

ANALYSIS OF TWO-STATE MULTIVARIATE PHENOTYPIC CHANGE IN ECOLOGICAL STUDIES

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Abstract. Analyses of two-state phenotypic change are common in ecological research. Some examples include phenotypic changes due to phenotypic plasticity between two environments, changes due to predator/non-predator character shifts, character displacement via competitive interactions, and patterns of sexual dimorphism. However, methods for analyzing phenotypic change for multivariate data have not been rigorously developed. Here we outline a method for testing vectors of phenotypic change in terms of two important attributes: the magnitude of change (vector length) and the direction of change described by trait covariation (angular difference between vectors). We describe a permutation procedure for testing these attributes, which allows non-targeted sources of variation to be held constant. We provide examples that illustrate the importance of considering vector attributes of phenotypic change in biological studies, and we demonstrate how greater inference can be made than by evaluating variance components with MANOVA alone. Finally, we consider how our method may be extended to more complex data.

Key words: geometric morphometrics; multivariate; phenotypic; vector.

INTRODUCTION

The covariation of phenotypic traits has received considerable attention in recent evolutionary and ecological studies. The concept of *phenotypic integration* (Wagner and Altenberg 1996, Pigliucci 2003)—the constraint of independent change in phenotypic traits because of correlations that are genetic, developmental, physiological, or functional—illustrates that an organism's phenotype is not easily truncated to one trait or variable that describes its form, nor can individual variables be analyzed separately. Most researchers consider an organism's phenotype as a multivariate set of variables, and the covariation of traits an important analytical consideration. Although considerable advances have been made to quantify an organism's phenotype using multivariate data, many interesting questions in biology are primarily concerned with changes of an organism's phenotype, rather than phenotypic variation per se.

Studies of two-state phenotypic change are frequent in ecological research and include examples of analysis of reaction norms in phenotypic plasticity research (e.g., Pigliucci et al. 1999, Pigliucci and Kolodynska 2002), analysis of phenotypic shifts between predator and non-predator environments (e.g., Reznick et al. 1997, Langerhans et al. 2004), analysis of phenotypic divergence with environmental change (e.g., Losos et al. 1997, Collyer et al. 2005), character displacement via competitive interactions (e.g., Adams and Rohlf 2000, Adams

2004), and sexual dimorphism (e.g., Shine 1994, Collyer et al. 2005). In these studies, two-factor univariate or multivariate analyses of variance (ANOVA or MANOVA, respectively) are frequently used to test for differences in phenotypic change between different operational taxonomic units (OTUs) under varying conditions (e.g., Adams 2004, Langerhans et al. 2004). A statistically significant interaction between OTU and another categorical factor, such as environment type, indicates that OTUs differ in their patterns of phenotypic change. Understanding the reason for a significant interaction is simple with univariate data: OTUs differ in the amount of phenotypic change between environments. Interpreting a significant interaction effect with multivariate data, however, is less straightforward.

As with univariate data, a statistically significant interaction in MANOVA between OTU and another categorical factor, such as environment type, indicates that OTUs differ in their patterns of phenotypic change. However, with multivariate data, phenotypic change between OTUs is not as easily interpreted because a significant interaction term fails to indicate whether OTUs differ in the magnitude of phenotypic change or the direction of phenotypic change, implied by the way multivariate variables covary. Various ad hoc approaches have been used to consider heterogeneity in phenotypic change among OTUs, mostly by evaluating eigenvector similarity among different covariance matrices. These approaches, however, are inconsistent with statistical inference made from two-factor MANOVA for testing differences between OTUs (see *Discussion*).

In the current paper, we outline a general approach for the statistical comparison of multivariate vectors of

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phenotypic change, building from (1) the use of linear models and MANOVA for evaluating two-factor interactions, (2) the description of phenotypic change vectors (calculated from least-squares means) as quantities specified by a magnitude (length) and a direction, and (3) permutation methods that allow randomization of factor interactions without destroying significant main effects in linear models (Gonzalez and Manly 1998). Our approach works with fixed and random effects, covariates, and non-normally distributed response variables. We provide examples with multivariate shape data that illustrate the utility of this method for inferring biological explanations from statistical results. Finally, we conclude by suggesting how this method could be generalized to multi-state phenotypic comparisons.

MULTIVARIATE VECTORS OF PHENOTYPIC CHANGE

To illustrate the meaning of multivariate phenotypic change vectors, we consider two closely related allopatric plant species, one occurring in nutrient rich habitats, the other occurring in nutrient poor habitats. An experiment is performed where experimental replicates of both species are cultured in common gardens that mimic nutrient rich and nutrient poor environmental conditions. After some time, phenotypic traits such as flower number, leaf size, number of branches, etc., are measured on each individual plant, and a two-factor MANOVA is performed to statistically evaluate the variance and covariance of variables associated with the species-environment interaction. If a significant interaction of factors is found, one concludes that the phenotypic change between the two environments differs between the two species.

For univariate phenotypic variables, the magnitude of phenotypic change is the level of difference between environments, and the direction of phenotypic change is easy to infer as either an increase or decrease in mean trait value from one environment to another (Fig. 1A–C). With multiple, potentially correlated traits, however, describing phenotypic change is more complicated (Fig. 1D–H). Fig. 2 illustrates the multivariate case, where the multivariate mean of each population in each environment is described as $\bar{\mathbf{Y}}_{ij}$, a (row) vector of means for population i in environment j for p traits. Thus, the phenotypic change vector is simply a difference in multivariate mean vectors: $\Delta\bar{\mathbf{Y}}_i = \bar{\mathbf{Y}}_{ij} - \bar{\mathbf{Y}}_{ik}$, for population i in environments j and k . The magnitude of difference between two means is calculated from the Euclidian distance of a phenotypic change vector: $D_E = \|\Delta\bar{\mathbf{Y}}_i\| = (\Delta\bar{\mathbf{Y}}_i \Delta\bar{\mathbf{Y}}_i^T)^{1/2}$, where \mathbf{T} represents a vector transpose. This value is the length of the phenotypic change vector described from the multivariate means (Fig. 2). A statistical measure of D_E can be assessed as a pairwise comparison within MANOVA by converting it to a generalized Mahalanobis (1936) distance that can be used in a Hotelling's (1931) generalized T^2 test (see e.g., Legendre and Legendre 1998).

To discern if the level of phenotypic change of one population is greater than another, the test statistic $|D_{E_1} - D_{E_2}|$ is calculated (e.g., Adams and Rohlf 2000). To discern if two populations differ in the direction of their phenotypic change vectors, their vector correlation is calculated as the inner product of the two vectors scaled to unit length:

$$VC = \left(\frac{\Delta\bar{\mathbf{Y}}_1}{D_{E_1}} \cdot \frac{\Delta\bar{\mathbf{Y}}_2^T}{D_{E_2}} \right)$$

(Cheverud and Leamy 1985, Schluter 1996, Klingenberg and Leamy 2001, Bégín and Roff 2003). The arccosine of this value is the angle, θ , between vectors, which describes the similarity of their direction (i.e., an angle of 0° describes parallel vectors; an angle of 90° describes orthogonal vectors; Fig. 2). These two test statistics can be evaluated by comparing them to distributions created from random pairs of vectors from a permutation procedure (see Appendix for more detail).

STATISTICALLY COMPARING PHENOTYPIC CHANGE VECTORS WITH A PERMUTATION PROCEDURE

The permutation procedure we outline is general, and can be implemented for designs incorporating fixed effects, random effects, covariate terms, and non-normally distributed response variables (see Appendix). In all cases, if significant fixed effects, or covariate effects are identified, the permutation procedure must also account for them (Manly 1997, Gonzalez and Manly 1998). This can be accomplished with a two-stage iterative procedure that uses linear models that lack and contain the interaction effect of interest, respectively. For simplicity, we illustrate our method with populations (POP) and environments (ENV) as fixed effects of interest. Testing the null hypothesis that observed $|D_{E_1} - D_{E_2}|$ or θ is not different than expected from random pairs of vectors is consistent with testing the null hypothesis that the POP \times ENV variance is 0, meaning that all effects in the linear model, other than POP \times ENV, must be held constant in the permutation procedure. First, estimates are made with a linear model that does not contain the POP \times ENV effect. The residuals from this model are then randomly assigned to linear estimates (calculated from regression coefficients that describe POP, ENV, and other effects) to reconstruct "random" phenotypic values. These random values are then used to calculate POP \times ENV means in the second stage of procedure, where the linear model contains the same effects plus the POP \times ENV effect. This procedure is repeated many times to create a distribution of random values from which the significance of observed values can be inferred. The computational details for this analysis are provided in the Appendix; example data and R code are available in the Supplement.

The advantage of the procedure described above is that it allows a test for differences in multivariate phenotypic change between OTUs, in a manner

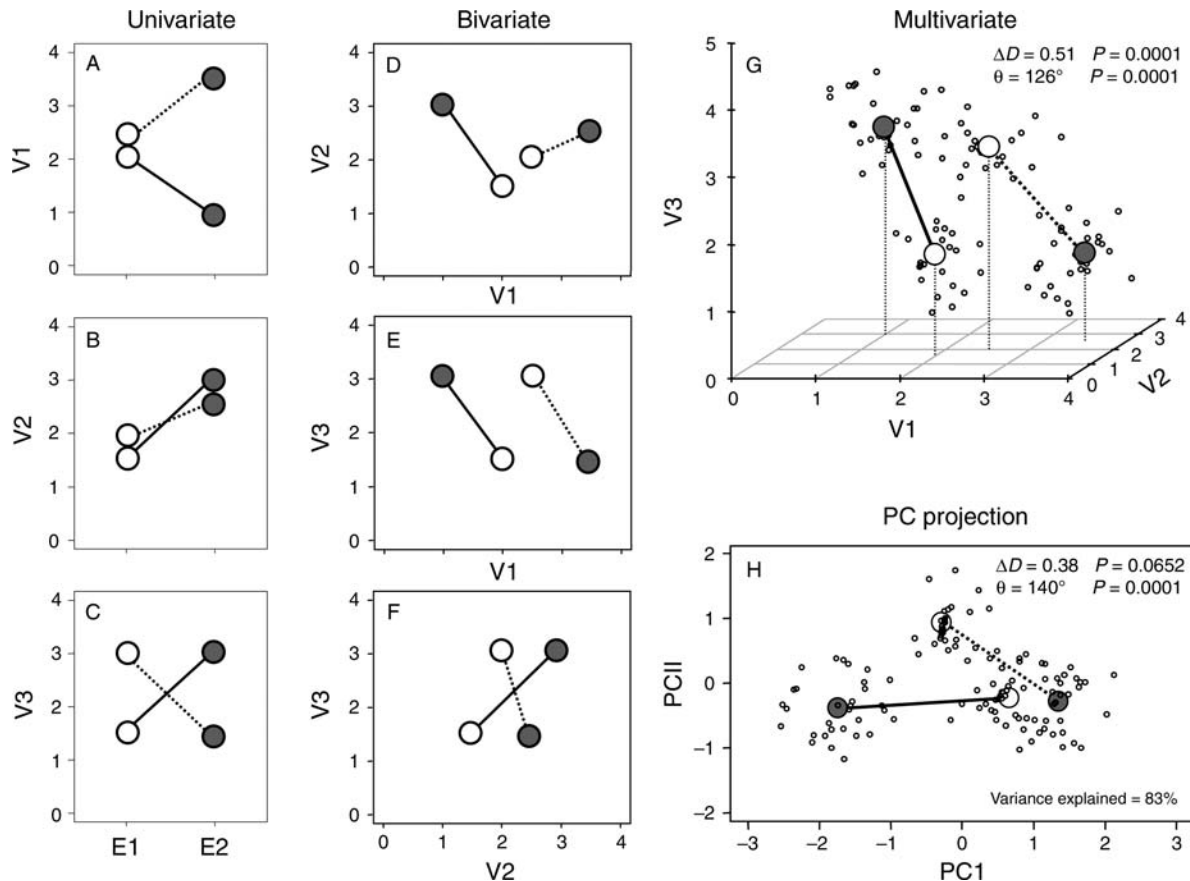


FIG. 1. Examples of phenotypic change vectors for three variables (V1–V3) shown in (A–C) univariate, (D–F) bivariate, (G) multivariate, and (H) principal component projection plots. In each example, phenotypic means for two operational taxonomic units (OTUs; solid and dotted lines) are shown in two environments (E1, open circles; E2, filled circles). For two variables (V1 and V3), the amount of phenotypic change is similar for both OTUs despite directional differences of phenotypic change (either increasing or decreasing values from E1 to E2). The other variable (V2) corresponds to an obvious difference in the magnitude of phenotypic change despite a similar direction of change (increasing value from E1 to E2). The data in (G) and (H) represent values described in (A–C) with simulated random error (30 values per group). Test statistics and P values from a permutation test (with 10 000 permutations), using the method described in this article, are shown. Projecting data onto the first two principal components (PC) results in a decrease in relative vector length difference (ΔD), increase in angle between vectors (θ), and different empirical P values.

consistent with tests of $\text{POP} \times \text{ENV}$ variance, without abandoning the covariance of multiple traits (i.e., by assuming independence and incorrectly testing each individual variable separately). We illustrate with two examples how this method has certain advantages for comparing phenotypic change between OTUs that can be described as complex phenotypes, where it is not possible to make a phenotypic description without using multivariate data. In the first example, we compare vectors of head shape change between two species of *Plethodon* salamanders that occur in sympatry and allopatry. The computational steps of this analysis are also provided in the Appendix. In the second example, we examine sexual dimorphism in body shape between two populations of pupfish (*Cyprinodon*). Both examples use landmark-based geometric morphometric methods (Rohlf and Marcus 1993, Adams et al. 2004) to generate multivariate shape variables. Multivariate analyses are required because each shape variable does not convey

any independent biological meaning (see Adams and Rosenberg 1998, Rohlf 1998, Zelditch et al. 2004).

EXAMPLE 1: CHARACTER DISPLACEMENT IN *PLETHODON* SALAMANDERS

This example uses morphological data reported in Adams (2004), which describes the head shapes of two species of *Plethodon* salamanders that occur both in allopatry and sympatry in the Balsam Mountains and Great Smoky Mountains, USA. Previous analyses suggested that ecological character displacement via aggressive interference led to evolved significant differences in head shape between *P. jordani* and *P. teyahalee* in sympatry (see Adams 2004). Landmark data were collected from two-dimensional Cartesian coordinates of 12 anatomical reference points located on the head and jaw (Fig. 3A). These data were recorded from photos of 336 individual salamanders using TPSDIG software (Rohlf 2001). The landmark configurations

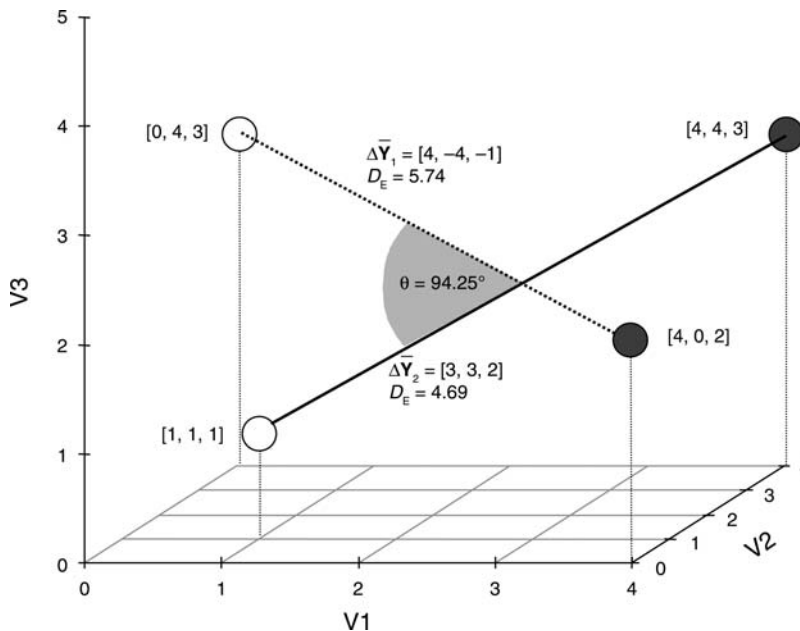


FIG. 2. Example of phenotypic change vector attributes. Two OTUs are represented (solid and dotted lines) between multivariate phenotypic means in two environments (open and filled circles). Phenotypic change vectors ($\Delta\bar{\mathbf{Y}}$) are described, as are their lengths (Euclidean distance, D_E), and the angle between them (θ). Values for each mean [V_1 , V_2 , V_3] are shown.

describe the shape, location, orientation, and size of salamander heads and jaws. To consider shape variation independent of non-shape variation, a generalized Procrustes analysis (GPA; Rohlf and Slice 1990) was performed, which scales, translates, and orients configurations via a generalized least-squares superimposition procedure. From the aligned coordinates, a set of 18 shape variables was generated (see Adams 1999 and 2004 for details) that were used to test differences in shape between species and locality type (i.e., allopatry vs. sympatry), as well as the covariation between shape and aggressive behavior.

In the original analysis of these data, a two-factor MANOVA was used to test for differences in head shape for species (SPE), locality type (LOC: allopatry vs. sympatry), and SPE \times LOC interaction. All effects were found to be significant, and a full randomization of the 336 individual salamanders was used in a permutation procedure to compare shape differences in sympatry to shape difference in allopatry (i.e., $|D_S - D_A|$). It was found that the shape difference in sympatry significantly exceeded the shape difference in allopatry (Adams 2004), but the permutation method was not specific for the SPE \times LOC interaction. Here, we apply the procedure described above, which holds constant the observed SPE and LOC effects and tests specifically the SPE \times LOC interaction. Phenotypic change vectors were calculated for each species, which described the phenotypic shift resulting from interspecific competition (i.e., the difference between sympatric and allopatric multivariate means). Differences in vector magnitudes and directions were considered with a permutation procedure

that included 9999 random permutations and the observed value. The significance of an observed value was assessed as the probability of finding a more extreme value by chance (P_{rand}) from the 10 000 values generated.

Results

The species' phenotypic change vectors (between sympatric and allopatric means) were not statistically different in length ($P. \text{jordani}$: $D_{Pj} = 0.087$; $P. \text{teyahalee}$: $D_{Pt} = 0.099$; $P_{\text{rand}} = 0.172$). However, the angle between these vectors was significantly greater than expected by chance ($\theta = 47.71^\circ$; $P_{\text{rand}} = 0.0001$; Fig. 3A). Further, in sympatry, the shape difference between species ($D_S = 0.079$) was statistically greater than the shape difference in allopatry ($D_A = 0.016$; $P_{\text{rand}} = 0.0001$), a finding consistent with Adams (2004). Combining our findings with those of Adams (2004) reveals that the significant SPE \times LOC variation describes a difference in the direction of phenotypic change between the two species, but not a difference in the amount of phenotypic change exhibited. This provides additional insight into how head shape has diverged and evolved as a result of interspecific competition in sympatry.

EXAMPLE 2: SEXUAL DIMORPHISM IN WHITE SANDS PUPFISH

This example uses a subset of morphological data reported in Collyer et al. (2005). In the original study, body shape variation was assessed among populations of White Sands pupfish, *Cyprinodon tularosa*, which occur in four locations in southern New Mexico, USA. Two populations are native and have been presumably isolated since the desiccation of the Pleistocene Lake

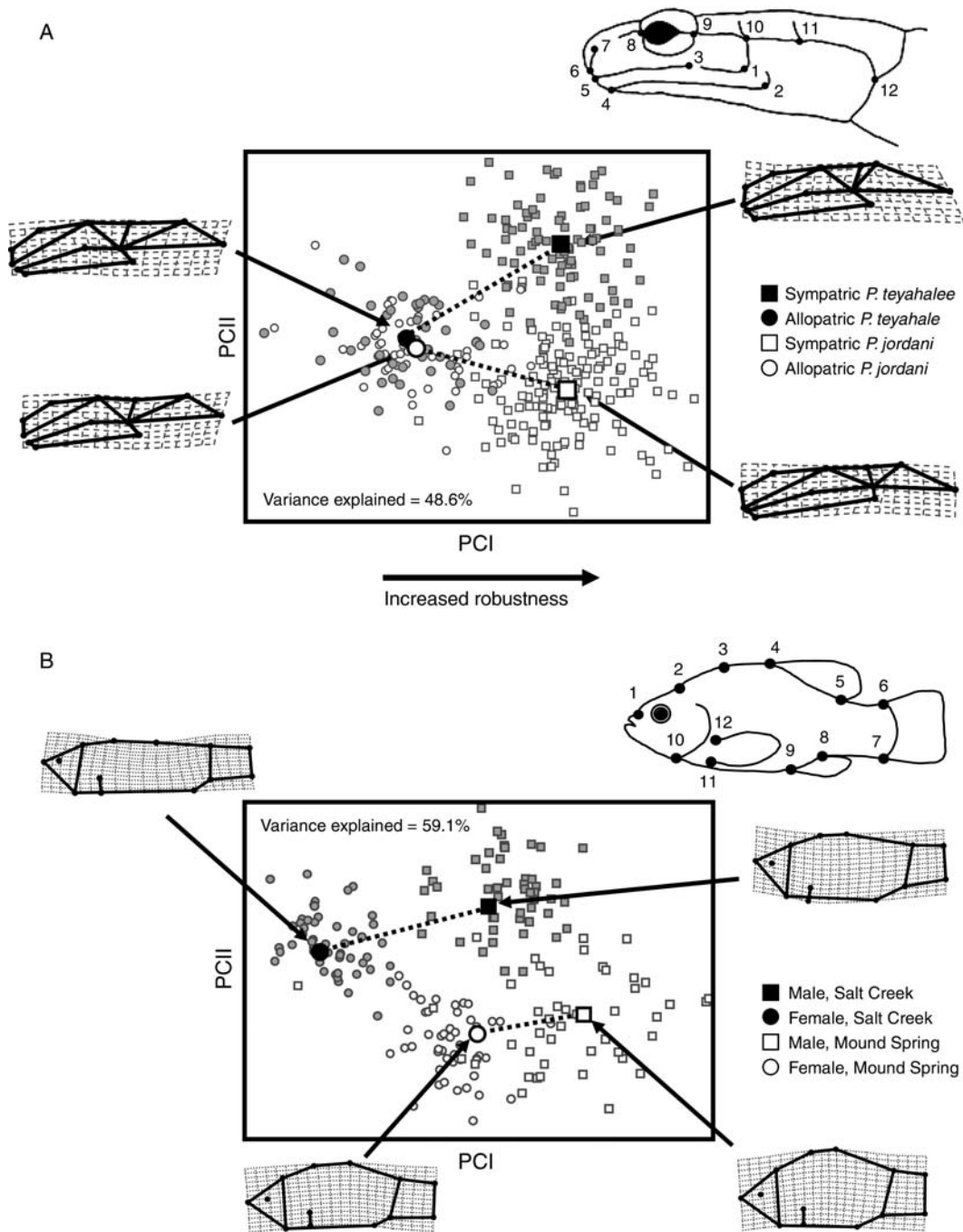


FIG. 3. Principal component plots for (A) *Plethodon* salamander (*P. jordani* and *P. teyahalee*) and (B) White Sands pupfish (*Cyprinodon tularosa*) shape variation. Dotted lines indicate phenotypic change vectors between least-squares means of shape for the interactions considered. Projection into the two-dimensional PCA space can visually alter relationships. For example, the angle between vectors in (B) is 22.88° for all variables but only 4.86° for the two dimensions shown (because less than 60% of the total variation is represented).

Otero in Salt Creek, a highly saline and fluvial habitat, and Malpais Spring, a brackish marsh habitat. Two other populations were established circa 1970 with fish from the Salt Creek population (Stockwell et al. 1998, Pittenger and Springer 1999). One population was

established in another saline environment, Lost River, and one population was established in a brackish environment at Mound Spring. The Mound Spring population was most divergent in body shape, having a more deep-bodied shape than its source population.

Streamlining due to water flow and salinity in the Salt Creek environment was suggested as the ecological mechanism that maintains this shape difference.

White Sands pupfish are also sexually dimorphic in body shape (Collyer et al. 2005). The previous study used (1) a permutation procedure with full randomization of individuals within female and male groups to test for differences in shape among populations and between males and females within populations, using Procrustes distance as a metric of shape difference (i.e., not accounting for shape variation due to fish size); and (2) a separate permutation procedure to generate random sets of shape vectors accounting for fish size to test differences in various vector directions, including sexual dimorphism vectors. Here we use the shape variables from the 195 Salt Creek and Mound Spring fish in the previous study to examine differences in sexual dimorphism between source (Salt Creek) and introduced (Mound Spring) populations.

Thirteen anatomical landmarks (Fig. 3B) were digitized from images of fish to collect morphometric data, using TPSDIG software (Rohlf 2001). GPA and TPS were used to generate a set of 22 shape variables with TPSRELW software (Rohlf 2003). We performed two-factor MANOVA on shape for the independent variables of a linear model including populations (POP), sex (SEX), and the POP \times SEX interaction. In addition, fish size, measured as the log of centroid size (CS, the square root of the sum of squared distances of landmarks from their configuration centroid) was used as a covariate of shape. Phenotypic change vectors were calculated which described sexual dimorphism in shape between multivariate least-squares means of POP \times SEX groups (i.e., at the average fish size). Differences in vector lengths and directions were considered each with a permutation procedure that included 9999 random permutations and the observed value. The significance of an observed value was assessed as the probability of finding a more extreme value by chance from the 10 000 values generated.

Results

MANOVA performed on shape variables indicated significant POP variance ($F_{22,169} = 27.92$, $P < 0.0001$), significant SEX variance ($F_{22,169} = 45.88$, $P < 0.0001$), a significant POP \times SEX interaction ($F_{22,169} = 6.16$, $P < 0.0001$), and a significant association of shape and log(CS) ($F_{22,169} = 27.70$, $P < 0.0001$). The magnitude of sexual dimorphism (Fig. 3B) in shape for Salt Creek ($D_{SC} = 0.068$) significantly exceeded the magnitude for Mound Spring ($D_{MO} = 0.044$; $P_{rand} = 0.0001$). Although the angle between sexual dimorphism vectors was small ($\theta = 22.88^\circ$), it was significantly greater than expected by chance with no POP \times SEX interaction ($P_{rand} = 0.0001$). Thus, for this example, significant POP \times SEX variation (while accounting for a common shape allometry) was associated with a clear difference in the magnitude of shape sexual dimorphism, and a slight, but significant difference in the direction of shape sexual dimorphism, between the two populations.

DISCUSSION

There is much new focus on phenotypic integration in ecological research, and the biological relevance of trait covariation in phenotypic evolutionary ecology (see discussions by Pigliucci 2004, Relyea 2004). As the push for utilizing multiple integrated traits in phenotypic research requires the concomitant formulation of specific hypotheses and tests (Pigliucci 2004), we argue that appropriate methods need to be developed to test such hypotheses. Analyzing differences in multivariate phenotypic change in terms of vector length and direction provides a logical link between phenotypic integration and the study of phenotypic change. As we have shown, significant two-factor interactions in MANOVA can result from differences in the amount of overall phenotypic change, or the way that multiple traits covary for different OTUs. We believe that discriminating between these two aspects of phenotypic change is essential for proper understanding of biological patterns in ecological and evolutionary research.

While previous methods have been proposed to examine the direction of multivariate vectors, they do not examine phenotypic patterns in a manner concordant with what is found in a multi-factor MANOVA design. Therefore, they are insufficient for identifying how patterns of phenotypic change differ between OTUs (as expressed through a significant interaction term). For instance, several approaches have compared similarity in eigenstructure of covariance matrices (e.g., Cheverud and Leamy 1985, Phillips and Arnold 1999, Klingenberg and Leamy 2001, Pigliucci and Kolodynska 2002), or between the eigenstructure of a covariance matrix with a predefined vector direction (e.g., Schluter 1996, Bégin and Roff 2003). However, as these approaches explicitly assess covariance structure similarity or principal component confidence bounds (with resampling procedures not specific to MANOVA interactions), they do not indicate why a significant interaction exists, especially because significant interactions inherently express difference, not similarity, among means. Further, testing differences in phenotypic change among OTUs with eigenstructure similarity methods is problematic when covariance matrices, and subsequently their PCs, are described for more than two states of phenotypic change. By contrast, our approach identifies differences for vectors explicitly described from multivariate means and is thus not limited in this capacity, and is amenable to analysis of multi-state phenotypic change.

Another frequently used approach in ecological studies is to project data onto a few principal component or canonical axes and examine these scores as multivariate surrogates. Our approach does not conflict with these data reduction methods, and can in fact be used on such reduced data. Nevertheless, it should be pointed out that data reduction through projection can alter perceived patterns of variation, thereby leading to incorrect assessments of patterns of phenotypic change (see e.g., Figs. 1 and 3). For example, the angle between

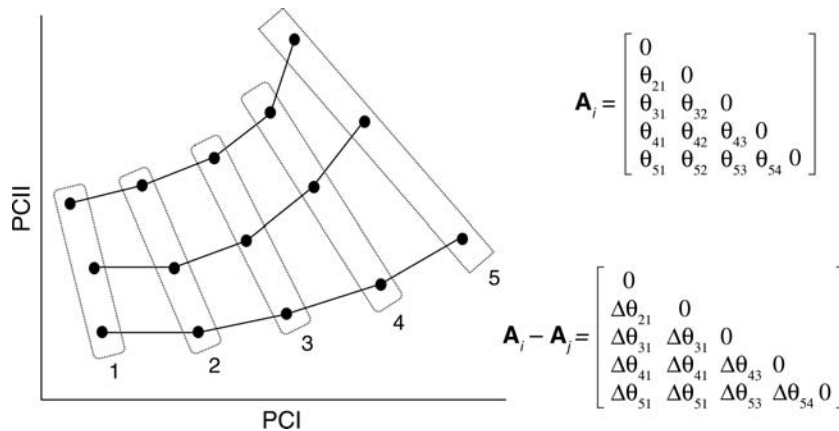


FIG. 4. Hypothetical two-dimensional representation of phenotypic change patterns for three OTUs in five environments. Environment means are labeled (1–5) and shown as groups (boxes). Solid lines represent phenotypic change vectors between adjacent environments. Association matrices (A) describe levels of phenotypic change of OTU i between different environmental means (θ represents either an angle or distance in this case) or the difference between two OTUs, i and j .

vectors in Fig. 3B is 4.86° if calculated only from data projected onto the first two PCs (compared to 22.88° for the full data). Excluding important information (in this case, approximately 40% of the total information) may lead to incorrect inferences. With our approach, however, excluding data is not necessary.

In our data examples, significant interactions in MANOVA corresponded to directional differences of phenotypic change for compared OTUs. However, in one case (pupfish example) the lengths of phenotypic change vectors were different, and in the other case (salamander example), they were not. Further, the directions of phenotypic change were much more different in the salamander example than in the pupfish example. By decomposing vectors of phenotypic change into length and direction attributes, and assessing the significance of differences in these attributes, we were able to describe the biological relevance of a significant interaction in MANOVA. In the salamander example, similar phenotypes in allopatry diverge in sympatry, and this divergence is described by the angular difference of shape change. In the pupfish example, the difference in shape sexual dimorphism was characterized more so by a difference in the amount of shape change. Examination of locations of shape means (Fig. 3B) illustrates that the difference in shape sexual dimorphisms results from females that were more male-like in shape in the Mound Spring population. Although similar MANOVA analyses on both example data sets produced significant interactions, specific hypothesis tests regarding attributes of phenotypic change revealed different conclusions for the reasons that significant interactions were observed.

Our examples focused on two-state factors such as sex. However, the comparison of phenotypic change vector length and direction can be extended to more complex designs. As an example, we consider three OTUs in two or more environments (e.g., Fig. 4). In cases where more than two OTUs are considered in two

environments, our procedure requires no alteration. First inferential tests are performed on variance components of fixed effects and interactions using MANOVA. Next, the magnitude and direction of phenotypic change vectors are explored as pairwise comparisons for each pair of OTUs (e.g., A vs. B, B vs. C, A vs. C). For the case of more than two environments, matrices of differences among phenotypic means could be described for each OTU. Such matrices could then be compared among OTUs.

For example, Fig. 4 displays OTUs that do not differ greatly in either the magnitude or direction of phenotypic change between adjacent environments. However, from environment 1 to environment 5, the cumulative contribution of between-environment differences produces different patterns for the three populations. This is most noticeable from the greater between-population distances in environment 5 than in environment 1. To assess such patterns one could describe an association matrix that expresses phenotypic change at different levels of comparison for each population (e.g., environment 1–environment 2 or environment 1–environment 5). Differences between OTUs could thus be considered as a matrix of difference between two association matrices, where the elements represent differences in length or angles between inter-environment vectors. In our example, differences in phenotypic change would be represented as progressively increasing values from the diagonal of such a matrix. Differences in matrix elements could be statistically compared to distributions of random differences using the same permutation procedure described in this paper. Alternatively, one could compare determinants of covariance matrices of differences among populations. Finally, one could also consider extending the concepts from geometric morphometrics (Rohlf and Marcus 1993, Adams et al. 2004) to estimate and compare the “shapes” of phenotypic change trajectories (sensu Adams and Cerney 2007).

The analysis of two-state phenotypic change described by multivariate vectors, as we developed in this paper, has four important characteristics. First, the attributes of phenotypic change defined by multivariate vectors capture pertinent biological description of phenotypic change. Specifically, the length of a vector describes the overall amount of phenotypic change and the direction of a vector describes the way multiple traits covary. Second, the permutation method we describe tests null hypotheses of vector attributes specifically for the variance associated with a two-factor interaction, thus holding main effects constant. An analogous procedure could be easily developed within the same framework that allows hypothesis testing for other variance components (e.g., holding species effects constant but randomize error with respect to environmental variance). Third, our method is easily employed with mixed linear models and generalized linear models because the permutation procedure is performed by altering the design matrix and re-describing models at each iteration of the procedure (see Appendix). Thus initial parameter estimates and error distributions remain unchanged. Finally, our method is easily generalized to more complex designs. These four characteristics suggest that this method of statistical evaluation of multivariate phenotypic change should have appeal for various disciplines within ecology and evolution that are concerned with measuring and comparing patterns or rates of phenotypic change among different groups.

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APPENDIX

STATISTICAL DETAILS AND COMPUTATIONAL STEPS FOR THE SALAMANDER DATA

Multivariate phenotypic data, \mathbf{Y} , can be expressed by the linear model, $\mathbf{Y} = \mathbf{X}\boldsymbol{\beta} + \boldsymbol{\varepsilon}$; where \mathbf{X} is an $n \times k$ design matrix for the k variables that model effects for the overall mean, fixed effects, interactions, and covariates; $\boldsymbol{\beta}$ is a $k \times p$ matrix of partial regression coefficients for p response variables; and $\boldsymbol{\varepsilon}$ is the $n \times p$ matrix of residuals. MANOVA is used to determine if fixed effects expressed in $\boldsymbol{\beta}$ are meaningful by evaluating the difference in model sums of square and cross-product (SSCP) matrices with likelihood ratio tests (e.g., Wilks' Λ ; Rencher 1995). The SSCP matrix for an effect, α , in the model is expressed as a difference between full and reduced model SSCPs ("reduced" refers to the full model without the effect, α): $\mathbf{H} = \hat{\boldsymbol{\beta}}_f^T \mathbf{X}_f^T \mathbf{Y} - \hat{\boldsymbol{\beta}}_r^T \mathbf{X}_r^T \mathbf{Y}$; where f and r correspond to full and reduced matrices, respectively, and superscript T indicates a matrix transpose. The error SSCP matrix, \mathbf{E} , is calculated for both full and reduced models by: $\mathbf{E} = \boldsymbol{\varepsilon}^T \boldsymbol{\varepsilon} = \mathbf{Y}^T \mathbf{Y} - \hat{\boldsymbol{\beta}}^T \mathbf{X}^T \mathbf{Y}$. Although several test criteria are available (see Rencher 1995), Wilks' Λ is frequently used and is calculated directly from SSCP matrices. Solving \mathbf{E}_r in terms of \mathbf{E}_f yields $\mathbf{E}_r = \mathbf{E}_f + \mathbf{H}$. Thus

$$\Lambda = \frac{|\mathbf{E}_f|}{|\mathbf{E}_f + \mathbf{H}|} = \frac{|\mathbf{E}_f|}{|\mathbf{E}_r|}$$

and has an expected value of 1 under the null hypothesis that $\mathbf{E}_r = \mathbf{E}_f$ (i.e., $\alpha = 0$). Wilks' Λ can be converted to a test statistic that approximately follows an F distribution (Rencher 1995) to infer the significance of α .

An alternative way of thinking about the significance of α is a modified multivariate generalization of the "residual randomization" method described by ter Braak (1992) and Gonzalez and Manley (1998). This method uses the reduced model to determine predicted values ($\hat{\mathbf{Y}}_r = \mathbf{X}_r \hat{\boldsymbol{\beta}}$), $\boldsymbol{\varepsilon}_r$, and \mathbf{E}_r . Residuals in $\boldsymbol{\varepsilon}_r$ are randomized such that $\hat{\mathbf{Y}}_r$ and \mathbf{E}_r remain constant. Randomized residuals ($\boldsymbol{\varepsilon}_r^*$) are added to predicted values to produce random values ($\mathbf{Y}^* = \hat{\mathbf{Y}}_r + \boldsymbol{\varepsilon}_r^*$). The full model can then be used to calculate predicted values from the random data (but holding non-targeted fixed effects constant). Repeating this procedure many times produces an empirical null distribution of Wilks' Λ for α .

Within this framework, adjusted least-squares means for the full model ($\bar{\mathbf{Y}} = E[\mathbf{Y}_i | \mathbf{X}_i, \boldsymbol{\beta}_i]$) can be used to calculate the test statistics described in this article, $|D_{E_1} - D_{E_2}|$ and θ . With each iteration, random versions of these statistics can be described which hold $\hat{\mathbf{Y}}$ constant, meaning that distributions of random test statistics are concordant with the null distribution of Wilks' Λ for α . The empirical P values of observed statistics are thus the probabilities of finding an equal or more extreme value from the randomly generated distributions. This method has the additional appeal that it works for linear mixed models, and generalized linear models: $\mathbf{Y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\boldsymbol{\gamma} + \boldsymbol{\varepsilon}$ and $\mathbf{Y} = g^{-1}(\mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\boldsymbol{\gamma}) + \boldsymbol{\varepsilon}$, respectively; where \mathbf{Z} is an $n \times r$ design matrix for the $r \times p$ random effects in the matrix, $\boldsymbol{\gamma}$ (e.g., additive genetic effects, maternal effects, or nested populations), and $g^{-1}(\cdot)$ is the inverse of a differentiable monotonic link function for the generalized model (McCulloch and Searle 2001). Random distributions are generated by alteration of \mathbf{X}_f . Thus, models containing random effects, non-continuous, or non-normally distributed data can be used provided $\boldsymbol{\beta}$ is estimable.

We use the salamander data here as a computational example. The entire data consist of 18 shape variables but, for simplicity, we demonstrate our method with only three variables (S1–S3), which yield similar results. The data constitute an $n = 336$ by $p = 3$ matrix, \mathbf{Y} (i.e., salamanders are rows, shape variables are columns):

n	Species	Locality	S1	S2	S3
1	<i>P. jordani</i>	sympatry	0.067	-0.022	-0.027
2	<i>P. jordani</i>	sympatry	0.039	-0.004	0.008
⋮	⋮	⋮	⋮	⋮	⋮
151	<i>P. jordani</i>	allopatry	-0.012	-0.003	0.042
152	<i>P. jordani</i>	allopatry	-0.001	0.030	-0.023
⋮	⋮	⋮	⋮	⋮	⋮
199	<i>P. teyahalee</i>	sympatry	0.046	0.014	0.033
200	<i>P. teyahalee</i>	sympatry	0.042	0.030	0.010
⋮	⋮	⋮	⋮	⋮	⋮
294	<i>P. teyahalee</i>	allopatry	-0.076	-0.025	-0.017
295	<i>P. teyahalee</i>	allopatry	-0.088	0.042	-0.009
⋮	⋮	⋮	⋮	⋮	⋮

The design matrices, \mathbf{X}_f and \mathbf{X}_r for these data are as follows (note these matrices would also contain a vector of 1s for the intercept):

<i>n</i>	Species	Locality	species	locality	SPE × LOC	species	locality
1	<i>P. jordani</i>	sympatry	1	1	1	1	1
2	<i>P. jordani</i>	sympatry	1	1	1	1	1
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
151	<i>P. jordani</i>	allopatriy	1	-1	-1	1	-1
152	<i>P. jordani</i>	allopatriy	1	-1	-1	1	-1
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
199	<i>P. teyahalee</i>	sympatry	-1	1	-1	-1	1
200	<i>P. teyahalee</i>	sympatry	-1	1	-1	-1	1
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
294	<i>P. teyahalee</i>	allopatriy	-1	-1	1	-1	-1
295	<i>P. teyahalee</i>	allopatriy	-1	-1	1	-1	-1
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮

$$\mathbf{X}_f = \begin{bmatrix} 1 & 1 & 1 \\ 1 & 1 & 1 \\ \vdots & \vdots & \vdots \\ 1 & -1 & -1 \\ 1 & -1 & -1 \\ \vdots & \vdots & \vdots \\ -1 & 1 & -1 \\ -1 & 1 & -1 \\ \vdots & \vdots & \vdots \\ -1 & -1 & 1 \\ -1 & -1 & 1 \\ \vdots & \vdots & \vdots \end{bmatrix} \quad \mathbf{X}_r = \begin{bmatrix} 1 & 1 \\ 1 & 1 \\ \vdots & \vdots \\ 1 & -1 \\ 1 & -1 \\ \vdots & \vdots \\ -1 & 1 \\ -1 & 1 \\ \vdots & \vdots \\ -1 & -1 \\ -1 & -1 \\ \vdots & \vdots \end{bmatrix}$$

Least-squares means are calculated from both design matrix formats (see Manly 1997), yielding the following species–locality type means (in the order presented above):

$$\bar{\mathbf{Y}}_f = \begin{bmatrix} 0.024 & -0.029 & 0.003 \\ -0.058 & -0.005 & -0.008 \\ 0.020 & 0.049 & 0.004 \\ -0.063 & -0.002 & -0.011 \end{bmatrix} \quad \bar{\mathbf{Y}}_r = \begin{bmatrix} 0.024 & -0.021 & 0.004 \\ -0.058 & -0.030 & -0.009 \\ 0.019 & -0.036 & 0.003 \\ -0.062 & -0.026 & -0.010 \end{bmatrix}$$

The difference in means between matrices reflects the difference due to the species–locality type interaction effect. For $\bar{\mathbf{Y}}_f$, phenotypic change vectors, $\Delta\bar{\mathbf{Y}}_i$ (between sympatry and allopatriy) are calculated by subtracting the second row from the first row, and the fourth row from the third row, within $\bar{\mathbf{Y}}_f$. The vector lengths of $\Delta\bar{\mathbf{Y}}_i$ are calculated as $DE = \|\Delta\bar{\mathbf{Y}}_i\| = (\Delta\bar{\mathbf{Y}}_i \Delta\bar{\mathbf{Y}}_i^T)^{1/2}$ and the angle between $\Delta\bar{\mathbf{Y}}_i$ is calculated as

$$\theta = \cos^{-1} \left(\frac{\Delta\bar{\mathbf{Y}}_1 \cdot \Delta\bar{\mathbf{Y}}_2^T}{DE_1 DE_2} \right).$$

For the three shape variable included in this example $D_{P_j} = 0.086$, $D_{P_t} = 0.098$, and $\theta = 47.0^\circ$ (differing only slightly from the values reported for the full set of 18 shape variables). Randomizing residuals (i.e., holding $\bar{\mathbf{Y}}_r$ constant) for 10 000 permutations produced distributions of random vector length differences and random angles, given observed species and locality type effects. The empirical probabilities of finding greater values than $|0.098 - 0.086|$ and 47.0° provide a basis for inferring the significance of phenotypic change vector difference (see *Example 1: Character displacement in Plethodon salamanders: Results*).

SUPPLEMENT

Example data and R code (*Ecological Archives* E088-045-S1).