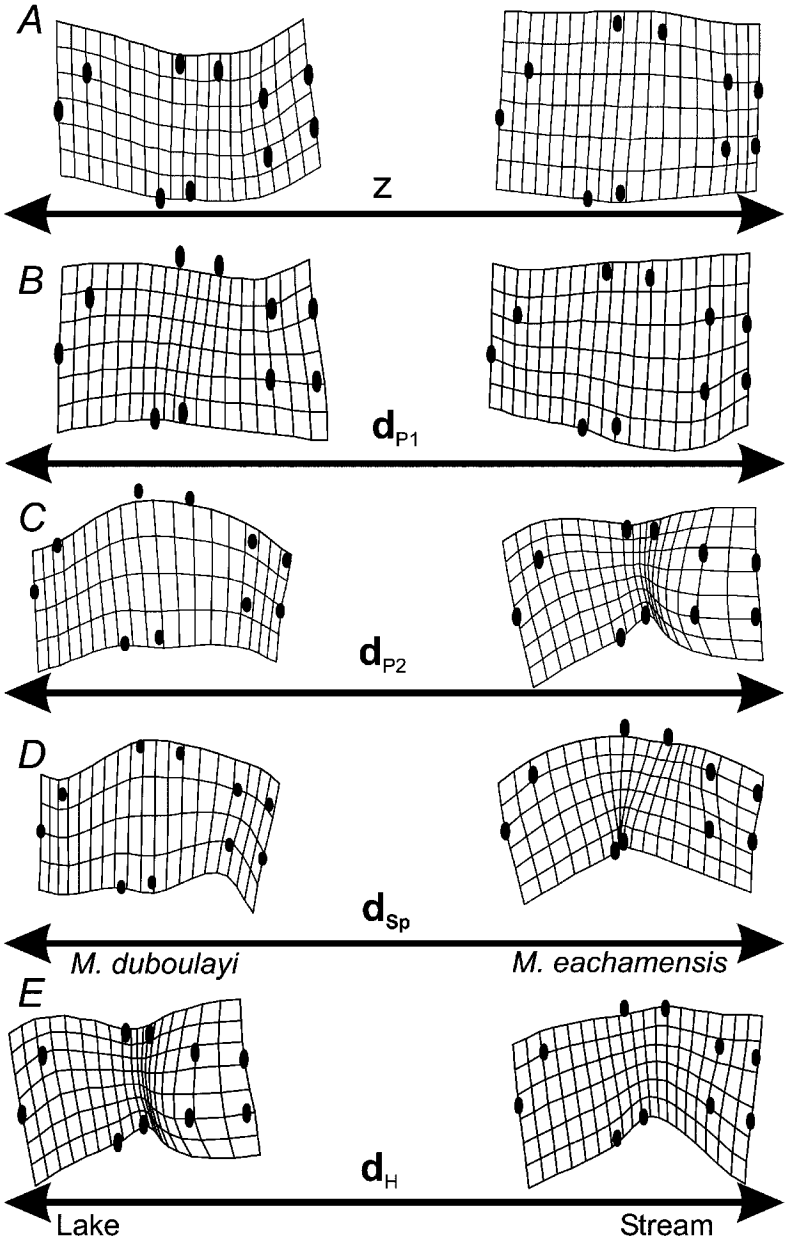

Figure 3: Shape variation along **z** (*A*); the first population divergence vector, $\mathbf{d}_{P1}$ (*B*); the second population divergence vector, $\mathbf{d}_{P2}$ (*C*); the species divergence vector, $\mathbf{d}_{Sp}$ (*D*); and the habitat divergence vector, $\mathbf{d}_{H}$ (*E*). From table 2, we calculated principal component scores for each of the 160 fish in the phenotypic data set and identified fish representing the extremes described by the eigenvectors. The average fish, landmark coordinates, and thin-plate splines were generated as for figure 2. Images on the left are the deformations of the mean fish to give the fish with the smallest principal component score for that eigenvector, while images on the right are the deformations to give the fish with the highest score. Because **z** describes total phenotypic variance while the other vectors describe only the portion of this variance attributable to particular sources (population, species, or habitat), the deformation in *A* is scaled by a factor of 1; in *B*–*E*, deformations are scaled by a factor of 2. Refer to figure 1 for landmark position information. Thin-plate splines were not used in hypothesis testing.

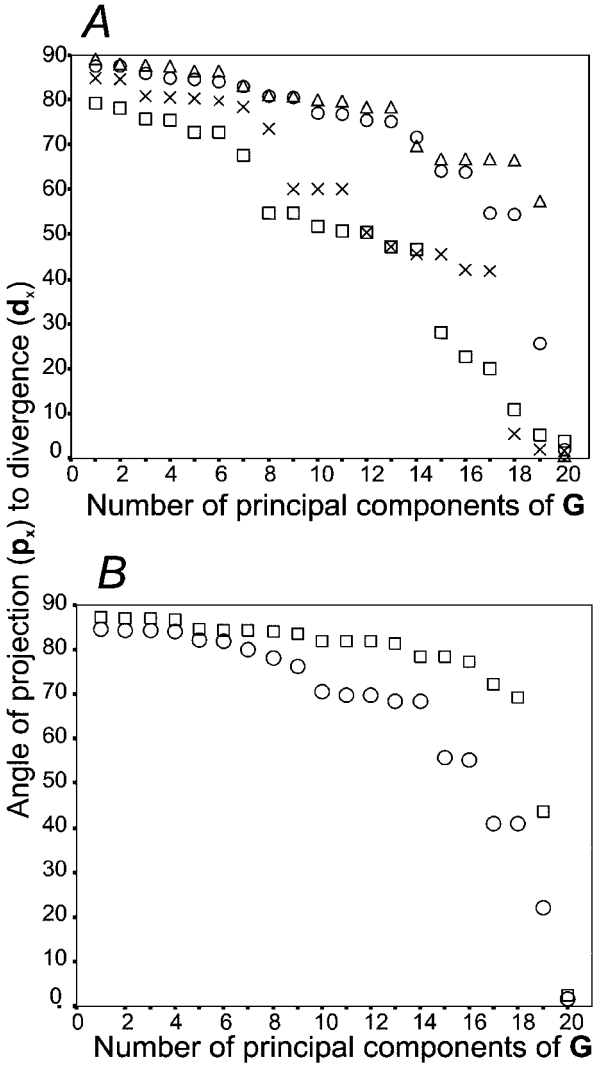

Figure 4: Angles between projections of genetic variance and the vectors of phenotypic divergence. Principal components of **G** were added sequentially to the subspace of **G** in ascending order of their eigenvalues, starting with $\mathbf{g}_{20}$ and ending with $\mathbf{g}_{max}$. *A*, Vectors of replicate population divergence: *triangles* = $\mathbf{d}_{P1}$, *circles* = $\mathbf{d}_{P2}$, *squares* = $\mathbf{d}_{P3}$, *crosses* = $\mathbf{d}_{P4}$. *B*, Vector of species divergence (*squares* = $\mathbf{d}_{Sp}$) and vector habitat divergence (*circles* = $\mathbf{d}_{H}$).

Melanotaenia duboulayi have been diverging for 2 myr (McGuigan et al. 2000).

Divergence between replicate populations was roughly proportional to the amount of genetic variance; the first eigenvector of replicate population divergence described a direction close to that described by $\mathbf{g}_{max}$, while $\mathbf{d}_{P2}$ was associated with $\mathbf{g}_{max}$ and $\mathbf{g}_2$. The more minor replicate population divergence vectors, $\mathbf{d}_{P3}$ and $\mathbf{d}_{P4}$, were not associated with $\mathbf{g}_{max}$ and $\mathbf{g}_2$ at all, but with eigenvectors of **G** accounting for less of the genetic variance. The proportionality of **G** to divergence is a direct prediction of models that describe evolution as a consequence of genetic drift (Lande 1979; Arnold et al. 2001). Our results are therefore consistent with the hypothesis that populations drifted apart, but this hypothesis remains to be directly tested. Applying our analytical approach to populations known to have diverged through drift will test whether the direction of drift is as tightly constrained by **G** as suggested by the apparent proportionality of $\mathbf{d}_P$ to $\mathbf{g}_{max}$ through $\mathbf{g}_3$ and over what time frames this constraint might hold.

Genetic Basis of Locomotor Morphology

Consistent with the results of a common garden experiment (McGuigan et al. 2003), we observed additive genetic variance in the morphology of rainbow fish. The quantitative genetic covariance among traits was highly structured, suggesting substantial integration and modularity of rainbow fish morphology. Modularity can be considered a hierarchical phenomenon (e.g., Wolf et al. 2001). Here, the first level of the hierarchy described the integration of the whole body; all traits were highly positively intercorrelated at the genetic level, as reflected in their strong positive contributions to $\mathbf{g}_{max}$. In a review of 27 published estimates of **G** for morphological traits, Björklund (1996) identified the dominant influence of size as a consistent pattern. It has long been acknowledged that genetic variance for size can generate large positive covariance of morphological traits irrespective of any other association (i.e., shape covariation; e.g., Wright 1932; Crespi and Bookstein 1989). Genetic covariation of traits might result from linkage disequilibrium or pleiotropy, with the latter the more common cause (Lande 1980; Falconer and Mackay 1996). In vertebrates, including fish, growth hormone and insulin-like growth factor pathways influence adult body size (reviewed by Duan 1998) and are therefore strong candidates for pleiotropic genes generating the observed additive genetic variance for body size in rainbow fish. The close association of directions of divergence with $\mathbf{g}_{max}$ suggests that allele frequencies at "size" loci will have diverged among rainbow fish species and populations.

At the next level in the hierarchy are the modules of caudal peduncle length and body. We observed opposing contributions to the first shape vector of **G** ($\mathbf{g}_2$) from caudal peduncle length versus body. Gene expression in early fish development suggested that different genes are responsible for the differentiation of the body versus the caudal peduncle (Ahn and Gibson 1999*a*, 1999*b*; Morin-Kensicki et al. 2002). Concordance of the quantitative genetic and molecular developmental patterns highlights the mutually informative nature of these two areas of biology. We analyzed genetic correlations estimated from size-

corrected midparent values and family means (data not presented) and observed a negative genetic correlation between caudal length and the rest of the body once the overall effect of size was removed. Such a negative relationship might be mediated through pleiotropic effects of genes controlling resource allocation during development (Riska 1986).

Conclusions

Current evidence for a close association between measures of evolutionary constraint and divergence is mixed (Begin and Roff 2003). Experimental studies have tended to find a close association between $\mathbf{g}_{max}$ and divergence when it has been explicitly examined (e.g., Schluter 1996; Arnold and Phillips 1999; Begin and Roff 2003). Our experimental system and analytical approach allowed us to partition phenotypic divergence into that produced within versus among hydrodynamic habitats such that we could distinguish phenotypic divergence as a consequence of hydrodynamic adaptation from divergence driven by other processes. Variation in the strength of association with **G** among divergences due to different evolutionary processes suggests that partitioning phenotypic variance to each factor is necessary when exploring the relationship between phenotypic divergence and the genetic basis of the traits. Studies exploring the role of genetic constraints on adaptive evolution need to carefully consider the potential role of genetic drift in generating associations between eigenvectors of **G** and directions of divergence, because selection need not have been the only process driving divergence among populations under different selection regimes.

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