

ORIGINAL ARTICLE

# A method for analysis of phenotypic change for phenotypes described by high-dimensional data

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The analysis of phenotypic change is important for several evolutionary biology disciplines, including phenotypic plasticity, evolutionary developmental biology, morphological evolution, physiological evolution, evolutionary ecology and behavioral evolution. It is common for researchers in these disciplines to work with multivariate phenotypic data. When phenotypic variables exceed the number of research subjects—data called ‘high-dimensional data’—researchers are confronted with analytical challenges. Parametric tests that require high observation to variable ratios present a paradox for researchers, as eliminating variables potentially reduces effect sizes for comparative analyses, yet test statistics require more observations than variables. This problem is exacerbated with data that describe ‘multidimensional’ phenotypes, whereby a description of phenotype requires high-dimensional data. For example, landmark-based geometric morphometric data use the Cartesian coordinates of (potentially) many anatomical landmarks to describe organismal shape. Collectively such shape variables describe organism shape, although the analysis of each variable, independently, offers little benefit for addressing biological questions. Here we present a nonparametric method of evaluating effect size that is not constrained by the number of phenotypic variables, and motivate its use with example analyses of phenotypic change using geometric morphometric data. Our examples contrast different characterizations of body shape for a desert fish species, associated with measuring and comparing sexual dimorphism between two populations. We demonstrate that using more phenotypic variables can increase effect sizes, and allow for stronger inferences.

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## INTRODUCTION

An interesting coevolution of two fields has transpired over the past few decades. In evolutionary biology, conceptual challenges to visualizing multivariate phenotypic change in response to natural selection have received considerable attention (Lande, 1979, 1980, 1981; Lande and Arnold, 1983; Phillips and Arnold, 1989; Brodie *et al.*, 1995; Schluter, 2000; Blows, 2007). At the same time, the ‘Procrustes paradigm’ (Adams *et al.*, 2013) evolved from its conceptual beginnings (Rohlf and Slice, 1990; Rohlf and Marcus, 1993), revolutionizing the way biologists describe and compare organismal shape, using geometric morphometric (GM) methods. Consistent between these two growing disciplines was the need for methods to analyze multivariate phenotypic data. Hence, various multivariate analyses were also developed, for example, to measure and test the association between matrices of multivariate phenotypes and other variables (Rohlf and Corti, 2000), to measure and compare multivariate vectors (Adams and Collyer, 2007; Collyer and Adams, 2007) or trajectories (Adams and Collyer, 2009; Collyer and Adams, 2013) and to assess such patterns in a phylogenetic framework (Adams and Felice, 2014; Adams, 2014a, b). Despite the convergence of different disciplines to spur development of analytical methods for multivariate phenotypic data, there is an interesting dichotomy between the disciplines.

In evolutionary biology, the ‘multivariate phenotype’ of an individual can be defined as a vector of either known or assumed

to be related trait values. By this definition, there is no precise indication that the traits, themselves, must be related in context, but just potentially correlated. For example, one might describe a multivariate phenotype with both morphological and life history values, rather than just multiple morphological values, as life history traits and morphological traits are likely to be correlated (Huttenegger and Mitteroecker, 2011). This is the emphasis of phenotypic integration (Arnold, 2005) that natural selection acts upon multiple, functionally related traits, and adaptation is an inherently multivariate process (Blows, 2007). Thus, the multivariate phenotype in evolutionary biology is a set of phenotypic traits that are potentially correlated in some way, and multivariate analyses are used to appropriately account for such correlations, although, hypothetically, individual variables could be analyzed separately.

In contrast, the data from GM methods are necessarily multivariate and explicitly require multivariate analysis. The phenotypic trait, organismal shape, is characterized by potentially many shape variables (derived from Cartesian coordinates of anatomical landmarks). None of these shape variables are interesting, individually, but collectively they define organismal shape as a ‘multidimensional trait’ (Klingenberg and Gidaszewski, 2010; Adams, 2014b). Whereas the previous definition of a multivariate phenotype emphasizes that multiple phenotypic traits are potentially correlated, the multidimensional trait is a single trait comprising multiple variables that are

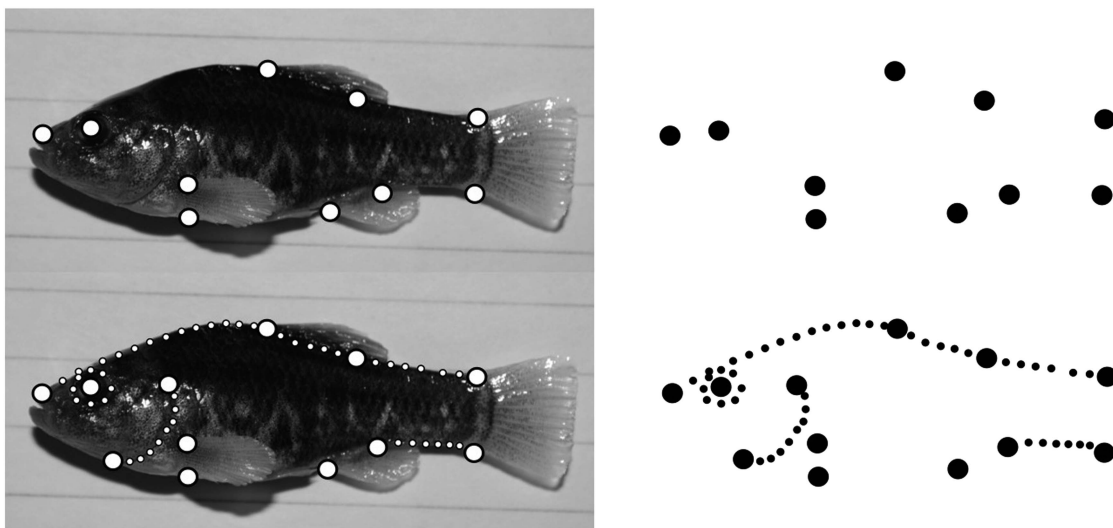
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certainly correlated in some way. Analysis of the single variables of a multidimensional trait, like shape, would be foolhardy, as they do not independently describe organismal shape. Only collectively, are the variables meaningful. In terms of the data, a multidimensional trait is a multivariate phenotype—both are vectors of variable scores—but in terms of biological questions, a multidimensional trait is more precise definition of a trait that requires full complement of its multiple variables to define it. Despite the precision in definitions that discern between multivariate phenotypes or multidimensional traits, hypothesis tests for both are concerned with assessing the amount of phenotypic change in a multivariate data space, associated with a gradient of ecological or evolutionary change. Linear models (or generalized linear models) are required for estimating the coefficients of phenotypic change for phenotypic variables. Hypothesis tests such as multivariate analysis of variance (MANOVA) are used to evaluate the significance of such coefficients.

Why then is the distinction between multivariate phenotype and multidimensional trait worth making? The latter emphasizes an analytical challenge, which is becoming increasingly common in the field of GM (Adams *et al.*, 2013), and other disciplines, when it might be preferable or even necessary to define a multidimensional trait with more variables than there are subjects to analyze. The current efficiency of digitizing equipment and computing power of computers permits collecting, for example, thousands of surface landmarks to define organismal shape. It might seem intuitively reasonable that using more anatomical information than less means having a greater ability to discern among different shapes (Figure 1) but, paradoxically, increasing the number of variables can decrease statistical power or preclude hypothesis testing about shape differences, altogether, using parametric multivariate tests (as parametric tests use probability distributions based on error degrees of freedom). Removing variables for multidimensional traits is not an option, and using, for example, fewer landmarks in the case of GM approaches, compromises the integrity of the morphological description used for comparative studies (see Adams, 2014b).

‘High-dimensional’ data are multivariate phenotypic data that use more variables to describe a phenotype than the number of phenotypes to analyze. High-dimensional data present a roadblock for analysis if typical (that is, parametric) statistical methods are used. Comparative analysis of high-dimensional data, in general, has received considerable recent attention. Especially in the field of community ecology, nonparametric methods have been developed based on test statistics derived from multivariate distances (Anderson, 2001a, b; McArdle and Anderson 2001). These methods have great appeal, as they do not rely on data spaces where Euclidean distances are the only appropriate metric of intersubject differences and can, therefore, be generalized to many different data types. (However, choice among different metrics or pseudometrics has consequences for statistical power; see Warton *et al.*, 2012.) Probability distributions for test statistics of these methods are derived from resampling experiments, using full randomization of raw phenotypic values, randomization of raw phenotypic values within strata or residuals from linear models (Anderson, 2001b; McArdle and Anderson, 2001). An acknowledged challenge for nonparametric (np)-MANOVA is the appropriate method for generating probability distributions for test statistics for factorial models (Anderson, 2001b). As discussed below, various independent studies have confirmed the benefit of using resampling experiments with residuals from linear models for multivariate data, especially for multifactor models with factor interactions. The purpose of this article is to synthesize different aspects of methodological development, plus introduce some new perspectives to establish a paradigm for analyzing high-dimensional phenotypic data. Although the intent is to offer a paradigm of general interest to several evolutionary biology disciplines, including phenotypic plasticity, evolutionary developmental biology, morphological evolution, evolutionary ecology and behavioral evolution, and should have appeal for any phenotypic data, we present examples specifically using data obtained from GM methods. We also demonstrate that the paradigm presented is commensurate with other recent methodological advances.



**Figure 1** Landmark configurations used for data analysis. The top configurations comprise 10 ‘fixed’ anatomical landmarks, indicating fin insertions, the dorsal tip of the premaxillary and the center of the eye. The bottom configurations are the same 10 landmarks plus two additional landmarks and 44 ‘sliding’ semi-landmarks that are used to estimate the curvature of the dorsal crest, caudal region and operculum, as well as the relative size and position of the eye. Landmark configurations on the right are shown in the absence of the source photograph, indicating the more realistic characterization of body shape with 56 landmarks.

## MATERIALS AND METHODS

### Conceptual development

We provide here a general description of a method for analyzing phenotypic change in high-dimensional data spaces, and also provide additional analytical details in the Supplementary Information. The term ‘phenotypic change’ can take on different meanings. For example, performing a hypothesis test to determine whether two taxa have different phenotypes attempts to ascertain whether the phenotypic change between means of the two taxa is significantly  $> 0$ . However, we intend that analysis of phenotypic change requires a factorial approach, where at least one factor indicates a categorical assignment of subjects into distinct groups (for example, taxa, population, sex) and at least one factor (or covariate) describes an interesting gradient for phenotypic change (for example, environmental difference, ecological difference, growth). Thus, analysis of phenotypic change refers to a statistical approach to determine whether two or more groups have consistent or differing phenotypic change along a gradient. Generally, this is a statistical assessment of a factor or factor–covariate interaction.

Many users of statistical software for MANOVA might not be aware that underlying the results is a methodological paradigm for calculating effects. MANOVA starts with an initial linear model, such as,

$$\text{Phenotype} \sim \text{Taxon} + \text{Environment} + \text{Taxon} \times \text{Environment}$$

which would be an appropriate model for determining whether different taxa have consistent or different changes in phenotype across an environmental gradient. Most users are probably aware that if the *Taxon*  $\times$  *Environment* interaction is significant, then it is less appropriate to concern oneself with the main effects, *Taxon* and *Environment*, as taxa have varied responses to different environments (that is, phenotypic change between environments is not the same among taxa). However, many users might not be aware that the test statistic and the *P*-value used to determine whether the *Taxon*  $\times$  *Environment* interaction is significant are calculated from a comparison of the ‘full’ model above and a ‘reduced’ model, namely,  $\text{Phenotype} \sim \text{Taxon} + \text{Environment}$ . The ‘size’ of the *Taxon*  $\times$  *Environment* effect, or any effect in the full model, is based on the difference in error produced by two models: one that contains the effect and one that lacks it. Thus, the methodological paradigm for multifactor MANOVA is an *a priori* decision to either add model terms sequentially, performing a comparison of initial and final models with each term addition, or iteratively compare the marginal difference between the full model and ones reduced by each term; processes known as calculating the sequential and marginal sums of squares, respectively (Shaw and Mitchell-Olds, 1993). There are also other methods, especially for models with three or more factors.

Although there are various multivariate coefficients for measuring effect size, and the choice of model comparison paradigm can alter their values (as well as *P*-value estimated from them), the default approach for most statistical programs is to estimate the probability of a type I error from integration of parametric probability density functions, like those that generate *F*-distributions. A necessary step is to convert multivariate coefficients to approximate *F*-values (Rencher and Christensen, 2012). The parameters of the *F*-distribution are transformations based on both linear model parameters and number of phenotypic variables, but rely on the former being larger than the latter. When the number of phenotypic variables exceeds the number of error degrees of freedom of the linear model (the number of observations minus the number of model parameters), parametric MANOVA cannot be performed. However, there is no such limitation in estimating coefficients for the linear model.

Arnold (2005) posited that it behooves evolutionary biologists to become skilled in linear algebra, as the conceptual development of the field is based on linear models, and bypassing the portions of important formative articles that contain matrix equations is tantamount to being ‘lost in translation’. Similarly, relying on the results of MANOVA without understanding the paradigm of linear model comparisons can cause problems with analyzing phenotypic change, not the least of which is to throw away phenotypic variables for the sake of attaining results. We, and others (Anderson and Legendre, 1999; McArdle and Anderson, 2001; Anderson, 2001b; Wang *et al.*, 2012), approach MANOVA as a multifaceted approach for providing probability distributions for test statistics based on the comparison of linear models. One does not need to use default computer program statistics or parametric methods for

probability distributions; rather, understanding the paradigm of linear model comparison allows one to make better-informed choices about the appropriate test statistics to use, and the method for generating probability distributions. The following is our description of this general paradigm for np-MANOVA, employing a probability distribution generation method that resamples linear model residuals, known as the randomized residual permutation procedure (Freedman and Lane, 1983; Collyer *et al.*, 2007; Adams and Collyer, 2007, 2009; Collyer and Adams, 2007, 2013).

*Step 1: describe the null model.* The phenotypic values of *p* variables, for *n* observations comprise a  $n \times p$  matrix, **Y**. If *p* is larger than *n*, **Y** is a matrix of high-dimensional data. A linear model can be used to estimate the relationship of values in **Y** with values from independent variables, such that,  $\mathbf{Y} = \mathbf{X}\mathbf{B} + \mathbf{E}$ , where **X** is a  $n \times k$  design matrix, **B** is an  $k \times p$  matrix of for the *k*–1 model coefficients plus an intercept (vector of 1s) and **E** is an  $n \times p$  matrix of residuals (Rencher and Christensen, 2012). In the case of the null model, **X** is only a vector of 1s, and the estimated  $1 \times p$  vector of coefficients,  $\hat{\mathbf{B}}$ , is solved as  $\hat{\mathbf{B}} = (\mathbf{X}^T\mathbf{X})^{-1}(\mathbf{X}^T\mathbf{Y})$ , where the superscripts <sup>T</sup> and <sup>–1</sup> indicate matrix transposition and inversion, respectively, and the symbol, ^, indicates estimation. (Solving  $\hat{\mathbf{B}}$  using generalized least squares is discussed in the Supplementary Information.) In the case of the null model,  $\hat{\mathbf{B}}$  is the centroid (multivariate mean). A  $n \times p$  matrix of ‘fitted’ values is found as  $\mathbf{X}\hat{\mathbf{B}}$ , and the residuals are found as  $\hat{\mathbf{E}} = \mathbf{Y} - \mathbf{X}\hat{\mathbf{B}}$ . The matrix of fitted values is a matrix of the centroid repeated *n* times. The  $p \times p$  matrix of sums of squares and cross-products for the null model is found as  $\hat{\mathbf{S}} = \hat{\mathbf{E}}^T\hat{\mathbf{E}}$ . This square-symmetric matrix contains the sum of squares (SS) for each variable along the diagonal, and the summed cross-products of each variable pair in the off-diagonal elements. The total SS can be calculated as the trace of  $\hat{\mathbf{S}}$  that is also equal to the trace of the  $n \times n$  matrix,  $\hat{\mathbf{E}}\hat{\mathbf{E}}^T$ . The diagonal of  $\hat{\mathbf{E}}\hat{\mathbf{E}}^T$  represents the squared distances of the *n* observations from the centroid; thus, SS is a measure of dispersion equal to the sum of squared distances of observations, making this method commensurate with nonparametric approaches based on distances (Goodall, 1991; McArdle and Anderson, 2001; Anderson, 2001a). Furthermore, the number of phenotypic variables is inconsequential for this statistic, based on this measure of SS.

*Step 2: describe the first-factor model and compare it with the null model.* The choice of first factor or covariate is arbitrary, but should not be made without consideration. We propose that if the analysis contains a continuous covariate such as organism size, which is measured at the level of the subject (unlike, for example, population, taxon), this variable should be added first. For simplicity, we will ascribe the covariate or factor as *A*. The procedure is followed as in step 1, except that the design matrix contains a vector of 1s for the intercept and  $k_A$  additional columns. If *A* is a covariate,  $k_A$  equals 1. If *A* is a factor (for example, categorical grouping variable),  $k_A$  equals *g*–1 for the *g* levels of groups. This design matrix is called  $\mathbf{X}_F$  because it represents the ‘full’ complement of model parameters, whereas the null design matrix,  $\mathbf{X}_r$ , is ‘reduced’ by the parameters that model the effect, *A*. Both  $\hat{\mathbf{S}}$  and SS can be calculated as in step 1 but, more importantly,  $\hat{\mathbf{S}}_A$  can be calculated as  $\hat{\mathbf{S}}_A = \hat{\mathbf{E}}_r^T\hat{\mathbf{E}}_r - \hat{\mathbf{E}}_F^T\hat{\mathbf{E}}_F$ , which is the same as  $\hat{\mathbf{S}}_A = (\hat{\mathbf{E}}_r - \hat{\mathbf{E}}_F)^T(\hat{\mathbf{E}}_r - \hat{\mathbf{E}}_F)$ , and whose trace is the SS of the effect of the parameters in *A*,  $SS_A$ . In other words, the effect of *A* is tantamount to the change in error between two models that contain and lack the parameters for *A*.  $SS_A$  is also a measure of dispersion that is the sum of squared distances of predicted (fitted) values from the centroid (the trace of  $\hat{\mathbf{E}}_F^T\hat{\mathbf{E}}_F$  or  $\hat{\mathbf{E}}_F\hat{\mathbf{E}}_F^T$  is the sum of squared distances of observations from their predicted values, the multivariate error of the full model).

*Step 3: describe the second-factor model and compare it with the first-factor model.* The design matrix,  $\mathbf{X}_F$  in step 2 becomes  $\mathbf{X}_r$  in step 3. All calculations in step 2 are repeated in step 3 to produce  $\hat{\mathbf{S}}_B$  and  $SS_B$ . The important caveat of this sequential method of calculations is that  $SS_B$  is the effect of *B*, after accounting for the effect of *A*.

*Step 4: describe the interaction model and compare it with the second-factor model.* The design matrix,  $\mathbf{X}_F$  in step 3 becomes  $\mathbf{X}_r$  in step 4. All calculations in step 3 are repeated in step 4 to produce  $\hat{\mathbf{S}}_{AB}$  and  $SS_{AB}$ . The important caveat of this sequential method of calculations is that  $SS_{AB}$  is the effect of

the interaction between *A* and *B*, after accounting for the main effects of *A* and *B*.

**Step 5: develop statistics.** The *SS* of each effect calculated in steps 2–4 are sufficient to use as test statistics, based on a resampling experiment (randomization test). However, it might be of interest to convert these values to variances, coefficients of determination, or *F*-values (see Supplementary Information). Any calculation of test statistics is a linear or nonlinear transformation of *SS*, as model parameters and *n* are constants (Anderson and Ter Braak, 2003). Therefore, the rank order of *SS* for the effects or test statistics calculated from them will be exactly the same in a resampling experiment, meaning *P*-values calculated as percentiles from empirical probability distributions will also be exactly the same.

**Randomized residual permutation procedure (RRPP).** RRPP is a procedure that uses a resampling experiment to randomize the residual (row) vectors of a matrix of residuals from a reduced model to calculate pseudorandom values for estimation of effects from a full model (Collyer *et al.*, 2007; Adams and Collyer, 2007, 2009; Collyer and Adams, 2007, 2013). The advantage of this approach, compared with randomizing vectors of raw phenotypic values, is that it holds constant the effects of the reduced model. For example, randomizing residuals of the second-factor model to generate pseudorandom values for estimation of parameters in the interaction model, many times, allows generation of a probability distribution of the interaction effect, holding constant the main effects. RRPP does not assume that alternative effects are inconsequential.

One important criterion though is how to implement RRPP when not one but three matrices of randomized residuals are required for evaluating the two main effects and interaction effect of a factorial or factor–covariate model. One might just choose to perform RRPP three separate times. However, this would mean that the random permutations of the three resampling experiments would be different, especially if the number of random permutations is small, and this might lead to an increase in probability of a type I error (as this would be the same as performing three separate tests). This problem can be alleviated by simply concatenating the matrices of residuals from steps 1 to 3. In every random permutation, matrices of this concatenated matrix are shuffled, meaning the placement of the three residual vectors for each observation is exactly the same. Pseudorandom values are calculated by partitioning the randomized concatenated residuals into their original  $n \times p$  dimensions and adding residuals to fitted values of corresponding models. Steps 2–5 are repeated for each random permutation, generating sampling distributions of statistics for each model effect. These sampling distributions are also probability distributions, as the percentile of observed statistics indicates the probability of observing a larger value, by chance, from the random outcomes of reduced (null) models.

**Generalization.** Perhaps the best indicator that a multivariate generalization is appropriate is that using univariate data produces the same results expected from univariate analyses. Using the paradigm above is exactly the same as performing analysis of variance on a linear model for a univariate-dependent variable, using sequential sums of squares. The only potential difference is that probability distributions are empirically generated rather than using parametric *F*-distributions. As discussed elsewhere (Anderson and Ter Braak, 2003), this is an appropriate method of probability distribution estimation, especially because it relaxes assumptions required for parametric distributions (especially concerning normally distributed error). Randomizing residuals produces type I error rates closer to exact tests than other randomization procedures. This paradigm will thus produce expected analysis of variance results. However, this approach has two major benefits. First, RRPP allows one to estimate relative effect sizes as standard deviations of sampling distributions (Collyer and Adams, 2013). Therefore, one can compare the size of effects both within and among different studies. Second, test statistics can be calculated with any number of phenotypic variables. The Supplementary Information contains some additional steps for calculating different types of statistics—which one might wish to consider for high-dimensional data—but in any case, the number of variables is not a limiting criterion. The following examples

illustrate why using more variables might be preferable than using fewer, in addition to demonstrating how this np-MANOVA paradigm works.

### Example 1: sexual dimorphisms in body shape for different populations of a desert fish

For this example, landmark data were collected from 54 museum specimens of Pecos pupfish (*Cyprinodon pecosensis*). These fish are inhabitants of the Pecos River and associated aquatic habitats in eastern New Mexico, USA. The 54 specimens comprise fish collected from a large marsh system (16 females and 13 males) and a small sinkhole (12 females and 13 males). In the former, *C. pecosensis* is part of a larger fish community (with four other species), in which at least one other species can be considered a predator of *C. pecosensis*. In the latter, *C. pecosensis* cooccurs with two other fish species that can be considered competitors. Sexual dimorphism has been noted in other species of *Cyprinodon* (Collyer *et al.*, 2005, 2007, 2011). Predators could hypothetically mitigate sexual dimorphism in *Cyprinodon* body shape. In the presence of predators, males are likely to exhibit streamlined body shapes with deep, compressed caudal regions associated with active predator avoidance (Langerhans *et al.*, 2004). Such a body shape would be more similar to the generally more streamlined body shapes of females. In contrast, males in predator-free environments might exhibit deeper, laterally compressed body shapes, associated with defense of breeding territories. Previous research on a congener using a common garden experiment has shown that body shape is heritable, but phenotypic plasticity in body shape can be associated with environmental gradients, such as salinity (Collyer *et al.*, 2011). We hypothesized that phenotypic plasticity in male body shape might be mediated by predation that would have consequences for the amount of sexual dimorphism in different *C. pecosensis* populations. This example represents one comparison of one predator (marsh) population and one antipredator (sinkhole) population, using one sample from each. It is not intended to be a comprehensive examination of sexual dimorphism, but rather illustrate the utility of the analytical paradigm, especially for small sample sizes.

Body shape was characterized in two different ways. A landmark configuration of 12 ‘fixed’ anatomical landmarks and 44 sliding semi-landmarks (that is, 112 variables from the Cartesian coordinates of the points) was digitized on the left lateral surface of photographs of fish specimens (Figure 1). A simpler configuration of 10 of the 12 fixed landmarks was also defined. The Cartesian coordinates of these landmarks were used to generate ‘Procrustes residuals’ via generalized Procrustes analysis (Rohlf and Slice, 1990). The generalized Procrustes analysis centers, scales to unit size and rotates configurations using a generalized least squares criterion, until they are optimally invariant in location, size and orientation, respectively. The aligned coordinates are the Procrustes residuals that can be used as shape variables themselves, or projected into a space tangent to the shape space, where shape variables are often described as the eigenvectors for these projections (Adams *et al.*, 2013). Our analyses used Procrustes residuals, but we visualized shape variation from projection of shapes onto principal components (PC) of shape variation.

For analysis of phenotypic change, we were interested in the model, *Body Shape* ~ *Population* + *Sex* + *Population* × *Sex*. We performed np-MANOVA using RRPP with 10 000 permutations on both types of landmark configurations to compare results between the two configuration types. (Additional analyses were also performed to compare the np-MANOVA to parametric MANOVA, and to compare RRPP with a randomization test using raw phenotypic values. The details of these analyses plus results are provided in the Supplementary Information.) The *post hoc* pairwise comparisons of group means were also performed, using the exact same random permutations of RRPP.

### Example 2: sexual dimorphisms in body shape allometry for different populations of a desert fish

In this example, the same data were used as in the previous example, but the intent was to consider the influence of phenotypic change associated with body size (static body shape allometry) among the *Population* × *Sex* groups. Body size was calculated from landmark configurations as centroid size (CS), the square root of summed squared distances of landmarks from the configuration centroid (Bookstein, 1991). The linear model used was *Body*

$Shape \sim \log(CS) + (Population \times Sex) + \log(CS) \times (Population \times Sex)$ . As in the previous example, np-MANOVA analyses, using RRPP with 10 000 permutations, were performed on both the 10- and 56-landmark configurations. A *post hoc* test of pairwise differences between least squares means was also performed, as in example 1, as np-MANOVA revealed that population by sex groups had common shape–size allometries (see Results).

All analyses in both examples were performed in R, version 3.0.2 (R Core Team, 2014). Generalized Procrustes analysis and thin-plate spline analysis (to generate transformation grids) were performed using the package geomorph, version 2.1, within R (Adams and Otárola-Castillo, 2013; Adams *et al.*, 2014). Any np-MANOVA effects or pairwise differences were considered significant if their *P*-values were less than a type I error rate of  $\alpha = 0.05$ . Because the RRPP method introduced here performs the exact same random placement of residuals for every test statistic calculated, we do not consider the inferences to be separate tests, but rather separate inferences from the same test (see Supplementary Information for details). For simplicity, we report coefficients of determination, effects sizes (*Z*-scores) and *P*-values here, but additional statistics plus parametric statistics (where appropriate) are provided in the Supplementary Information.

## RESULTS

### Example 1

The np-MANOVA analyses performed with RRPP indicated that main effects were significant for both 10- and 56-landmark

**Table 1 Nonparametric multivariate analysis of variance (np-MANOVA) statistics based on a randomized residual permutation procedure (RRPP) with 10 000 random permutations**

Source	d.f.	10 Landmarks			56 Landmarks		
		R <sup>2</sup>	Z	P	R <sup>2</sup>	Z	P
Population	1	0.1378	11.2771	0.0001	0.1625	12.2766	0.0001
Sex	1	0.2140	14.4609	0.0001	0.2798	16.4307	0.0001
Population × sex	1	0.0250	2.2338	0.8697	0.0613	6.2732	0.0098

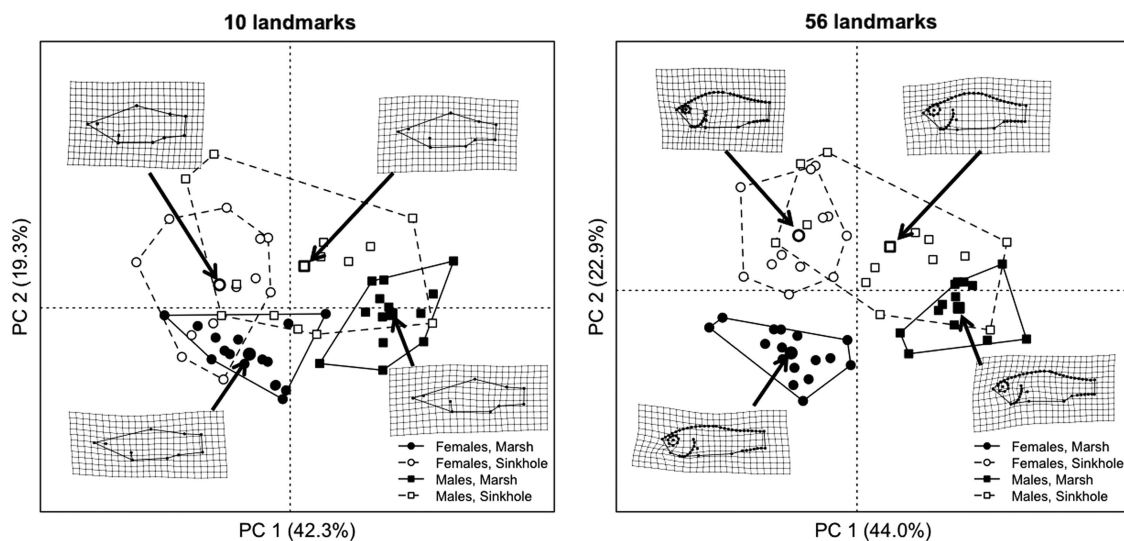
The error degrees of freedom were 50. Effect sizes (*Z*) are standard deviations of observed *SS*-values from sampling distributions of random values found via RRPP. Each *P*-value are the probability of finding a random value larger than the observed value. See Supplementary Information for additional statistics.

configurations, but the interaction between population and sex was only significant for the 56-landmark configuration (Table 1). Effect sizes were all larger using the 56-landmark configurations. These results were consistent with PC plots of shape variation (Figure 2). In the 56-landmark case, means were more distinct, as evidenced by the comparatively smaller dispersion of individual shapes relative to the distances between means. Unexpectedly, sexual dimorphism was larger in the case of the marsh pupfish, and marsh females were most divergent, based on 56-landmark configurations. The *post hoc* test of pairwise distances indicated that sexual dimorphism for marsh fish was the only significant pairwise shape difference, after accounting for general shape differences between populations and between males and females (Table 2). No pairwise differences in shape were significant for the 10-landmark configurations. Thus, *post hoc* tests performed as expected, based on the results of np-MANOVA.

Transformation grids (Figure 2) indicated that the divergent body shapes of marsh females revealed in the 56-landmark configurations were strongly the result of opercular curvature (landmarks defining the ventral curvature of the head). Although both configurations indicated that females had more streamlined body shapes than males, and that sinkhole fish had relatively shorter caudal regions (indicated by divergence along the second PC), only the analysis on the 56-landmark configuration was able to detect subtle differences in head shape. Hence, it revealed greater sexual dimorphism in body shape for Marsh pupfish, and a larger effect size for the population by sex interaction. In essence, more variables increased effect size, in this case (we found that np-MANOVA with RRPP also provided more ‘honest’ results than parametric MANOVA or np-MANOVA with randomization of raw data, as explained in the Supplementary Information).

### Example 2

In the second example, results of the np-MANOVA were rather consistent between the 10- and 56-landmark configurations, and the effect sizes for each model effect were comparable, except for the noticeably larger effect for the population by sex effect for the 56-landmark configuration (Table 3). In both cases, an interaction between  $\log(CS)$  and the population by sex groups was not



**Figure 2** PC plots of shape variation. PCs are the first two eigenvectors of Procrustes residuals projected into a space tangent to shape space. The relative amount of shape variation explained by PC is shown. Individual shapes are shown as well as convex hulls. Transformation grids (scaled  $\times 2$ ) are shown to facilitate and understand shape change among groups. These shapes correspond to mean values, shown as bolder symbols.

**Table 2** Pairwise Procrustes distances and *P*-values based on a randomized residual permutation procedure (RRPP) with 10 000 random permutations associated with nonparametric multivariate analysis of variance (np-MANOVA) in Table 1

	10 Landmarks				56 Landmarks			
	<i>M, F</i>	<i>M, M</i>	<i>S, F</i>	<i>S, M</i>	<i>M, F</i>	<i>M, M</i>	<i>S, F</i>	<i>S, M</i>
Marsh, female		0.1329	0.5428	0.6862		<b>0.0056</b>	0.0560	0.6891
Marsh, male	0.0430		0.5862	0.3623	<b>0.0458</b>		0.5826	0.4272
Sinkhole, female	0.0309	0.0554		0.9042	0.0328	0.0454		0.9885
Sinkhole, male	0.0344	0.0334	0.0307		0.0385	0.0280	0.0245	

Abbreviations: *M, F*, Marsh, female; *M, M*, Marsh, male; *S, F*, Sinkhole, female; *S, M*, Sinkhole, male. Values below diagonal are distances; above diagonal are *P*-values. Bolded values are significant at  $\alpha=0.05$ .

**Table 3** Nonparametric multivariate analysis of variance (np-MANOVA) statistics based on a randomized residual permutation procedure (RRPP) with 10 000 random permutations

Source	<i>d.f.</i>	10 Landmarks			56 Landmarks		
		<i>R</i> <sup>2</sup>	<i>Z</i>	<i>P</i>	<i>R</i> <sup>2</sup>	<i>Z</i>	<i>P</i>
log(CS)	1	0.2257	18.4582	0.0001	0.2480	18.4387	0.0001
Pop. $\times$ sex	3	0.2238	13.9847	0.0001	0.3251	18.7852	0.0001
log(CS) $\times$ (Pop. $\times$ sex)	3	0.0508	3.4257	0.9499	0.0351	3.0552	0.9872

Abbreviations: CS, centroid size; Pop., population.

The error degrees of freedom were 46. Effect sizes (*Z*) are standard deviations of observed.

*F*-values from sampling distributions of random values found via RRPP. *P*-values are the probability of finding a random value larger than the observed value. See Supplementary Information for additional statistics.

**Table 4** Pairwise Procrustes distances and *P*-values based on a randomized residual permutation procedure (RRPP) with 10 000 random permutations associated with nonparametric multivariate analysis of variance (np-MANOVA) in Table 3

	10 Landmarks				56 Landmarks			
	<i>M, F</i>	<i>M, M</i>	<i>S, F</i>	<i>S, M</i>	<i>M, F</i>	<i>M, M</i>	<i>S, F</i>	<i>S, M</i>
Marsh, female		0.0005	0.0002	0.0001		0.0001	0.0001	0.0001
Marsh, male	0.0333		0.0001	0.0001	0.0356		0.0002	0.0006
Sinkhole, female	0.0348	0.0506		0.0162	0.0363	0.0365		0.0404
Sinkhole, male	0.0333	0.0357	0.0244		0.0378	0.0268	0.0182	

Abbreviations: *M, F*, Marsh, female; *M, M*, Marsh, male; *S, F*, Sinkhole, female; *S, M*, Sinkhole, male.

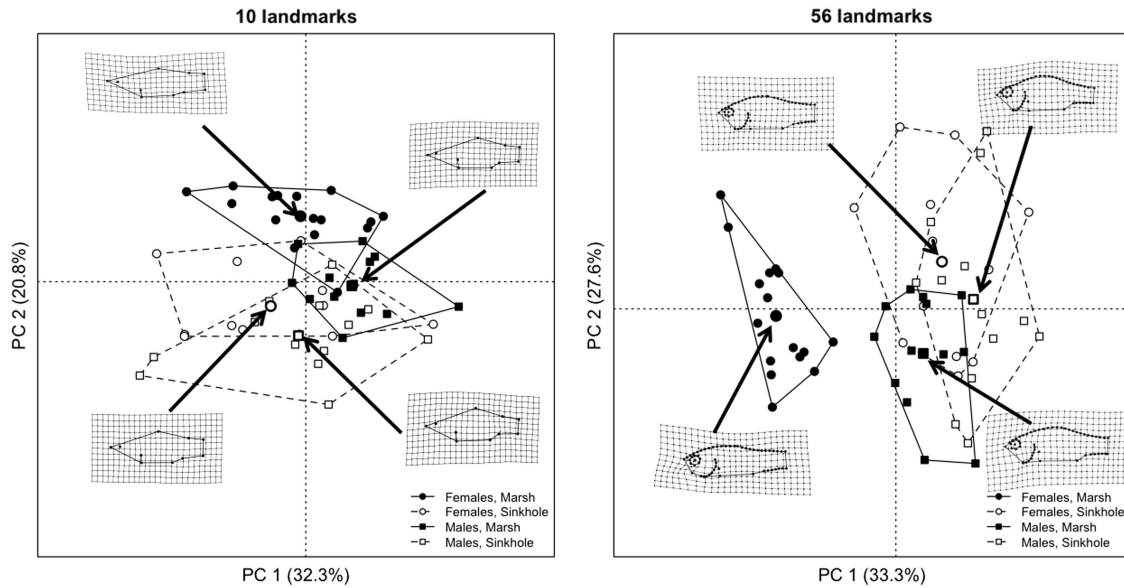
The reduced model for RRPP was log(centroid size (CS)) and the full model was log(CS) + (population  $\times$  sex). Values below diagonal are distances and above diagonal are *P*-values. All values are significant at  $\alpha=0.05$ .

significant, indicating a common shape–size allometry among groups. For both the 10- and 56-landmark configurations, all pairwise distances between least squares means (assuming a common allometry) were significant (Table 4). Results from the *post hoc* test and PC plots (Figure 3) confirmed that greater sexual dimorphism was found in marsh pupfish because of the divergent head shapes of female fish. This result was also consistent with the analysis in example 1. However, accounting for shape allometry increased the ability to detect shape differences among any groups, for both 10- and 56-landmark configurations.

## DISCUSSION

The examples above, plus the additional analyses in the Supplementary Information, highlight three important attributes of a paradigm for analysis of phenotypic change using np-MANOVA and RRPP. First, the effect sizes and *P*-values of np-MANOVA statistics are reasonable and intuitive based on PC plots of multi-dimensional trait variation. In the case where parametric MANOVA could be applied (10-landmark configurations), np-MANOVA

provided more conservative results (less likely to reveal significant effects; see Supplementary Information). One could see np-MANOVA as a safeguard against inferential errors that are likely caused by parametric MANOVA when assumptions are not met, or see parametric MANOVA as having greater statistical power. However, the latter is unlikely. First, statistical research on various univariate and multivariate linear model designs indicates that RRPP provides asymptotically appropriate *P*-values that are closest to an exact test (Anderson, 2001b). Second, just by the nature of converting multivariate test statistics like Pillai's trace to *F*-values, as *p* approaches the  $n - k$  degrees of freedom in model error, the denominator degrees of freedom for the *F*-distribution decrease (Rencher and Christensen, 2012) that is effectively a decrease in statistical power without an increase in effect size. Third, based on our results, effect sizes increased by using more variables, suggesting an increase in statistical power, although parametric MANOVA could not be used. Indeed, estimation of statistical power curves for known effects and type I error rates using np-MANOVA and RRPP will be an exciting next phase of research.



**Figure 3** PC plots of allometry-free shape variation. All descriptions are the same as in Figure 2, but PCs were derived from Procrustes residuals after regression of shape on log centroid size.

The second attribute worth noting is that np-MANOVA with RRPP found larger effect sizes for important effects when high-dimensional data were used. It seems that np-MANOVA performed with RRPP might be one solution to the ‘curse of dimensionality’, analogous to other distance-based approaches that, irrespective of the number of variables used, one can produce  $n \times n$  analogs of sums of squares and cross products matrices, namely  $(\hat{\mathbf{E}}_r - \hat{\mathbf{E}}_f)(\hat{\mathbf{E}}_r - \hat{\mathbf{E}}_f)^T$ , whose diagonal elements are the  $n$  squared distances of predictions between full and reduced models. These squared distances indicate which observations correspond to a larger effect. Inclusion of more phenotypic variables rather than less is more likely to increase the sensitivity to detect subtle but perhaps important phenotypic differences, much like the opercular curvature noted in our examples, that would be missed with the 10-landmark configuration. The trace of  $(\hat{\mathbf{E}}_r - \hat{\mathbf{E}}_f)(\hat{\mathbf{E}}_r - \hat{\mathbf{E}}_f)^T$  is the effect SS that can only increase by including more phenotypic variables. Therefore, adding more variables should have no negative consequence on the effect size. For example, using more than 56 landmarks to characterize the same aspects of curvature in our examples should not reduce effect sizes, but could increase them. In a theoretical sense, there should be no paradox because of an inverse relationship between variable number and statistical power. However, in an applied sense, adding more variables might increase the propensity for measurement error that could have an adverse effect.

Therefore, the third important attribute is that the sampling distributions empirically produced by RRPP allow one to estimate the effect size of observed effects from the distributions of random results. In our examples, even when effects were consistently important (significant) between different landmark configurations, the 56-landmark configurations led to larger effect sizes. In one case, we found a significant and larger effect with the 56-landmark configurations that was not detectable with the 10-landmark configurations. If statistical assessments of effects are not constrained by variable number, such as with np-MANOVA, an increase in effect size should be tantamount to an increase in statistical power (although simulation studies are needed to confirm this).

The merits of different resampling methods have been debated, but not in the context of trait dimensionality, especially for characterizing similar multidimensional traits, as we have done here. Anderson and

Ter Braak (2003) provide both a nice summary of different resampling methods and a demonstration that randomization of residuals from reduced models (Freedman and Lane, 1983) has greater statistical power than alternative methods. Their simulations applied to specific nested effects. To date, analyses of type I error rates and statistical power have not been considered for RRPP applied to multifactor or factorial models of multidimensional traits, and examples presented here are the first to specifically target a comparison of different trait dimensionalities for the same general phenotypic trait (body shape). In addition, the present study introduces a method for considering not only a statistical test of interaction terms but also all possible effects in a factorial model by replicating random placement of residuals for multiple reduced–full model comparisons. This development should maintain the same type I error rate across model effects and *post hoc* pairwise comparisons. Further statistical research on type I error rates and statistical power is needed, but the np-MANOVA with RRPP paradigm should generalize the goals of MANOVA to any linear model design, including linear models with mixed effects and generalized least squares estimation of model coefficients (see Supplementary Information).

RRPP using concatenated residual matrices, to test multiple model effects, is a development that solves a substantial problem with current implementations of np-MANOVA procedures. In essence, nonparametric methods should be no different as a paradigm than parametric methods. Whether sequential or marginal sums of squares and cross-products are used in parametric approaches, multivariate test statistics are derived from the matrix,  $\hat{\mathbf{S}}_f^{-1}(\hat{\mathbf{S}}_r - \hat{\mathbf{S}}_f)$ , that expresses the effect of parameters that differ between full and reduced models, relative to the error produced by the full model (Rencher and Christensen, 2012). Because  $\hat{\mathbf{E}}_r$  is held constant during RRPP, the trace of  $\hat{\mathbf{S}}_f^{-1}(\hat{\mathbf{S}}_r - \hat{\mathbf{S}}_f)$  is merely a transformation of the trace of  $(\hat{\mathbf{S}}_r - \hat{\mathbf{S}}_f)$ , meaning SS as described above is a statistic commensurate with evaluating  $\hat{\mathbf{S}}_f^{-1}(\hat{\mathbf{S}}_r - \hat{\mathbf{S}}_f)$ . Except for the special case that  $\hat{\mathbf{E}}_r$  is the matrix of residuals from the null model ( $\mathbf{X}_r$  contains only an intercept), randomizing ‘raw’ phenotypic values (full randomization) does not provide the appropriate null model for calculating test statistics (Anderson and Ter Braak, 2003). In other words, randomizing raw values produces random versions of both  $\hat{\mathbf{S}}_r$  and  $\hat{\mathbf{S}}_f$  not

accounting for the established coefficients in  $\hat{\mathbf{B}}_r$ . Thus, randomizing raw phenotypic values does not preserve reduced model effects and is, therefore, not commensurate with the paradigm used by parametric MANOVA methods. As shown in the Supplementary Information, this can have devastating consequences for inferences made. Unaware acceptance of default probability distribution generation by np-MANOVA software is a likely reason for analytical malfeasance. At the time of this analysis, for example, the default setting for the *adonis* function in the *vegan* package (version 2.0.10) for R is a full randomization of raw data, advocated as having better ‘small sample characteristics’ (Oksanen *et al.*, 2013). However, stratified resampling is possible in this program, which means randomizing vectors of values within strata. For example, male and female phenotypes can be randomized within populations. Stratified resampling is an obvious solution to multifactor models without interactions. Performing np-MANOVA with RRPP on sequential models extends the concept of stratified resampling to factor or factor–covariate interactions, and alleviates the concern of inflated type I error rates because of improper sampling distributions based on suboptimal null models (Anderson and Legendre, 1999; Anderson, 2001b).

The important work of Anderson (2001a) introduced a method of np-MANOVA based on distance-based metrics and pseudometrics to accommodate multivariate data in which Euclidean distances among observations might not be appropriate (that is, when response data are not necessarily continuous). A link between distance-based approaches and MANOVA was established using linear models applied to scores of principal coordinates analysis (Gower, 1966) based on appropriate principal coordinates (McArdle and Anderson, 2001). Therefore, np-MANOVA using RRPP is possible with non-Euclidean distance-based characterization of disparity among observations, by using either principal coordinates analysis or nonmetric multidimensional scaling as a method of data transformation. In addition, np-MANOVA with RRPP should be adaptable to linear models with mixed effects and generalized least squares coefficient estimation (see Supplementary Information). Provided one can assign logical reduced and full models, RRPP produces ‘correct exchangeable units’ under a null hypothesis (Anderson and Ter Braak, 2003). The examples in this article illustrate a paradigm for evaluating all model effects, but the methodology could be applied to specific effects only or suites of effects. Understanding the paradigm enables researchers to choose any nested models they wish to compare.

Although np-MANOVA with RRPP is a methodological approach that should be commensurate with pairwise non-Euclidean distances estimated from, for example, count data or presence/absence data (for example, via using principal coordinate scores as data; see McArdle and Anderson, 2001), we do not wish to advocate that this approach should supersede other methodological approaches that offer potentially better statistical properties. For example, Warton *et al.* (2012) demonstrated that multivariate analyses based on pairwise distances ignores important mean–variance associations for count data, leading to erroneous analytical results. In these cases, generalized linear models should be used. Methods for employing generalized linear models for high-dimensional data, especially ecological ‘abundance’ data, have been developed (Warton, 2011). Currently, the R package, *mvabund* (Wang *et al.*, 2012), offers options to use generalized linear models on high-dimensional data, plus choose from among several resampling methods, including bootstrap resampling of residuals, for hypothesis tests (based on methods described by Davison and Hinkley, 1997; chapters 6 and 7). Similarly, hypothesis tests using generalized linear models offer some similar challenges to those presented in this article, namely, selecting an appropriate resampling

algorithm for factor interactions (Warton, 2011). Although np-MANOVA with RRPP might seem intuitively adaptable to generalized linear models, two constraints limit its feasibility. First, several definitions of ‘generalized’ residuals are possible under generalized linear models (Pierce and Schafer, 1986; Davison and Hinkley, 1997). Second, the pseudovalues generated by RRPP might preclude parameter estimation in random permutations (for example, if they are not binary or integers). Research to explore the feasibility and statistical power of using generalized residuals from reduced models to generate sampling distributions of test statistics of full models—which produce appropriate pseudovalues—would be an interesting future direction. Nonetheless, np-MANOVA with RRPP and the ‘model-based’ approach to multivariate analysis of abundance data (Warton, 2011) are rather commensurate in their approaches to general linear models and generalized linear models, respectively, in that both (1) offer solutions for statistical analysis of high-dimensional data by (2) using resampling algorithms with residuals.

We also do not wish to inadvertently suggest that because np-MANOVA with RRPP is not constrained by an  $n \gg p$  expectation, that it is a salvo for estimation error because of small sample sizes, non-multivariate normality or heteroscedasticity. One should not confuse statistical issues with proper parameter estimation. The paradigm presented here targets the former issue and not the latter. Warton *et al.* (2012) demonstrated that hapless use of pairwise distance-based MANOVA (Anderson, 2001a, b) can lead to inferential errors if linear model assumptions (evaluation of normality and homoscedasticity) are ignored. Diagnostic analyses performed on the examples that we presented here (see Supplementary Information) suggest that the inferences should be made with caution.

The greater point we intended to make is that it is important to remember in quantifying and comparing phenotypic change among different groups that taking a simpler approach to accommodate statistical limitations could mean compromising the description of phenotype. Parametric degrees of freedom do not constrain natural selection, so why should describing the phenotypic response to natural selection be constrained? The evolutionary biologist who is willing to allow a high-dimensional definition of phenotype is capable of making additional discoveries. In the examples we used, we expected to find reduced sexual dimorphism in the marsh habitat, as predators would mediate body shape by causing similar ecological roles between males and females, namely, streamlined body shape associated with predator avoidance swimming behavior. Based on a simpler definition of body shape, we did not find this to be the case, although we did observe consistent sexual dimorphisms and differences between habitats. However, in our higher-dimensional definition of body shape, we found the counterintuitive result of greater sexual dimorphism in the marsh habitat, associated with females having different head shapes based on opercular curvature. This finding did not obscure inferences we could make about the relative lengths of caudal regions between habitats or the tendency for deeper-bodied shapes of males, but it reveals morphologically fascinating results we had not considered.

Having an analytical paradigm that is not constrained by variable number equips researchers studying phenotypic evolution with the capacity to simultaneously consider both subtle and general aspects of phenotypic change, and should have positive influence on the types of questions that can be asked in evolutionary biology research. We presented examples using morphometric data that define multi-dimensional traits. These examples have obvious appeal to researchers in the various fields of evolutionary biology concerned with phenotypic evolution. These examples should also highlight the use of



factorial models that are common in quantitative genetics research (that is, to address genotype by environment interactions). Extending np-MANOVA and RRPP to models with generalized least squares estimation of parameters (see Supplementary Information) permits analysis of high-dimensional phenotypic data using genetic covariance matrices, as is typical with ecological genetics and evolutionary genetics research. However, we also expect that the fields of comparative genomics, functional genomics and proteomics will also continue to benefit from development of analytical tools for comparative analyses for high-dimensional data. Recent methodological developments have improved the ability to extend the generalized linear model to high-dimensional data (Warton, 2011; Warton *et al.*, 2012), allowing for collective analysis of multiple noncontinuous variables (for example, discrete or categorical variables). The methods introduced here enable collective analysis of multiple continuous variables, plus allow multiple effects in factorial models or factor-covariate interactions to be evaluated with proper null models. These commensurate research directions will hopefully spur a synthesis for the analysis of high-dimensional data, irrespective of variable type. In this synthesis, the inclusion of the most biological information possible for an organism might be embraced rather than discouraged because of statistical limitations, for as we have shown, inferential ability can be positively associated with the amount of biological information used.

#### DATA ARCHIVING

Data available from the Dryad Digital Repository: doi:10.5061/dryad.1p80f.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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