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Univariate and Multivariate Approaches to the Analysis of Ecotoxicological Data

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ABSTRACT

The possible effects of agricultural chemicals on the reproductive biology of non-target species are a major concern of environmental groups, government agencies, and the general public. Particular concern focuses on the effects of agricultural chemicals on avian species. Current regulatory guidelines recommend that a number of variables related to reproductive success be measured in avian field studies. These include both original and derived variables. The traditional statistical testing procedure (univariate analysis of variance on all variables) is not an appropriate design when comparing many closely related and intercorrelated variables. By definition α (= 0.05) of the variables, on average, will be significant due to chance alone when the variables are independent and no treatment effect is real. When characters are correlated, one cannot assess the overall error rate, reducing statistical decision-making to a state of futility. Multivariate procedures are more appropriate in these cases, because the algorithms incorporate intercorrelation into the analysis. We discuss the utility of three kinds of multivariate procedures (multivariate analysis of variance, discriminant function analysis, and principal component analysis) for the evaluation of treatment effects on avian reproductive success. We present guidelines for the use and interpretation of these procedures, using examples based upon the analysis of morphological data, and evaluate their advantages and disadvantages compared to traditional univariate procedures.

KEY WORDS

data analysis, multivariate statistics, ecotoxicology, discriminant function analysis, principal component analysis

INTRODUCTION

The study of natural systems is based upon a long history of careful observation and induction. Modern environmental science has placed a strong emphasis on the hypothetico-deductive approach, a trend noted by several contemporary philosophers of science.[1,2] Hypothesis testing in ecotoxicology demands rigor in the collection of data, and these data generally are quantitative. Accuracy and precision are important because variability at a number of levels is inherent to these systems. This results in a

concomitant dependence on the application of statistical methodology to resolve quantitative hypotheses in ecotoxicological research.

A broad spectrum of research questions characterizes the emerging field of ecotoxicology. A convenient classification of this spectrum considers those questions that are variable specific in nature to be distinct from those that examine a suite of variables that together estimate a composite characteristic which may have biological significance. Examples of variable-specific questions include: (1) the effect of different exposures, species, age groups, or sexes on the uptake of a particular chemical compound; (2) the effect of different chemical vehicles (such as aerosols vs granules) on the uptake of a particular toxicant; (3) the detection of a toxicant (or a degradation product) in the tissues of potential target species; and (4) the effect of various doses of a chemical compound on the mortality of organisms. In each of these examples, the focus is on a single dependent variable or on a limited set of dependent variables in their own right. In contrast, composite characteristics estimated by a suite of variables include: (1) the behavior of organisms, in which behavior is described by several variables such as flying, resting, foraging, and aggression; (2) physiological or biochemical status as measured by heart rate, blood pressure, and brain or serum acetylcholinesterase activity; and (3) reproduction as estimated by clutch size, and hatching or fledgling success. Within each of these suites, variables generally are related to each other in varying degrees; this relationship is expressed quantitatively as a correlation. Multivariate procedures are especially efficacious when dealing with intercorrelated variables. When research protocols involve the measurement of several variables of interest taken on the same experimental unit, investigators are confronted with a choice between univariate and multivariate statistical approaches.

Multivariate statistical procedures are not new, and procedures such as multivariate analysis of variance (MANOVA) have been used since the 1930s.[3] They have been used by systematists for many years,[4,5] and have become increasingly common in phytosociology[6-10] as well. James and McCulloch[11] review the application and interpretation of multivariate procedures in ecology and systematics. Multivariate procedures are not yet commonly used in ecotoxicology even though they have much to offer. This chapter reviews several multivariate procedures that should prove useful to ecotoxicologists, and discuss their advantages and disadvantages in relation to univariate methods. We illustrate the utility of a multivariate analysis of biological data with examples from systematics and evolutionary biology. This review should assist researchers and regulatory agencies in the design of appropriate guidelines for future research protocols.

MULTIVARIATE STATISTICAL PROCEDURES FOR ECOTOXICOLOGY

There are several distinctions between univariate and multivariate statistical procedures. Univariate procedures evaluate hypotheses concerning a single dependent variable, whereas multivariate procedures test hypotheses based upon several dependent variables acting simultaneously. Multivariate analogues pertain to many univariate designs. The differences between these analyses are highlighted in numerous good discussions suitable for the statistically naive as well as for the more advanced user.[3,12-18] The authors illustrate the major differences in assumptions and inference by comparing univariate (ANOVA) and multivariate (MANOVA) analyses of variance.

Hypothesis Tests and Assumptions

ANOVA tests the null hypothesis that the means of a single dependent variable are equal in k groups. The test of significance is based upon the F statistic. MANOVA tests

Table 1. Assumptions of ANOVA and MANOVA[3]

ANOVA	MANOVA
Random sampling	Random sampling
Statistical independence of observations	Statistical independence of observations
Samples in each group drawn from a population that is normally distributed	Samples in each group drawn from a population that exhibits multivariate normality
Equality of within-group variances (homoscedasticity)	Equality of within-group covariance matrices

the null hypothesis that the mean weighted linear combinations of p variables in multidimensional space (group centroids) are equal in the k groups.[3] No single test criterion is universally accepted, but several are commonly used, such as Wilks's lambda criterion (Λ), Pillai's trace criterion (V), Roy's greatest characteristic root criterion (Θ), and the Lawley-Hotelling trace criterion (τ).[15] Fortunately, results from these statistics are frequently similar,[19] particularly if departures from the null hypotheses are slight.[20] MANOVA is generally robust with respect to moderate violation of its basic assumptions, but each test criterion performs somewhat differently in relation to the major assumptions,[3] and each criterion is differentially sensitive to the distribution of discriminating variance of the variables (trace).[15] The major assumptions of univariate and multivariate procedures are analogous (Table 1). The major differences are MANOVA requirements for multivariate normality (univariate normality for all variables does not guarantee multivariate normality) and for equality of variance-covariance matrices (again, not guaranteed by the homogeneity of all variances in the separate ANOVAs). The equality of variance-covariance matrices actually requires two conditions: within-group variances must be statistically equal for all variables, and the correlation for any two variables must be statistically equal in all groups involved in the analysis.[3]

When to Choose MANOVA in Ecotoxicology

The decision to choose MANOVA over ANOVA will depend, to a large degree, on the nature of the hypothesis and selection of variables. For example, if the major interest in a study is to evaluate the levels of six different chemical products in the bodies of collected birds from several exposure and control areas, then it may be appropriate to analyze the levels of each of the chemicals with separate ANOVAs. Remediation may require knowledge of which chemicals are actually in higher concentration in tissues from exposed vs control sites. Even though all six measurements were taken on the same experimental unit, and may well be intercorrelated, the real interest will likely be in the levels of each of the six chemicals across the sites, and not in the overall differences of some new composite variable. However, even in this case caution must be taken to avoid an excessively high type I error because the same null hypothesis (no difference among sites) will be tested for each variable. In this case, some correction of α (probability of incorrectly rejecting a true null hypothesis) will be necessary, either using the Bonferroni method or the Dunn-Šidák method.[21] An exception to this would be a case in which, because of potentially grave effects on human health or wildlife, it is critically important not to underestimate any potentially significant effects. In such a case, conservative tests might be inappropriate. Moreover, under some circumstances, a multivariate approach may be more powerful than a series of univari-

ate tests (i.e., a MANOVA can be significant when all of the separate ANOVAs were nonsignificant) and may represent the most efficacious approach for identifying areas of concern.

If ANOVA seems to be the method of choice, then a series of steps should be followed in subsequent analyses (Figure 1). These include tests of the critical assumptions, such as randomness of sampling, normality, and homoscedasticity. Based upon the results of these tests, a decision must be made to use parametric procedures or an equivalent nonparametric analysis.

Both parametric and nonparametric methodologies have provisions for the analysis of *a priori* comparisons, a more powerful option for tests of significance that is rarely employed. It is particularly applicable in designs in ecotoxicology, where the groups consist of a control and several levels of application of a pesticide, or a control and several modes of application of the same pesticide. Investigators can test a restricted set of orthogonal or nonorthogonal comparisons at a fixed experiment-wise error rate, usually set at $\alpha = 0.05$. When the design does not allow for *a priori* comparisons (rarely) or all pairwise comparisons are of interest, then one of a number of *a posteriori* or multiple comparison procedures can be used. Statistical power can be substantially reduced, however, especially when a large number of comparisons is involved. Concise discussions of these procedures are readily encountered in a variety of sources.[21,22] Collyer and Enns[23] provide a readable and useful guide to ANOVA, including *a priori* and *a posteriori* analyses as performed in commonly used statistical packages (BMDP, SAS, and SPSS-X).

If the objective of the study is to evaluate the effect of a chemical exposure on a number of variables measuring clutch, nesting, and fledgling success, then MANOVA

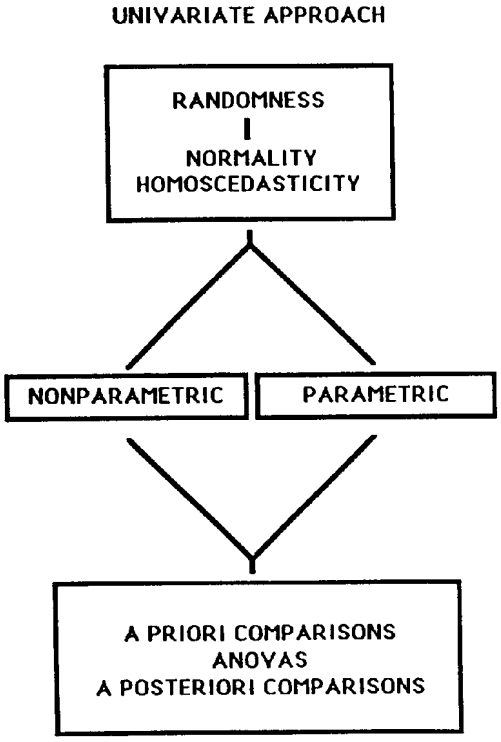

FIGURE 1. Pathway of procedures and decisions that should be followed when carrying out an ANOVA.

would be a better choice as these variables likely will be intercorrelated and no single variable is of concern by itself (Figure 2). (If all of the dependent variables are orthogonal, then the MANOVA approach simply yields the sum of the F ratios of the individual ANOVAs.[15]) MANOVA derives a number of composite variables that may have biological significance as a measure of overall reproductive success. A significant MANOVA among sites would mean the sites differed in overall reproductive success. The analysis can take several directions, depending upon results of initial test assumptions (Figure 3).

As discussed above, the critical assumptions for MANOVA are similar to those of ANOVA (Table 1). No commonly available tests are universally applied to assess multivariate normality; in any case, MANOVA is considered robust with respect to deviations from this assumption. Violations of the assumption of the homogeneity of within-group variance-covariance matrices can be more severe. All four of the test statistics discussed earlier are robust with respect to unequal matrices if sample sizes are equal and large, and the number of variables is small. The effect on the number of type I errors when these conditions do not apply and variance-covariance matrices are unequal is difficult to predict.[3] Because this assumption is frequently violated, precautions with regard to sample sizes should be incorporated into experimental design.

Whenever high communality (intercorrelation) characterizes a character suite, the pooled within-group variance-covariance matrix may be singular, and a MANOVA cannot be performed. (This occurs when one of the variables is a linear combination of the other variables.) Two solutions are reasonable. One or more variables suspected of being redundant can be deleted from the character suite and the reduced data set analyzed as before. Alternatively, the original character correlation matrix can be subjected to principal component analysis (PCA). Each experimental unit can be char-

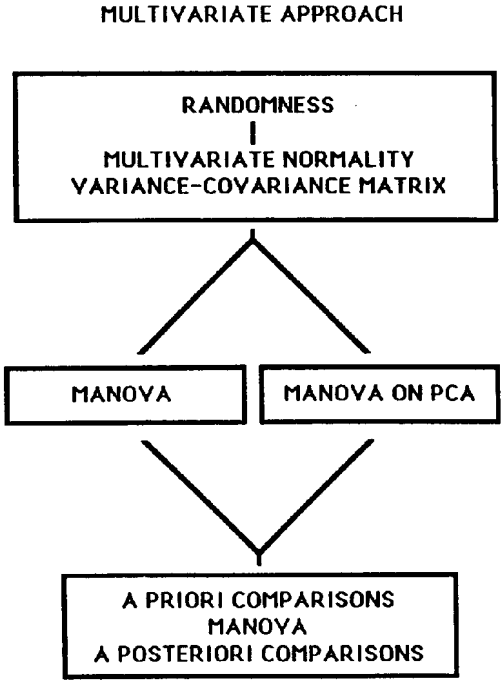

FIGURE 2. Pathway of procedures and decisions that should be followed when carrying out a MANOVA.

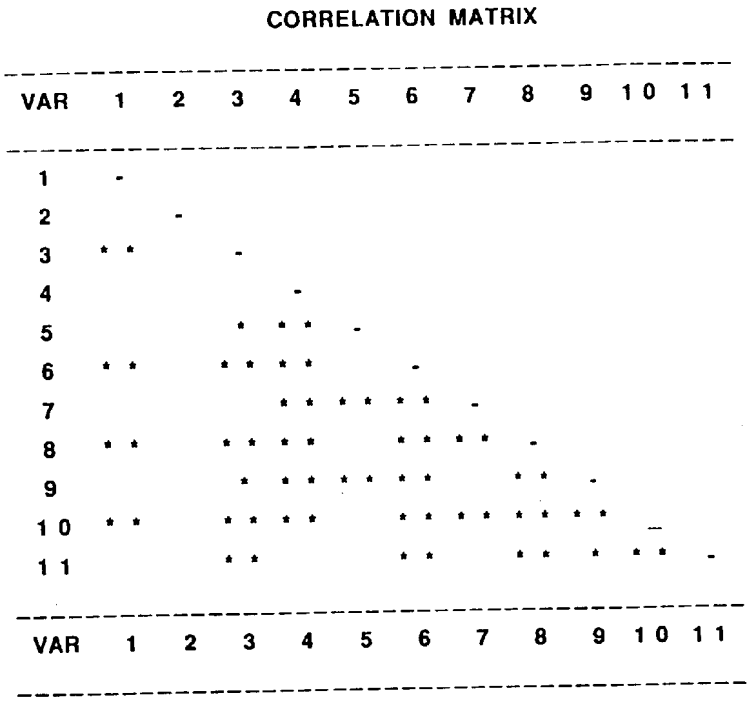

FIGURE 3. The character correlation matrix from a study on the effects of a pesticide application on starling reproduction. Characters are original and derived measures of various aspects of reproduction. Of 55 correlations, 31 were statistically significant.

acterized by a new set of uncorrelated variables (principal component axes) in which individual component scores represent each individual in a reduced number of dimensions but interindividual distances (= differences) are preserved. Principal component scores can then be subjected to MANOVA as suggested by Willig and Hollander;[24] an example of its application is presented in the section on case studies.

The same consideration should be given in MANOVA designs to the potential use of *a priori* and *a posteriori* comparisons with knowledge of their effects on experimentwise error rates.[25,26]

A researcher often is interested in evaluating the relative importance of the original dependent variables in contributing to group differences. Some authors have recommended performing univariate ANOVAs on all dependent variables if the MANOVA omnibus test is significant[12,15] under the concept of protection levels. Interpretation still can be misleading because of intercorrelation, and the table of pooled within-group correlation matrices should be presented along with such an analysis.[14] It is safer to restrict the evaluation of the relative importance of dependent variables to a more qualitative interpretation of the standardized discriminant function coefficients and the factor loadings. Standardized discriminant function coefficients are analogous to beta values in a multiple regression equation and present many of the same problems of interpretation. The loadings are correlations between the original set of dependent variables and the derived variables (discriminant function axes). Large correlations indicate that a given variable is important in accounting for group differences.[14]

MANOVA vs Discriminant Function Analysis

MANOVA and discriminant function analysis are two closely related multivariate procedures that are frequently confused and misapplied. The major distinction is that MANOVA is a multivariate test that assesses group differences based upon multiple dependent variables while holding the experiment-wise error rate constant. Examination of the derived, synthetic variables (also referred to as multiple criterion variables) can reveal complex biological effects not readily apparent from the examination of the individual dependent variables. The emphasis in discriminant analysis is to derive a synthetic variable, the discriminant function, that maximally distinguishes among groups already assumed or known to be different, and to use this criterion as a basis for predicting group membership. Two basic questions are involved: can we adequately discriminate among groups, and which linear combination of dependent variables is the best discriminator?

MANOVA will generally be the procedure of choice in ecotoxicology. Predicting group membership does not seem to be a priority in most ecotoxicological field studies, where the primary question is determining the effect of levels of a pesticide application on a suite of dependent variables. An exception may be a study in which data are available on a suite of reproductive or physiological variables from two aquatic sites: one pristine and one polluted by a chemical spill. Concern exists over the extent of the impact, and a sample of organisms is taken downstream from the spill. A discriminant analysis is conducted on the polluted and pristine sites as *a priori* groups, with the downstream cases assigned as unknowns; use of the predicted group memberships of the cases from the unknown site would assess the geographic scale of impact on these organisms resulting from the spill. If most unknown cases are assigned to the polluted group, it would indicate that the pollution had already moved downstream. Remediation would be suggested for sites with cases classified with the polluted group but not a priority for sites classified with the control.

CASE STUDIES

Adequacy of ANOVA vs MANOVA

Willig et al.[27] presented a critical analysis of univariate and multivariate approaches in the analysis of geographic variation in the morphology of bat populations. This research is analogous to an ecotoxicological study of variation in reproductive success in passerine populations across several levels of pesticide application. In morphometrics, proponents of a univariate approach conduct a set of analyses on each of a suite of variables. ANOVA is computationally simple and easy to understand, but it embodies several disadvantages. When several variables are compared, ANOVA is an inappropriate model of group difference. Significance is variable dependent, and group-specific contrasts (*a priori* or *a posteriori*) may not converge across individual variables. More importantly, morphological characters can be highly intercorrelated. The multivariate model for statistical analysis would seem most appropriate. However, some systematists attempt to avoid the problem by establishing a number of variables in advance that must be significant in order to consider the treatment groups distinct. This number is called the criterion variable. The overall error rate (OER) can be calculated if all variables are independent by the formula:

$$OER = 1 - \left[\prod_{i=1}^{c} (1 - \alpha_i) \right]$$

where α_i is the level of significance for character i in the ANOVA for a particular species and c equals the total number of variables in the analysis. However, when intercorrelation is present this formula is incorrect, and the decision becomes arbitrary.

MANOVA is a more appropriate model in this case because it incorporates the structure of the intercharacter correlation matrix, gives a single statement of group differences, and is frequently more powerful than a suite of ANOVAs. Disadvantages include the computational difficulty of MANOVA, the complicating factor of Rao's Paradox,[28] and the lack of association of particular characters with group differences. Also, singularity of the error matrix may prevent the analysis in some situations.

The question to resolve is whether univariate results emulate multivariate results. More specifically, are inferences drawn from a suite of univariate comparisons the same as those drawn from a single multivariate comparison? Willig and colleagues[27] tested the reliability of the arbitrary selection of univariate criterion values (percentage of significant ANOVAs) based upon a study of sexual and geographic variation in 12 cranial and 10 postcranial characters analyzed from both ANOVA and MANOVA perspectives. Comparison of results revealed little concordance between the results of the two analyses. In one case, none of the 10 postcranial characters were significant, yet the MANOVA was significant. At the other extreme, 11 of 12 cranial characters were significant based upon ANOVA, but nonsignificant using the multivariate approach. Essentially all possible combinations of significant and nonsignificant ANOVAs and MANOVAs were observed in their data. When a given percentage of significant ANOVAs was selected as a criterion value, the ANOVA approach yielded less than satisfactory results. Using the MANOVA outcome as the "correct" conclusion, no criterion value had a >85% success rate. The best success was obtained when the criterion was between 17 and 30% of the conducted comparisons. This occurred because very low criterion values led to exaggerated conclusions concerning group differences (a kind of type I error), whereas high criterion values increase the probability of "type II error." MANOVA is clearly the preferred statistical methodology for the morphological data examined by Willig et al.[27] In fact, any data set with a highly intercorrelated character set will best be analyzed by MANOVA. The authors expect that this will commonly be the case in many ecotoxicological studies as well.

The MANOVA/PCA Approach and the Question of Rao's Paradox

When ecotoxicological research involves the measurement of suites of characters, the MANOVA approach is preferred over the univariate approach. The MANOVA approach is not without difficulties. Hypotheses of group equality cannot be evaluated via MANOVA if the error matrix is singular, and high character intercorrelations can lead to the presence of Rao's Paradox.[28,29] Rao's Paradox is a situation in which some (or many) univariate analyses detect group differences, yet the MANOVA yields nonsignificance. The only way to completely avoid Rao's Paradox is to analyze characters that are orthogonal, an unlikely situation. A solution is to utilize principal component analysis, a data reduction procedure that constructs a set of orthogonal axes that may be considered independent characters for the ANOVA.[13,17,18] Willig and Hollander[24] analyzed the previously discussed data set on Brazilian bats to evaluate this approach. The character correlation matrix was subjected to a PCA, and a new set of variables were derived (the principal component axes). These orthogonal axes are linear combi-

nations of the original character set. The principal component scores of each case (an individual bat in this analysis) were then used as the dependent variables.

If the disparity between the ANOVA and MANOVA results was attributable to Rao's paradox, then the MANOVAs run on principal component scores should have consistently higher significance levels than the MANOVAs run on the original characters. The results strongly supported this hypothesis. In nine cases in which Rao's Paradox may be important, the MANOVA/PCA procedure resulted in lower probability levels than the original MANOVA. Clearly, character correlation can decrease the power of the MANOVA.

When analyzing ecotoxicological data via MANOVA, investigators must be aware of this effect. Most commonly used packages of statistical procedures (e.g., SAS, SPSS-X, BMDP) will give ANOVA results as part of the MANOVA output.[25,30,31] If many ANOVAs are significant, but the MANOVA is not, then the original character matrix should be subjected to a PCA. The MANOVA should then be conducted on the principal component scores in order to evaluate the potential effects of Rao's Paradox. Willig and Hollander[24] recommend adhering to the MANOVA/PCA results for drawing conclusions. Investigators must be careful to use PCA and not factor analysis for this approach. These two methods use different algorithms and are frequently included in the same chapter in manuals for statistical software packages.

A Method for Assessing Variables of Importance

An important attribute of MANOVA is the generation of a new composite variable that may better summarize the biological differences among groups. A disadvantage is that it is more difficult to assess the importance of the original variables in the discrimination. Willig and Hollander[32] present a model that assesses the importance of individual variables in the discrimination of groups and measures the degree to which a group of differences are consistent. The model was developed to deal with research questions in systematics, but applications to ecotoxicology are shown here.

The original research question posed by their study was to evaluate which variables were important in accounting for sexual dimorphism in bat populations and determine the degree to which this sexual dimorphism is constrained by phylogeny. Sexes of each species for a given locality were compared using a stepwise discriminant analysis.[25] The initial analysis finds the best linear combination of variables for separating the sexes. The loadings of the original variables on the discriminant function axis are frequently used to assess their importance, but some variables important in discrimination can receive low loadings if they have a high communality with a more "important" variable.

In Willig and Hollander's[32] method, the importance of the original variables is determined in a series of steps. First, the original variables are correlated with the discriminant function scores for each individual. Second, this correlation coefficient is then squared to provide a measure of the proportion of the variation in the discriminant function scores that are attributable to variation in the original variable. This gives the importance of the discrimination of the original variable. Third, the polarity of the axes are standardized to facilitate the comparisons, such that males have high loadings and females low loadings. Therefore, the sign of the correlation indicates the direction of the effect; a positive correlation coefficient would mean that males are larger than females for that variable. A clear analogy to ecotoxicology would be in the comparison of reproductive effects on control and treatment fields after the application of a pesticide.

A study of sexual dimorphism will involve the calculation of only one discriminant

axis. The method can be extended to scenarios which have more than two groups and more than one axis. Consider the formula:

$$I_i = \sum_{j=1}^{n-1} (\lambda_i)^2 (r_{i,j})^2$$

where I_i is the importance of variable i; n is the number of *a priori* groups; λ^2 is the proportion of variation accounted for by discriminant function i; and $r^2_{i,j}$ is the proportion of variation among scores for discriminant axis j accounted for by variation in variable i. The I values are scaled such that:

$$n - 1 > I_i > 0 \text{ or } 1 > I_i/(n - 1) > 0$$

Bar diagrams (Figure 4) provide a convenient way of viewing the I values. A comparison of the graphs for the same species from different areas in the bat study by Willig and Hollander provided a means of assessing character-specific phylogenetic con-

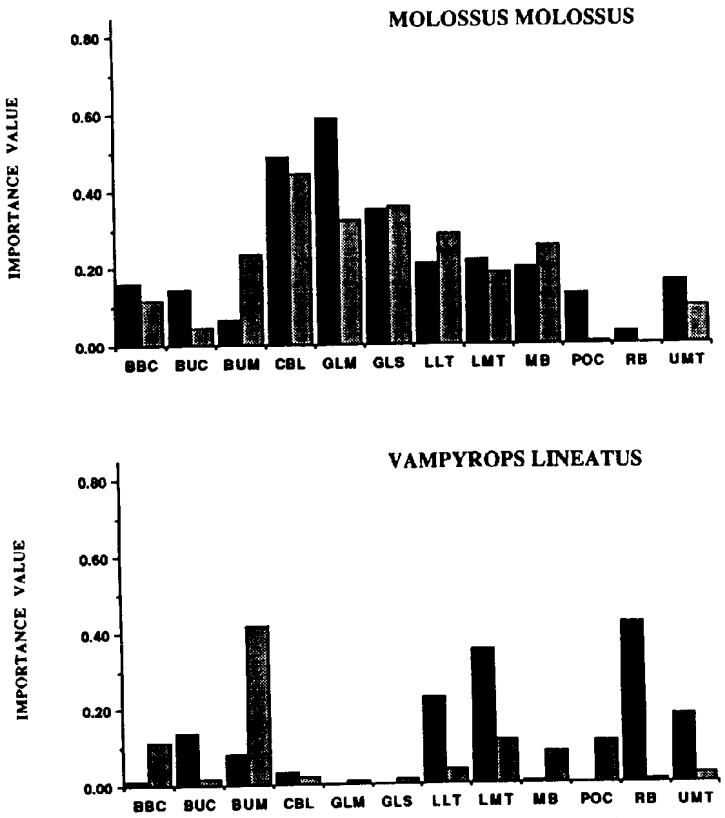

FIGURE 4. Bar diagram in which the importance value of a particular variable (x-axis) is represented by the height of the bars.[32] In ecotoxicology, the height of the bars may represent the effects of a pesticide on several variables related to reproductive success of a passerine. For *Molossus molossus*, significant concordance between two sites (solid, arid thorn scrub; shaded, savanna) in the expression of secondary sexual dimorphism is illustrated by the similar shapes of the distributions and a significant correlation between importance values. For *Vampyrops lineatus*, a lack of concordance is illustrated by dissimilar frequency distributions between sites and the absence of a significant correlation.

straints on the expression of secondary sexual dimorphism (see Figure 4 for discussion). This has a potentially important application in wildlife toxicology. For instance, bar graphs could be prepared from studies conducted with the same experimental design and on the same target species (for example, starlings), but at a number of different sites. Comparison of such graphs will reveal whether the same biological variables are affected by the chemical under a range of environmental conditions. This will determine whether the chemical has a specific physiological effect across a wide geographic area or affects the variables in a habitat-specific fashion. These results will assist in developing management plans for the timing and method of application in order to minimize deleterious impacts.

A MODEL RESEARCH PROTOCOL

Based upon the above scenarios, we envisage a model protocol to direct analyses in studies for which the MANOVA option is appropriate. MANOVA is performed on the original data, being careful to use the appropriate experimental design. A common error is to fail to use repeated measures designs when they are required. The design should also incorporate *a priori* comparisons if appropriate. In many cases, nonorthogonal contrasts should be seriously considered.[25] Rather than allowing the statistics to totally constrain the ecotoxicological questions of interest, the questions themselves should drive the experimental design.[26] For example, when a single control site exists along with several sites that differ in exposure to a pesticide, nonorthogonal contrasts of each exposed site to the control may constitute the most useful approach to hypothesis testing. If, on the other hand, a dose-response to different levels of a pesticide application is suspected, then polynomial contrasts are most appropriate. Finally, if sites are expected to respond to pesticide application as a step-cline or in a threshold-like fashion, then nonorthogonal comparison of adjacent sites may be the *a priori* design of choice. Fortunately, these designs are available in many statistical packages (e.g., SPSS-X).

As part of the MANOVA output, the battery of ANOVAs on the original characters should be requested. The MANOVA and ANOVA results should be compared in order to detect the possibility of Rao's Paradox. If evidence of the Paradox is found, then the original character correlation matrix should be subjected to PCA, and a MANOVA conducted on the principal component scores of the cases as the new variable set. If the MANOVA/PCA results confirm the suspicion of Rao's Paradox, then they should be used to draw inferences concerning group differences. As a final step, the original variables should be analyzed with stepwise discriminant function analysis and followed by the calculation of I values according to the method of Willig and Hollander.[32] Bar diagrams (Figure 4) will facilitate interpretation of which original variables are most important in distinguishing among groups.

SUMMARY

Although univariate analysis of variance is widely used in ecotoxicological research, many studies are more appropriately analyzed via multivariate techniques, such as multivariate analysis of variance, discriminant function analysis, and PCA. We illustrated the applications of multivariate approaches to ecotoxicology using a number of case studies.

All of the case studies presented deal with multivariate approaches to resolving questions in systematics. The authors chose examples from outside the realm of ecotox-

icology, largely to emphasize the importance of intellectual interchange across disciplines. A substantial body of knowledge, theory, and methodology exists outside of the discipline of toxicology and has immediate and direct application to research on the effects of toxic substances on wildlife. Cooperative research endeavors involving scientists from basic and applied disciplines hold great promise not only in ecotoxicology, but in other areas of environmental science as well.

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