

# A general hypothesis-testing framework for stable isotope ratios in ecological studies

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**Abstract.** We propose a framework for hypothesis-testing of stable isotope ratios in ecological studies. Statistical procedures are based on analysis of nested linear models and a residual permutation procedure (RPP) that is employed to evaluate probabilities associated with test statistics. We used simulated examples and a real data set to illustrate the utility and generality of the method. First, we developed a test for differences in centroid location and dispersion of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values within and among groups of isotopic data. Second, we evaluated magnitude and direction of change in centroid position (termed “path”) of a pair of isotopic samples separated in space/time relative to paths of other paired sample sets. Third, we compared attributes of path trajectories (size, direction, and shape) over sample sets containing more than two samples to provide a quantitative description of how patterns of isotopic ratios change in response to spatial and temporal gradients. Examples are limited to the bivariate case ( $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$  biplots), but the statistical method can readily be applied to univariate and multivariate cases.

**Key words:**  $\delta^{13}\text{C}$ ;  $\delta^{15}\text{N}$ ; food web; functional groups; niche; residual permutation; stable isotopes.

## INTRODUCTION

Stable isotope ratios are widely employed in ecological studies of nutrient flow through food webs and ecosystems (Fry 2006), niche breadth (Layman et al. 2007) and other applications (e.g., animal migration; Kelly and Finch 1998). Recently, summary statistics and analytical methods have been proposed to characterize dispersion of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) isotope ratios in bivariate space (Layman et al. 2007), and to evaluate directional changes in central tendency among samples taken at different times or locations (Schmidt et al. 2007). In this paper, we built on previous work to develop a framework for statistical comparison of isotopic ratios among groups of samples. Such samples can be composed of individuals within a population or species, feeding guilds, or within entire communities or ecosystems, and can include abiotic constituents (e.g., water and soils) from which isotopic ratios can be confidently determined.

In many ecological studies, stable isotope data are represented as bivariate plots with  $x$ -axis equal to  $\delta^{13}\text{C}$  and  $y$ -axis equal to  $\delta^{15}\text{N}$ . By convention, delta notation refers to the ratio of heavy to light isotope abundance in a sample relative to that of a standard, and is reported in parts per mil (‰; Sharp 2006). Isotopic composition

varies among biotic and abiotic components of an ecosystem in systematic ways (e.g., via metabolic isotopic fractionation) that reflect sources and transfer dynamics of energy and nutrients through a food web (Fry 2006). For example, carbon isotopes are conservative tracers of energy sources for consumers, and nitrogen isotopes are indicators of trophic positions of organisms in the food web. Although much less common in food web studies, isotope ratios of other elements (e.g., hydrogen, sulfur, and oxygen) can be employed to reveal insights into biogeochemical processes of interest, including microbial processes, and cycling of pollutants. All examples presented in this paper are based on  $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$  biplots, and are evaluated in the context of a food web study where the primary objective is to distinguish isotopic signatures from samples that represent different functional groups (e.g., trophic guilds).

We focused on three aspects of analysis of C–N isotope data. First, we evaluated whether centroid location and dispersion metrics differed between two groups of isotopic samples as a potential indicator of differences in (trophic) niche breadth, resource use, and as a means to evaluate ontogenetic changes in resource use or movement of organisms between isotopically distinct habitats (e.g., Layman et al. 2007, Zeug et al. 2009). Second, we evaluated the magnitude and direction of change in central tendencies between two sample pairs (Schmidt et al. 2007) obtained at different locations or times. Third, we employed a method typically used in geometric morphometric studies (Rohlf

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and Slice 1990, Rohlf and Marcus 1993) for statistical discrimination and interpretation of *trajectories* of change in isotopic ratios for species or communities sampled at more than two points across an ecological gradient (e.g., longitudinal surveys of streams) or through time (e.g., Vander Zanden et al. 2003). Statistical procedures were encoded and executed in the statistical package R (*available online*).<sup>4</sup> Example R script is provided in the Supplement.

#### GENERAL STATISTICAL METHODOLOGY

Layman et al. (2007) employed six metrics to characterize dispersion of individual values in bivariate isotopic space that they viewed as measures of trophic resource space occupied by individuals in a community. These authors noted that their metrics alone do not provide hypothesis tests and indicated a need for appropriate statistical methods. In this paper, we developed a hypothesis-testing framework based on linear models and a residual permutation procedure (RPP) to generate null distributions to test for differences between centroids of two samples or differences between samples in the dispersion measures described by Layman et al. (2007).

We conducted a test for difference in centroid location by computing Euclidean distance between centroids (bivariate means) of a pair of isotope samples. The two centroids occupied different locations if the Euclidean distance between them was significantly greater than zero. Similarly, to test for differences in dispersion metrics between sample pairs, we computed the metric of interest (e.g., mean distance of observations to the centroid) for each sample separately, and then evaluated the absolute value of the difference of metrics between samples as a test statistic. The difference between samples for a particular metric was considered significant if the test statistic was greater than zero. All test statistics were compared to null distributions generated by a residual permutation procedure (RPP) that works by shuffling residual vectors (Freedman and Lane 1983, Anderson and ter Braak 2003, Adams and Collyer 2007, Collyer and Adams 2007), where each observation ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  pair) is described as a residual vector from the overall centroid and also as a residual vector from each group centroid (see Appendix for additional detail). We compared observed test statistics to null distributions obtained from 9999 random permutations of residuals from reduced linear models via the RPP procedure. The rank percentile of observed difference between groups was used as an empirical  $P$  value for the test of the null hypothesis.  $P$  values from RPP were compared (where appropriate) to those obtained using the parametric Hotelling's  $T^2$  test statistic. Hotelling's  $T^2$  is a multivariate analogue of the univariate  $t$  test that is suited for comparison of population mean vectors.

In addition to testing differences in centroid location and dispersion metrics, we employed RPP to compare responses to spatial and/or temporal gradients that are estimated by changes in centroid locations in isotopic space. Differences in trends are obtained by comparing three attributes associated with the path followed from start point to end point in each trajectory: (1) differences in path length, (2) differences in general path direction, and (3) differences in path shapes (Schmidt et al. 2007, Adams and Collyer 2009). Path length distance is the cumulative Euclidean distances summed across consecutive centroids in the trajectory. The test statistic for the difference between two groups is the absolute difference in path lengths between them (Collyer and Adams 2007, Adams and Collyer 2009). Path orientation or direction is described by the first principal component (PC1) estimated from the correlation matrix for each set of samples separately. The test statistic for the difference in path directions is the angle ( $\theta$ ) between PC1 vectors. If a trend contains only two centroids per group (as in *Example 2* below) then the trajectory is a vector and the path length is the Euclidean distance between centroids. Likewise, PC1 is the vector between centroids, and so an angle between PC1s ( $\theta$ ) is the angle between vectors of centroid differences (Adams and Collyer 2009). The shape difference between vectors is always null, and so comparing two-point trends is a simplified version of comparing multi-point trends (as in *Example 3* below).

Path shape itself cannot be estimated as a single value, but path shape differences are inner-products of differences (i.e., distances) calculated between trajectories after generalized Procrustes analysis (GPA; Rohlf and Slice 1990), which centers, scales, and rotates trajectories through a generalized least-squares superimposition to remove variation in position, size, and orientation, respectively. The distance between these "aligned" trajectories, usually referred to as Procrustes distance in geometric morphometric studies (Bookstein 1991), is invariant to size, orientation, and location differences and thus characterizes cumulative differences in the relative length and directions between consecutive points in the spatial trend (i.e., shape differences). The estimation of trajectory attributes (size, orientation, and shape) is referred to as "trajectory analysis" (Adams and Collyer 2009), and trajectory attributes are test statistics with probabilities evaluated via RPP (with additional analytical details in Adams and Collyer 2009).

#### EXAMPLES

Three simulated examples and one empirical example are presented below to illustrate the statistical procedures for specific cases that would be of interest in ecological studies of stable isotopes. In the examples based on simulated data, unless otherwise noted, individual  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values are drawn at random from a bivariate normal distribution. Each sample, which represents observations from a functional group or guild, contains 30 randomly drawn individual

<sup>4</sup> (<http://www.r-project.org/>)

observations of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . Each guild may contain a set of samples obtained at more than one site or time. Locations of centroids in bivariate isotopic space were chosen for illustrative purposes.

We also tested our approach on a stable isotope data set obtained from members of the aquatic food web in Lake Tahoe (USA), first reported in Vander Zanden et al. (2003), and reanalyzed in Schmidt et al. (2007). The Lake Tahoe data set contains eight fish species (one species, tui chub, is polytypic) sampled over five time periods: i, 1872–1894; ii, 1904–1913; iii, 1927–1950; iv, 1959–1966; and v, 1993–2000. We assigned five taxa (Lahonton cutthroat trout, lake trout, redbreasted sunfish, kokanee, tui chub [pelagic morph]) to a “pelagic” guild because they obtain dietary carbon from (predominately) phytoplankton, and we assigned four taxa (speckled dace, mountain whitefish, Paiute sculpin, tui chub [benthic morph]) to a “benthic” guild because they obtain carbon (predominately) from epilithic algae.

*Example 1.*—Example 1 illustrates procedures for evaluating and testing differences of centroid location and dispersion metrics among three functional groups or guilds (consisting of  $n = 30$  individual observations) represented in bivariate isotopic space. For each guild, we evaluated two of six dispersion metrics in Layman et al. (2007), namely, the mean Euclidean distance of individual observations to the sample centroid (CD) and the mean of the Euclidean distances to each observation's nearest neighbor (NND). We also evaluated a new measure, eccentricity ( $E$ ) that describes departure from isotropic scatter in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  dimensions. Eccentricity is calculated as  $E = (1 - \lambda_2/\lambda_1)$ , where  $\lambda_1$  and  $\lambda_2$  are eigenvalues for first and second principal components (estimated from a correlation matrix). A value of  $E = 0$  describes perfectly circular (independent) scatter;  $E = 1$  describes a perfectly linear relationship where direction of the line is described by principal component loadings.

In Example 1, guilds 1 and 2 have the same centroid location but differ in the distribution of points about the centroid, namely, guild 1 points are randomly distributed with  $\text{SD } \delta^{13}\text{C} = \text{SD } \delta^{15}\text{N} = 0.8$ , and guild 2 points are drawn at random from a skewed (and rotated  $45^\circ$ ) bivariate distribution where  $\text{SD } \delta^{13}\text{C} = 1.5 \times \text{SD } \delta^{15}\text{N}$  (Fig. 1A). Guild 2 is thus expected to have higher eccentricity than guilds 1 and 3. Guild 3 points are drawn from an identical distribution to that of guild 1, but centroids occupy different locations in isotopic space (Sample 1  $\delta^{13}\text{C} = -24.0\%$ ,  $\delta^{15}\text{N} = 7.0\%$ ; Sample 3  $\delta^{13}\text{C} = -18.0\%$ ,  $\delta^{15}\text{N} = 7.0\%$ ; Fig. 1A).

We also compared centroid locations, CD, NND, and  $E$  between pelagic and benthic feeding guilds in the Lake Tahoe data set. Fishes were assigned to guilds as described above and untransformed  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data (where pelagic  $n = 209$ , benthic  $n = 171$ ) were pooled across sample periods within guilds (Fig. 1B). For both the simulated and real data sets, we tested two sets of null hypotheses: (1) that Euclidean distance between each pair of guild centroids did not differ from zero, and

(2) the absolute value of the difference of CD, NND, and  $E$ , did not differ from zero when compared pairwise across guilds (see Appendix for mathematical detail and development of test statistics).

*Example 2.*—Here, we tested for differences in path length and path direction between isotopic samples taken from three distinct trophic guilds sampled at two sites. In this example, guilds 1 and 2 have equal path directions (PC1 vector angles  $\approx 45^\circ$  relative to  $\delta^{13}\text{C}$  axis for each), but path length of guild 1 is roughly half the value of path length for guild 2. Path lengths of guilds 1 and 3 are equal but path direction differs where the angle of PC1 of guild 3 is  $\approx 0^\circ$  relative to the  $\delta^{13}\text{C}$  axis (Fig. 1C). Thus, the angle between PC1s for guilds 1 and 3 ( $\theta_{1,3}$ ) is roughly  $45^\circ$ . Likewise, we compared path length and direction between time periods iv (1959–1966; pelagic  $n = 57$ , benthic  $n = 40$ ) and v (1993–2000; pelagic  $n = 56$ , benthic  $n = 25$ ) that pre- and post-dated (respectively) invasion by an exotic pelagic consumer (a mysid shrimp) into Lake Tahoe (Fig. 1D). For both simulated and real data, we tested the null hypotheses that (1) the difference of path length (the Euclidean distance between centroids within a guild) between sites does not differ from zero and (2) the difference of path direction (measured as the angle  $\theta$  between PC1 for each guild) does not differ from zero.

*Example 3.*—Example 3 is an extension of Example 2, where two guilds were sampled at three distinct sites instead of two. As in Example 2, sites could reflect samples obtained from distinct locations along an ecological gradient, or those taken across three time steps at the same location. The first and second null hypotheses (and test procedures) are the same as that in Example 2 except that path lengths are computed as summed Euclidean distances between sites 1 and 2, and then sites 2 and 3. Also, estimation of the first principal component vector, PC1 (shown in dashed lines on Fig. 1E) contains all three samples in the series. There is an additional null hypothesis that path *shape* does not differ between the two sets of samples; the null is not rejected if the Procrustes distance between the two trajectories does not differ from zero (see Appendix for more detail). Together, path length, direction, and shape describe the *trajectory* of a sample set, and so any differences in these characteristics between sample sets would lead to rejection of the general null hypothesis that trajectories do not differ between guilds 1 and 2. In the simulated example, guild 1 exhibits an initial increase of  $4\%$  in  $\delta^{15}\text{N}$  from site 1 to site 2, followed by no increase in the  $\delta^{15}\text{N}$  dimension at the third site (Fig. 1E). For guild 2,  $\delta^{15}\text{N}$  decreases  $2\%$  between sites 1 and 2, but does not change between sites 2 and 3. Delta values for carbon isotopes increase (become more enriched) for both sample sets, but path lengths differ between guilds 1 and 2. We also determined and then compared trajectories across feeding guilds in the Lake Tahoe data set across the five time periods listed above (Fig. 1F).

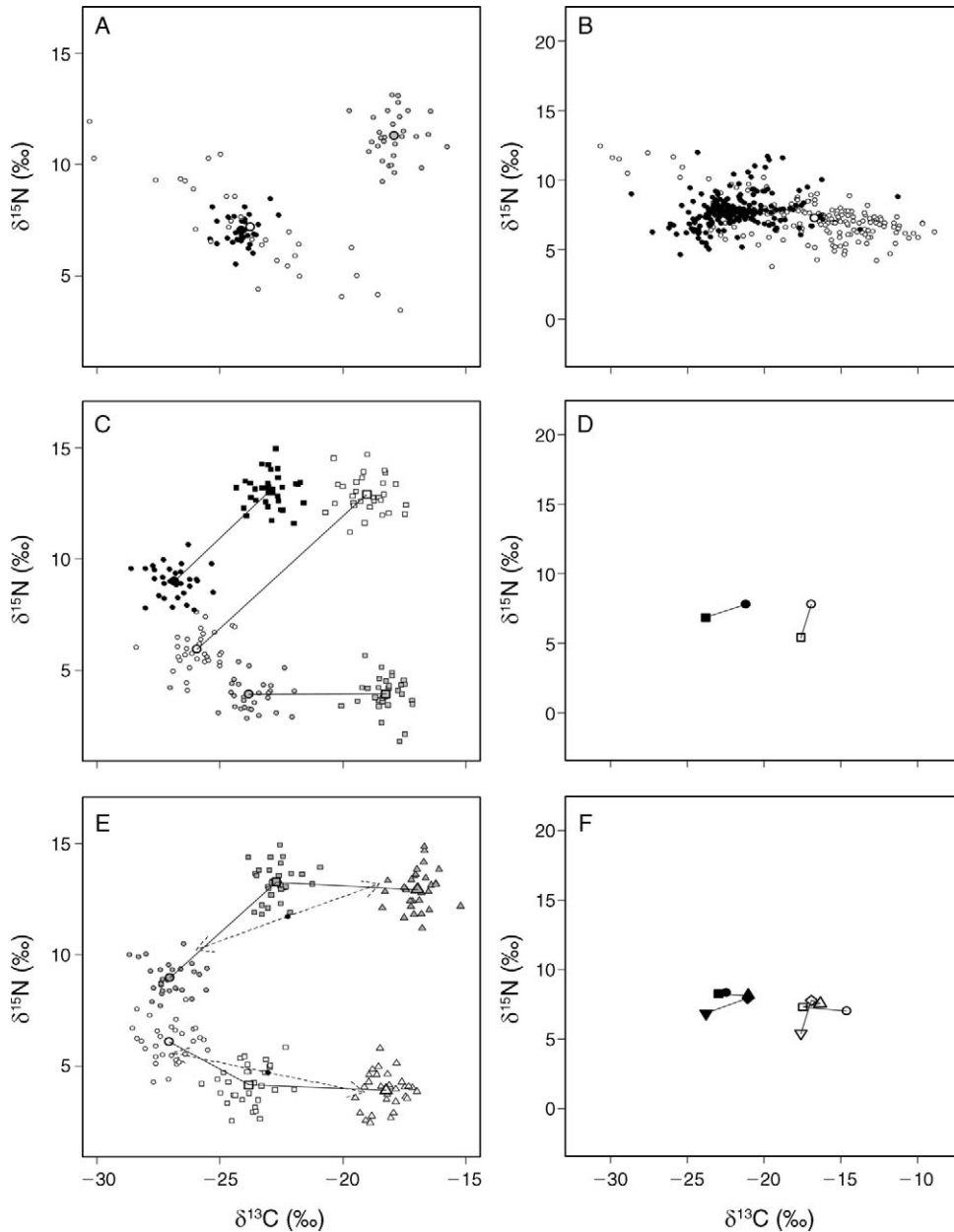


FIG. 1. Biplots of delta values where  $x$ -axis is  $\delta^{13}\text{C}$  and the  $y$ -axis is  $\delta^{15}\text{N}$ . (A) Three simulated samples of 30 individuals each identified in example where guild 1 is in black symbols, guild 2 is open, and guild 3 is gray. (B) Isotope ratios of fishes from Vander Zanden et al. (2003) pooled across five sample dates where the "pelagic" guild is black and the "benthic" guild is open (same designation as in panels D and F). (C) Three simulated guilds sampled at two sites as discussed in *Example 2*: guild 1 (black), guild 2 (open), and guild 3 (gray), with samples at site 1 (circles) and site 2 (squares). (D) Centroids and PC1 vectors across time steps that pre- (circles) and post- (squares) introduction of mysid shrimp into Lake Tahoe. (E) Two simulated guilds with samples obtained from three sites each as described in *Example 3*: guild 1 (open symbols), guild 2 (gray); with samples at site 1 (circles), site 2 (squares), and site 3 (triangles) for both guilds. Dashed lines represent PC1 vectors for each guild. (F) Path trajectories across five time steps in the Lake Tahoe data set, where centroids represent time periods i (circles), ii (squares), iii (triangles), iv (diamonds), and v (inverted triangles). Larger symbols in the center of data points in panels A, B, C, and E represent mean values (centroids) for each isotopic sample. Individual data points are removed from panels D and F to facilitate visualization of trajectories.

#### RESULTS AND DISCUSSION

Layman et al. (2007) noted conceptual and empirical similarities between analyses of stable isotope ratios in bivariate space and analyses of measurements in

geometric morphometrics and developed their metrics accordingly. In this paper, we also used analytical approaches from the geometric morphometrics literature as a template to develop a series of non-parametric

statistical tests for metrics of interest to ecologists who wish to use stable isotopes to distinguish sets of samples in time or space in two or more dimensions.

There has been some criticism of morphometrics-based approaches as applied to isotope data, especially with regard to summary metrics within samples and comparing them between samples (Hoeinghaus and Zeug 2008, Layman and Post 2008). Criticisms are based on two points: first that the magnitude of absolute differences between sources of energy and shifting baselines can confound summary metrics of higher trophic levels especially when there are multiple sources; and second that  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  axes may not scale equally, which would be required for meaningful comparison between food webs (Hoeinghaus and Zeug 2008). It is likely that careful sampling and study design coupled with appropriate data transformations may permit some resolution to these problems (e.g., Newsome et al. 2007, Layman and Post 2008). Our analysis can be conducted on isotope ratios or transformed data (e.g., proportional contributions from multiple sources as described in Newsome et al. 2007) as long as transformations are consistently applied across data sets and retain properties of the original data that permit analysis of Euclidean distances in uni-, bi-, or multivariate space.

Simulated Example 1 (Fig. 1A) demonstrates a hypothesis-testing procedure for differences in centroid location and dispersion metrics compared across three functional groups or guilds. Testing with RPP and Hotelling's  $T^2$  revealed that Euclidean distance between centroids differed significantly from zero for contrasts of guilds 1 and 3 (distance = 7.53,  $P = 0.0001$ ; Hotelling's  $T^2 = 1006.0$ ,  $P < 0.0001$ ) and guilds 2 and 3 (distance = 7.14;  $P = 0.0001$ ; Hotelling's  $T^2 = 915.5$ ,  $P < 0.0001$ ), but not guilds 1 and 2 (distance = 0.39,  $P = 0.585$ ; Hotelling's  $T^2 = 2.17$ ,  $P = 0.351$ ). Moreover, the absolute value of the difference of mean distance to centroid (CD) differed significantly from zero for guilds 1 and 2 ( $|\text{CD}_1 - \text{CD}_2| = 2.26$ ;  $P = 0.0001$ ) and guilds 2 and 3 ( $|\text{CD}_2 - \text{CD}_3| = 1.86$ ;  $P = 0.0001$ ), but did not differ from zero for guilds 1 and 3 ( $|\text{CD}_1 - \text{CD}_3| = 0.40$ ;  $P = 0.3376$ ). Likewise, the difference of nearest-neighbor distances (NND) differed significantly from zero for guilds 1 and 2 ( $|\text{NND}_1 - \text{NND}_2| = 0.41$ ;  $P = 0.0027$ ), was marginally significant for the contrast of guilds 2 and 3 ( $|\text{NND}_2 - \text{NND}_3| = 0.28$ ;  $P = 0.0525$ ), but as expected, was not significantly greater than zero between guilds 1 and 3 ( $|\text{NND}_1 - \text{NND}_3| = 0.13$ ;  $P = 0.3831$ ). Finally, differences of eccentricity values when calculated from guilds 1 and 2 ( $|E_1 - E_2| = 0.65$ ;  $P = 0.0012$ ) and guilds 2 and 3 ( $|E_2 - E_3| = 0.55$ ;  $P = 0.004$ ) were significantly different from zero, but the difference of  $E$  for guilds 1 and 3 was not different than zero ( $|E_1 - E_3| = 0.11$ ;  $P = 0.4169$ ).

For the Lake Tahoe data set (depicted in Fig. 1B), centroids of pelagic ( $\delta^{13}\text{C} = -21.95\text{‰}$ ,  $\delta^{15}\text{N} = 7.75\text{‰}$ ) and benthic ( $\delta^{13}\text{C} = -16.73\text{‰}$ ,  $\delta^{15}\text{N} = 7.24\text{‰}$ ) guilds occupied different locations in bivariate space (distance = 5.25,  $P = 0.0001$ ; Hotelling's  $T^2 = 245.6$ ,  $P < 0.0001$ ; Fig. 1B). Delta values for benthic consumers ( $\text{CD}_{\text{benthic}}$

= 3.50) were more widely dispersed than pelagic consumers ( $\text{CD}_{\text{pelagic}} = 2.03$ ) in bivariate space as indicated by a significant difference in CD ( $|\text{CD}_{\text{pelagic}} - \text{CD}_{\text{benthic}}| = 1.47$ ,  $P = 0.0001$ ). Likewise, eccentricity was significantly higher for benthic consumers (benthic  $E = 0.93$ ) than pelagic consumers (pelagic  $E = 0.77$ ), where  $|\text{pelagic } E - \text{benthic } E| = 0.16$ ,  $P = 0.0001$ . Mean nearest-neighbor distance did not differ between feeding guilds ( $|\text{pelagic NND} - \text{benthic NND}| = 0.065$ ;  $P = 0.1780$ ; Fig. 1B).

The first two measures of dispersion (CD, NND) were proposed by Layman et al. (2007). The third measure, eccentricity ( $E$ ), is new and provides insight into differences of the underlying distribution of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data. Like more widely used correlation or regression coefficients, a statistically significant value  $E$  also reveals covariation between  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (as in Fig. 1A, B), but differs from a correlation or regression coefficient in that it can also identify cases where one axis has significantly higher standard deviation than the other, even when there is no obvious covariation between C and N isotope ratios. This latter property of  $E$  may help identify cases where data transformations should be applied to avoid misinterpretation of dispersion data (e.g., Newsome et al. 2007, Hoeinghaus and Zeug 2008). Interestingly, both pelagic and benthic feeding guilds exhibited values of  $E > 0.5$  in the Lake Tahoe data set; this result is consistent with shifting N baseline for fish species that feed across isotopically distinctive carbon sources (e.g., isotopic generalists; Vander Zanden et al. 2003, Martínez del Rio et al. 2009).

In simulated Example 2 (Fig. 1C), we compared magnitude (absolute difference in path length) and path direction ( $\theta$ ) of change between centroids for two samples taken at different locations or times, and then tested for differences in these metrics among three guilds (again in pairwise fashion). The (absolute value of) difference of path lengths (difference = 6.12;  $P = 0.0001$ ), was significantly different from zero when compared between guilds 1 and 2, but the directions of PC vectors from each sample set were not different ( $\theta = 0.83^\circ$ ;  $P = 0.830$ ). Conversely, the difference of path lengths was not greater than zero when compared between guilds 1 and 3 (path length difference = 0.07;  $P = 0.572$ ), but  $\theta$  was ( $\theta = 45.13^\circ$ ;  $P = 0.0001$ ). Finally, the difference of path lengths (4.22;  $P = 0.0001$ ) and path directions ( $\theta = 45.97^\circ$ ;  $P = 0.0001$ ) were significantly greater than zero for contrasts of guilds 2 and 3 (Fig. 1C). In Lake Tahoe fishes, path lengths of trajectories between time periods iv and v (Fig. 1D) did not differ between feeding guilds (difference = 0.0049,  $P = 0.9936$ ) and path lengths for both were significantly greater than zero (pelagic  $P = 0.0001$ ; benthic  $P = 0.0085$ ) based on the test for differences in centroid location in Example 1. Paths differed significantly in direction ( $\theta = 54.03^\circ$ ,  $P = 0.0005$ ). Both guilds were characterized by depletion in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, but benthic fishes were not as depleted in  $\delta^{13}\text{C}$  values as pelagic fishes (Fig. 1D).

The approach outlined in Example 2 and our analytical results from the Lake Tahoe data set are consistent with those of Schmidt et al. (2007); both approaches detected a significant response of isotopic signatures in fishes after introduction of an invasive pelagic consumer. However, there are two differences in our respective approaches. While Schmidt et al. (2007) use species mean vectors as replicates, our method incorporates variance within and among functional groups (in this case trophic guilds) and allows for hypothesis-testing of differences in magnitude of change (i.e., path length) among sample sets based on nested linear models. It also allows for explicit comparisons of differences in path directions between functional groups.

Like Example 2, Example 3 illustrates a method to elucidate and compare trajectories of isotope ratio response across ecological or temporal gradients, but is a case where more than two isotopic samples are obtained per sample set. In this example, each trophic guild contains samples from three distinct sites with centroids calculated for each (Fig. 1C). The absolute difference of the sum of path lengths (across paths connecting centroids in each sample set) between guilds 1 and 2 differed significantly from zero (difference = 2.51;  $P = 0.0001$ ). Path directions (described by PC1 for each guild, dashed line in Fig. 1C) of guilds also differed significantly because the angle between them was significantly greater than 0 ( $\theta = 34.77^\circ$ ,  $P = 0.0001$ ). Finally, path shapes were different as expected, as indicated by the Procrustes distance between them being greater than zero (shape difference = 0.632;  $P = 0.0001$ ). For Lake Tahoe, we detected no differences in overall path length (difference = 1.544,  $P = 0.488$ ), trajectory direction ( $\theta = 5.413$ ,  $P = 0.787$ ), or trajectory shape (0.540,  $P = 0.3308$ ) between guilds, suggesting that both guilds responded similarly to changes in food web dynamics of Lake Tahoe across five distinct time steps. This result is consistent with the findings Schmidt et al. (2007), who used circular statistics in sequence across time steps to demonstrate no significant directionality to isotopic values prior to introduction of invasive mysid shrimp.

To our knowledge, comparisons of trajectory shape have never been employed in stable isotope studies and so may be the least familiar or intuitive approach presented here. However, testing trajectory shape differences among functional groups within a food web may be valuable for elucidating processes that control nutrient flow. For example, where isotopic values of basal energy sources shift in space or time, we would expect no difference in trajectories of consumers if baseline shifts are transferred through the food web similarly at all sites/times. Alternatively, if a predator exerts top-down pressure on a primary consumer, and thereby alters its energy source, then the consumer's trajectory may change independently of baseline shifts but rather in response to predator abundance or predation intensity.

Perhaps the most important advantage of trajectory shape analysis is that it permits evaluation of nonlinear responses to ecological or temporal gradients compared across food webs or among constituents within a food web. Such nonlinear responses might include isotopic responses to point source nutrient enrichment, distinct tipping points, and/or alternatively stable states across a spatial or temporal gradient. Isotopic values may also oscillate between two endpoints in space or time, which would result in a weak linear response, but potentially strong shape and path length differences from other functional groups of food webs of interest. Measures like centroid distance or convex hull area (as in Layman et al. 2007) might underestimate the response of a guild that shifts between two endpoints because there would be little spread around the overall centroid.

In their paper, Layman et al. (2007) focused primarily on distinguishing differences between entire food webs, but in our examples we extended their approach to distinguish differences of components of food webs (e.g., trophic guilds). However, the statistical methods employed in our paper could be applied to any set of isotopic samples where the investigator is interested in distinguishing differences in dispersion, location, magnitude, direction, or shape of responses across spatial or temporal gradients. We should note that our methods are not explicitly designed to evaluate energy source contributions to consumers as is done in a mixing model framework. There has been considerable development of quantitative and statistical resampling approaches to mixing models (Phillips and Gregg 2003, Phillips et al. 2005, Saito et al. 2007) for distinguishing proportional contributions of various sources to individuals or sample sets. Some workers have advocated the calculation of proportional contributions of sources prior to hypothesis-testing to avoid problems of multiple isotopically distinct sources and differences in scales of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (Newsome et al. 2007).

Like any statistical method, issues of statistical power to distinguish isotopic samples needs to be resolved in more real-life case studies. Nested linear models with residual permutation procedures are statistically consistent (Adams and Collyer 2007) and thus power and performance are dependent on sample sizes and the magnitude of difference between samples. What will be more important is to understand the limits of detection of significant differences between sets of samples, and whether those differences are biologically meaningful. Our approach will be most useful when combined with plots of actual (or appropriately transformed) isotopic data to aid in interpretation, as demonstrated by the Lake Tahoe data set (Fig. 1B, D, F).

The residual permutation procedure (RPP) we employed is similar to the multi-response permutation procedure (MRPP; Mielke and Berry 2007) recently used in stable isotope studies (see Zeug et al. 2009). MRPP permutes original observations (and thus discards significant fixed effects in the model) whereas RPP

permutes residuals from nested linear models (that are reduced to exclude interaction terms) while holding significant fixed effects constant. A key advantage of the RPP approach over MRPP is that it accounts for variation due to covariates and other nontargeted sources of variation with no procedural alteration (see Adams and Collyer 2007). *P* values from RPP were similar to those obtained from Hotelling's  $T^2$  in our examples, but implementation of RPP requires fewer underlying assumptions (e.g., does not require multivariate normality) and permits testing additional hypotheses, such as null hypotheses for trajectory attributes.

**Conclusions.**—Undoubtedly, analytical methods for stable isotope data will continue to advance at a rapid pace, as such data are used to evaluate long-term community responses to environmental change and other important applications in ecology and environmental biology. We have presented a hypothesis-testing framework that builds on previous work; the dispersion metrics of Layman et al. (2007) and the circular statistics approach of Schmidt et al. (2007), and a statistical framework used in ecological morphology studies (Adams and Collyer 2007, Collyer and Adams 2007). Analysis of path trajectories for two or more isotopic samples could be a very valuable procedure to study how food webs or ecosystem features respond to linear and non-linear spatial and temporal ecological gradients. Perhaps most importantly, our method provides a means to evaluate long-term trends in food web or ecosystem features in response to global climate change.

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#### APPENDIX

Statistical details for article examples in a general hypothesis-testing framework for stable isotope ratios in ecological studies (*Ecological Archives* E091-157-A1).

#### SUPPLEMENT

R code for conducting statistical tests of stable isotope data (*Ecological Archives* E091-157-S1).