Multivariate Analysis of Covariance in Morphometric Studies of the Reproductive Cycle

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A new approach for analysing morphometric data of reproductive cycles is proposed, involving multivariate analysis of covariance of the directly measured variables (e.g., total weight and gonadal weight), with body length being the covariate. Multivariate, univariate, and between-group tests can be used progressively if significant differences have been found previously. Seasonal variation and other factors of interest can be described with predicted means of the model, adjusted for covariate, rendering the use of indices such as condition factor and gonadosomatic index unnecessary. A special design of multivariate analysis of covariance, with a pooled covariate by factor interaction, can be used to test the fundamental assumption of homogeneous slopes (parallelism hypothesis) in the standard multivariate analysis of covariance. Data for an Iberian brackish water cyprinodontid fish are used to demonstrate the proposed method.

Une nouvelle approche pour l'étude morphométrique du cycle reproductif est proposée, basée sur l'analyse multivariable de la covariance des variables mesurées directement (p. ex. poids total et poids gonadal), la variable de longueur étant la covariable. Des analyses multivariables, univariables et intergroupes peuvent être utilisées progressivement si on détecte des différences significatives. La variation saisonnière, et d'autres facteurs d'intérêt, peuvent être décrits par les moyennes prédites par le modèle, ajustées pour la covariable, et ainsi l'utilisation d'indices comme le facteur de condition ou l'index gonadosomatique devient superflue. Un modèle spécial d'analyse multivariable de covariance, avec une interaction conjointe covariable–facteur, peut d'abord être utilisé pour analyser la supposition fondamentale de pentes homogènes (hypothèse de parallélisme) du modèle courant d'analyse multivariable de la covariance. Des données pour un poisson cyprinodontidé ibérique d'eaux saumâtres sont utilisées pour montrer l'usage de la méthode proposée.

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The study of reproductive cycles and life history is a standard practice in animal ecology. Morphometric methods used in such studies rely on the measurement of several body characteristics, e.g., total weight, gonadal weight, liver weight, eviscerated weight, and length. All these variables usually increase with growth or size of individuals, and some of them are measured to estimate other processes. For instance, gonadal weight is related to the maturity and spawning processes and depends on multiple factors, e.g., sex, maturity stage, individual size, season, or reproductive investment. Total or eviscerated weight is assumed to indicate condition, fatness, or well-being of animals, based on the hypothesis that heavier individuals of a given length are in better condition (e.g., Bagenal and Tesch 1978), and to be related to the physiological processes involved in reproduction and survival (e.g., Dobson 1992).

These morphometric variables have traditionally been studied with univariate procedures (if tested), using some of the many available indices (e.g., see Bagenal and Tesch 1978; Weatherley and Rogers 1978; Pitcher and Hart 1982; Roff 1983; Weatherley and Gill 1987; Bolger and Connolly 1989; Lindström and Alerstam 1992) such as the following: as Fulton's condition factor (K_1):

$$K_1 = 100WL^{-3}$$

Ricker's (1975) condition factor (K_2):

$$K_2 = 100WL^{-b}$$

Relative condition factor (K_3):

$$K_3 = 100W\,a^{-1}L^{-b}$$

Gonadosomatic index (GSI):

$$\mathrm{GSI} = 100GW^{-1}$$

where W is total weight, L is length, G is the gonadal weight, and a and b are parameters of the weight–length relationship

$$W = aL^b.$$

Problems with these indices have been well studied (see reviews in DeVlaming et al. 1982; Erickson et al. 1985; Bolger and Connolly 1989) and they include violation of several assumptions. Firstly, indices such as K_1 and GSI are ratios and therefore usually present the following statistical difficulties (Sokal and Rohlf 1969): (1) increased variability in comparison with that of the variables that were compounded into the ratio, (2) biased estimation of the true mean value of the ratio, (3) unusual, nonnormal, and possibly intractable distributions of ratios (but see Jolicoeur 1963), (4) tendency to obscure rather than elucidate the intervariable relationships. Secondly, a linear relationship, i.e, isometric growth, between the two variables involved is wrongly assumed, especially for indices such as K_1 and GSI. Thirdly, the indices are often incorrectly assumed to be independent of length. Furthermore, most indices assume that variation of condition is related only to variation of parameter *a* of the weight–length relationship, i.e., there exists homogeneity of parameter *b* (slope in the log-transformed form) among groups. This assumption is also present in the method for studying condition recently proposed by Patterson (1992).

The purpose of this investigation is to propose a new methodological approach to morphometric studies of the reproductive cycle based on the use of multivariate analysis of covariance (MANCOVA) of the directly measured variables (e.g., total weight and gonadal weight), with the length variable being the covariate. The seasonal variation and other factors of interest can then be described with the predicted means of the model, rendering the use of indices unnecessary. Data for an Iberian brackish water cyprinodontid fish, *Aphanius iberus,* are used to exemplify the use of the proposed method.

Methods

The method is an application of MANCOVA. The measured variables whose relationships with the reproductive cycle are to be investigated, e.g., total, gonadal, liver, and eviscerated weights, are considered dependent variables. The length variable is treated as the covariate. Variables that influence the reproductive cycle, e.g., season, sex, cohort, maturity stage, are considered to be factors.

Some assumptions of standard MANCOVA are independent sampling, multivariate normality of variables, homogeneity of variance–covariance matrices, linear relationship between the dependent and covariate variables, and homogeneity of slopes of dependent variable – covariate relationship.

Authors apparently disagree as to whether nonnormality is a serious violation of the multivariate case (Green 1980; but see also Orlóci 1990). In contrast, they generally agree that heterogeneity of variance–covariance matrices is a problem (Green 1980). Use of large samples, equal replication, appropriate (often logarithmic) transformations, few rather than many variables, and one of the more robust test statistics will probably eliminate any serious heterogeneity problem (Green 1980). Alternatively, if the homogeneity of variance–covariance matrices cannot be assumed, a weighted procedure could be applied (see McCullagh and Nelder 1983; Misra et al. 1990).

The log-transformation of the continuous variables (weight and length measurements) seems to fulfil some of those requirements. The distribution of body size is often lognormal (Misra and Carscadden 1987) and linearity is usually also achieved by log-transformation of variables (e.g., DeVlaming et al. 1982; Erickson et al. 1985; Misra and Carscadden 1987; Misra et al. 1990). Further justification for the log-transformation of morphometric variables relies on the allometric factor model and physical dimensions (see Bookstein et al. 1985).

The homogeneity of regression coefficients (slopes) of dependent variable – covariate relationships, i.e., the parallelism hypothesis, is a fundamental assumption of standard MANCOVA (see McCullagh and Nelder 1983) and is often overlooked in studies of the reproductive cycle (Bolger and Connolly 1989). This assumption can be tested with a special design of MANCOVA, analyzing the pooled covariate by factor interaction. For instance, Arnason et al. (1992) recently applied this same type of test in a model of the increase of fish weight variance. If the covariate by factor interaction is significant, standard MANCOVA (as for univariate analysis of covariance) should not be developed. An additional interesting design for these cases could be to nest the covariate within the factor, which allows fitting separate regressions within each of the factor levels. Otherwise, if the covariate by factor interaction is not significant, the standard MANCOVA design should be preferred.

MANCOVA provides multivariate and univariate tests of significance of the dependent variables for covariates and factors. For the cases with significant multivariate and univariate tests, we can soundly describe the variation (e.g., seasonal variation) by use of the predicted means for each cell that are adjusted for the effect of the covariate. Moreover, we can test differences between these adjusted estimates with some standard tests, e.g., Scheffé or Bonferroni.

Case Example

Data for *A. iberus* are used to demonstrate the proposed method. From February 1989 to January 1990, 615 fish were captured with dip nets of 9.0-mm stretched mesh at the small southern coastal lagoon (42°16′N, 3°9′E) in La Rubina salt marshes, Alt Empordà wetlands (Catalonia, Spain). The specimens were preserved on ice and transported to the laboratory where they were frozen. These specimens were later measured (standard length) to the nearest 0.1 mm, weighed to the nearest 0.1 mg, and dissected. Sex was determined by external characters and confirmed when necessary by gonad examination under a dissecting microscope. The liver and gonads of individuals were weighed to the nearest 0.1 mg. Some individuals (total 105) from the largest samples were released after determining sex and size in the field. Six to eight scales from the left side of the body between the lateral line and dorsal fin were removed and mounted dry between two slides for age estimation under a dissecting microscope. According to the reproduction pattern, we used 1 May as the date of birth. Additional information on the age and growth and general ecology of this population is available (García-Berthou and Moreno-Amich 1991, 1992).

The relationships between total, liver, and gonadal weights and standard length were clearly nonlinear, and the log-transformation of the data appeared to linearize them. Therefore, we used the log-transformation of total, liver, and gonadal weights as dependent variables and the log-transformation of standard length as the covariate. To avoid the use of negative values, we previously multiplied all variables by 10^6. This procedure (i.e., to multiply by a power of 10) is suitable when variables range from 0 to 1 (Sokal and Rohlf 1969). The factors considered were season (sample date), cohort (equivalent to age of the individual), and sex (immature, male, and female).

Unfortunately, a full factorial model cannot be applied to the data because of missing data. This was not entirely a sampling failure because some missing data are "structural," i.e., there are no individuals corresponding to some combinations of factors. For instance, the maturation of this species is extremely fast (some few months after hatching) and there are no immature individuals during part of the year, at least from March to April. Likewise, age 2+ individuals are very uncommon in this species (much less than 1% of total population size in winter) and all these fish seem to die during winter. For this reason, we divided the data into four sets: 1989 (i.e, 0+) cohort

TABLE 1. Preliminary design of MANCOVA for *A. iberus* data: degrees of freedom, F-statistics, and P-values. All variables (dependent and covariate) were log-transformed. Four independent data sets were analyzed: 1989 cohort mature fish, 1989 cohort immature fish, 1988 cohort males, and 1988 cohort females. Standard length is the covariate. Multivariate F-statistics correspond to Wilks' lambda statistic and are exact if their related degrees of freedom are both integers. The parallelism hypothesis, i.e., homogeneity of slopes, is tested with a pooled covariate by factor interaction.

	Multivariate			Univariate						
					Total wt.		Liver wt.		Gonadal wt.	
Source	df	*F*	*P*	df	*F*	*P*	*F*	*P*	*F*	*P*
				1989 cohort, mature						
Length	3, 92	174.97	<0.0005	1, 94	533.60	<0.0005	29.08	<0.0005	26.67	<0.0005
Sex	3, 92	0.26	0.847	1, 94	0.03	0.857	0.21	0.644	0.47	0.490
Date	21, 264.72	1.11	0.335	7, 94	0.60	0.748	1.39	0.218	1.59	0.147
Date × sex	21, 264.72	0.92	0.563	7, 94	0.65	0.708	0.62	0.731	1.32	0.247
Length × factors	45, 274.09	1.13	0.262	15, 94	0.76	0.712	1.32	0.206	1.36	0.181
				1989 cohort, immature						
Length	3, 4	2.08	0.245	1, 6	4.17	0.087	0.02	0.892	5.58	0.056
Date	15, 11.44	2.93	0.036	5, 6	3.12	0.099	4.04	0.059	1.49	0.316
Length × date	9, 9.89	1.05	0.466	3, 6	3.11	0.110	0.37	0.773	2.05	0.208
				1988 cohort, male						
Length	3, 35	15.04	<0.0005	1, 37	47.50	<0.0005	1.30	0.261	12.40	0.001
Date	30, 103.41	4.40	<0.0005	10, 37	2.76	0.012	4.98	<0.0005	5.13	<0.0005
Length × date	24, 102.11	3.61	<0.0005	8, 37	2.43	0.032	5.30	<0.0005	3.21	0.007
				1988 cohort, female						
Length	3, 41	4.32	0.010	1, 43	12.03	0.001	0.01	0.889	2.72	0.106
Date	21, 118.28	1.87	0.019	7, 43	0.31	0.941	2.97	0.012	1.09	0.385
Length × date	18, 116.45	1.83	0.029	6, 43	0.36	0.897	3.36	0.008	0.95	0.468

TABLE 2. Final design of MANCOVA for *A. iberus* data for which the parallelism hypothesis was not rejected: degrees of freedom, F-statistics, and P-values. See Table 1.

	Multivariate			Univariate						
					Total wt.		Liver wt.		Gonadal wt.	
Source	df	*F*	*P*	df	*F*	*P*	*F*	*P*	*F*	*P*
				1989 cohort, mature						
Regression	3, 107	927.20	<0.0005	1, 109	2803.26	<0.0005	193.34	<0.0005	115.72	<0.0005
Sex	3, 107	31.06	<0.0005	1, 109	5.24	0.024	1.67	0.199	81.96	<0.0005
Date	21, 307.80	2.84	<0.0005	7, 119	1.20	0.308	2.66	0.014	4.72	<0.0005
Date × sex	21, 307.80	0.97	0.497	7, 109	1.32	0.247	0.74	0.634	1.03	0.411
				1989 cohort, immature						
Regression	3, 7	98.33	<0.0005	1, 9	105.71	<0.0005	7.55	0.023	0.30	0.595
Date	15, 19.73	3.28	0.007	5, 9	1.60	0.253	6.05	0.010	0.41	0.827
				1988 cohort, female						
Date	21, 135.51	5.16	<0.0005	7, 49	7.44	<0.0005	—	—	1.13	<0.0005
Regression	3, 47	115.74	<0.0005	1, 49	287.58	<0.0005	—	—	56.01	<0.0005

mature individuals (males and females), 1989 cohort immatures, 1988 cohort males, and 1988 cohort females. For the mature fish in the 1989 cohort, we considered two factors: sample date and sex (male or female). For the other three data sets, we considered only the date factor. No analysis was developed on 1987 cohort data because very few individuals were caught. Statistical analyses used specimens for which all measurements were available, and were performed with the SPSS-X software package (SPSS Inc. 1988). For a posteriori contrasts of adjusted estimates, we applied joint Bonferroni univariate contrast, comparing adjacent dates (SPSS-X MANOVA subcommand: CONTRAST = REPEATED). A significance level of 0.05 was accepted for all analyses.

The pooled covariate by factor interactions were significant ($P < 0.05$) for all dependent variables of 1988 cohort males and for liver weight of 1988 cohort females (Table 1). Therefore

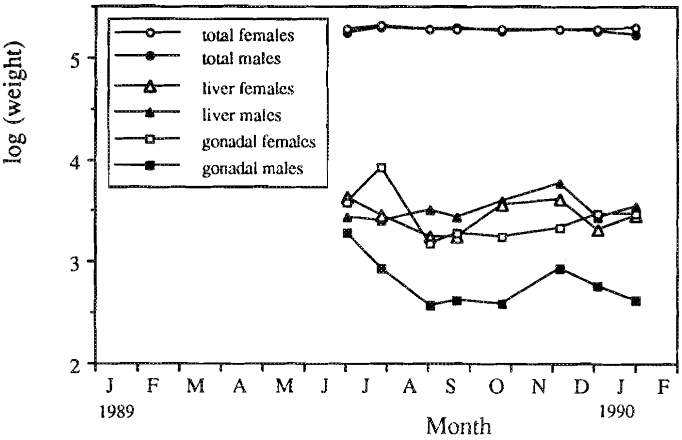

FIG. 1. Seasonal predicted means, adjusted for the effect of covariate in the sample, of log-transformed weights for 1989 cohort mature (male and female) *A. iberus*. Bonferroni difference contrast conclusions ($\alpha = 0.05$) for variables with significant variation (number corresponds to sample date rank, i.e., μ_1 is the June mean, μ_2 is the July mean, etc.): liver weight, $\mu_1 = \mu_2 = \mu_3 = \mu_4 < \mu_5 = \mu_6 > \mu_7 = \mu_8$; gondal weight, $\mu_1 = \mu_2 > \mu_3 = \mu_4 = \mu_5 = \mu_6 = \mu_7 = \mu_8$. All computations according to the MANCOVA design detailed in Table 2.

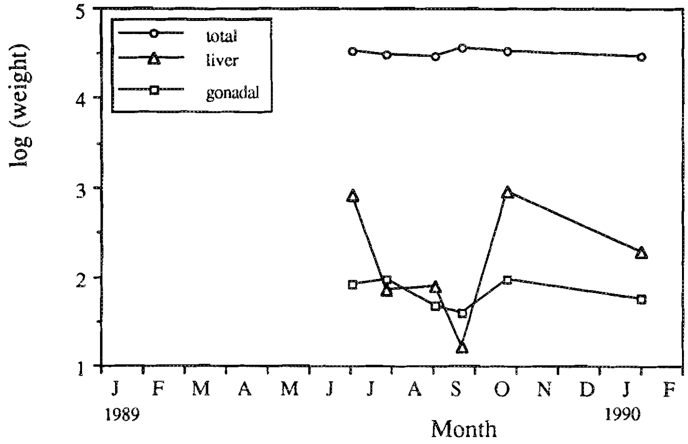

FIG. 2. Seasonal predicted means, adjusted for the effect of covariate in the sample, of log-transformed weights for 1989 cohort immature *A. iberus*. Bonferroni difference contrast conclusions ($\alpha = 0.05$) for variables with significant variation (number corresponds to sample date rank, i.e., μ_1 is the June mean, μ_2 is the July mean, etc.): liver weight, $\mu_1 > \mu_2 = \mu_3 = \mu_4 < \mu_5 > \mu_6$. All computations according to the MANCOVA design detailed in Table 2.

the standard MANCOVA design should not be developed for these cases. The results of final standard MANCOVA designs for the rest of *A. iberus* data are shown in Table 2.

The covariate effect (regression) is usually very significant. The exception is for gonadal weight of 1989 cohort immatures, which is nonsignificant. The reason for this may be that the gonads of immature individuals are very small and do not increase in size during growth. Note that in this case the use of the gonadosomatic index would seem inappropriate because log-transformed gonadal weight is independent of log-transformed standard length.

The date by sex interaction is clearly not significant for 1989 cohort mature fish and this facilitates the interpretation of main effects. The sex effect is significant for total and gonadal weights, after parcelling out the covariate effect. As male mean weight is smaller for both variables (Fig. 1), this means that females are heavier and have larger gonads than similarly sized males. The former feature may be related to sexual size dimorphism, which is quite strong in this species, and the latter to the usual larger reproductive effort of females. Furthermore, this significant sex effect without significant interaction justifies the independent analysis of males and females for the 1988 cohort. Variables with significant seasonal variation can be soundly described with Bonferroni contrasts, which should rigourously limit our inference statements. Thus, for the mature fish in the 1989 cohort (Fig. 1), liver weight increases in late October and decreases in late December, and gonadal weight decreases in late August. Therefore, mature young-of-the-year (i.e., 1989 cohort) individuals spawn before August, and their liver weight subsequently increases (supposed energetic reserve increase) in early Autum and decreases in winter.

Young-of-the-year immatures were captured (Fig. 2) from summer to winter, and they show a seasonal variation in liver weight quite similar to young-of-the-year matures. However, they do not show significant variation of gonadal weight, and probably only the young-of-the-year individuals born early in the reproductive season (perhaps mostly from 1+ and 2+ parents) reproduce the same year.

The liver weight variation was also similar for 1988 cohort males, with a maximum in late September (Fig. 3). The reproductive season, i.e., the period when gonadal weight is at a significant maximum, ranges from March to July.

The reproductive season for 1988 cohort females (Fig. 4), from late February to early June, is similar to that for males. The liver weight is minimal in winter, and variation in total weight is similar; this would usually be interpreted as a minimum in "condition." However, as total weight actually includes gonads, liver, and other viscera, it should be interpreted carefully. We think that the use of eviscerated weight (as in Palmer and Culley 1983; Stearns 1983) instead of total weight provides an alternative measure that is more representative of true condition.

Discussion

Further criticisms of classical indices may be added to those previously reported in the literature, e.g., the use of univariate procedures in a generally multivariate problem. There is an increased recognition that a series of univariate tests is as inappropriate for a multivariate problem as is a series of *t*-tests for a univariate multigroup problem (Green 1980; Hair et al. 1987). The procedure proposed in this paper takes this criticism into consideration by providing an overall multivariate test of significance, and only if significant results are encountered can this be followed up with univariate tests of significance and difference contrasts among groups. The usual subjective interpretation of seasonal variation is thus completely avoided on the basis of statistical theory.

The use of length as the covariate also needs two comments. Firstly, in morphometric studies of the reproductive cycle the effect of individual size needs to be subtracted from other variables. That length is the most appropriate variable for this purpose seems intuitively obvious because it depends less on environmental characteristics and physiological conditions than weight variables. However, gonadosomatic index and other similar indices use total weight to remove the individual size effect, which we believe may be faulty in most cases. For

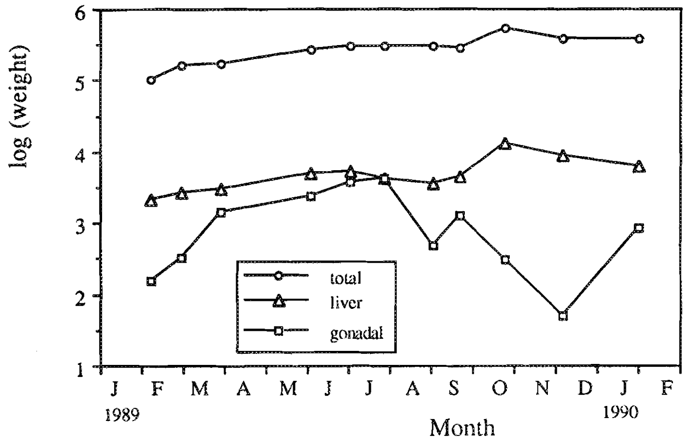

FIG. 3. Seasonal predicted means, adjusted for the effect of covariate in the sample, of log-transformed weights for 1988 cohort male *A. iberus*. Bonferroni difference contrast conclusions ($\alpha = 0.05$) for variables with significant variation (number corresponds to sample date rank, i.e., μ_1 is the February mean, μ_2 is the March mean, etc.): liver weight, $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6 = \mu_7 < \mu_8 > \mu_9 = \mu_{10} = \mu_{11}$; gonadal weight, $\mu_1 > \mu_2 < \mu_3 > \mu_4 = \mu_5 = \mu_6 > \mu_7 = \mu_8 = \mu_9 = \mu_{10} = \mu_{11}$. All computations according to the MANCOVA design detailed in Table 1.

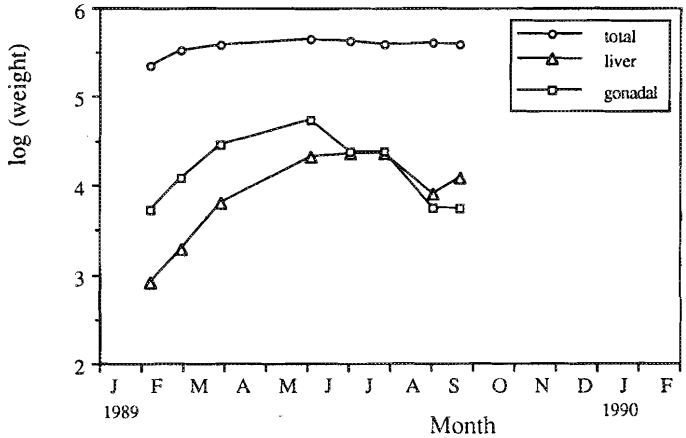

FIG. 4. Seasonal predicted means, adjusted for the effect of covariate in the sample, of log-transformed weights for 1988 cohort female *A. iberus*. Bonferroni difference contrast conclusions ($\alpha = 0.05$) for variables with significant variation (number corresponds to sample date rank, i.e., μ_1 is the February mean, μ_2 is the March mean, etc.): total weight, $\mu_1 < \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6 = \mu_7 = \mu_8$; liver weight, $\mu_1 < \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6 = \mu_7 = \mu_8$; gonadal weight, $\mu_1 < \mu_2 = \mu_3 = \mu_4 > \mu_5 = \mu_6 = \mu_7 = \mu_8$. Computations for liver weight according to the MANCOVA design detailed in Table 1, and other computations according to that of Table 2.

A. iberus data ($N = 269$) the coefficients of variation of (log-transformed) standard length and total, liver, and gonadal weights were 0.015, 0.069, 0.177, and 0.278, respectively. Therefore, standard length varies less than the other variables and describes "size" better than total weight.

Secondly, we believe that the recommendation of estimating general size by principal components analysis (or other ordination techniques) instead of considering a single variable as covariate (cf. Bookstein 1982; Bookstein et al. 1985; Crespi and Bookstein 1989; Dobson 1992; Klingenberg and Zimmermann 1992) does not apply either to the present case or most morphometric studies of reproductive cycles because too few metric variables are usually involved (Hair et al. 1987). Hair et al. (1987) pointed out that the researcher generally would not factor analyze a sample of fewer than 50 observations, and preferably, the sample size should be 100 or larger. Moreover, they suggested that, as a general rule, there should be four or five times as many observations as there are variables to be analyzed. Other studies fulfilling these requirements, i.e., involving many variables (and observations), should consider such an ordination approach both to estimate general size and to reduce the reproductive variables to a few components.

It also seems worthwhile to mention that the a posteriori contrasts applied in our example, comparing adjacent dates, helped to avoid the loss of power of common contrasts when there are ordered samples. The technique of isotonic regression (Gaines and Rice 1990) may improve the between-group comparisons of seasonal variation.

Particular attention to experimental design should be used when applying this procedure. Large equal sample sizes will provide both homogeneity of variances (as stated above) and reduced experimental error and will allow a more factorial approach if we are interested in several factors. Generalized linear models (including MANCOVA) for data with missing observations cannot easily be used because they involve particular procedures (McCullagh and Nelder 1983), and therefore, special care in sampling and data processing should be taken. Furthermore, we think that a full factorial model will not generally be possible because of missing data cells, some of them structural, and the best combination of data sets should then be chosen.

One of the central problems of classical indices is that both regression parameters may change in the bivariate relationships (e.g., the two parameters of the weight–length relationship). Therefore, valid interpretation of results is difficult if just one of the parameters is considered (Bolger and Connolly 1989). Our approach allows us to overcome this problem. The first preliminary MANCOVA design, analyzing the pooled covariate by factor interaction, tests if homogeneity of one of the parameters (slope) can be assumed. When the interaction is not significant, this can be followed by the standard MANCOVA design, i.e., without the covariate by factor interaction, to study variation due to the other regression parameter (intercept). We believe that the proposed procedure is the suitable statistical test suggested for condition analysis by Bolger and Connolly (1989). All the aforementioned MANCOVA analyses and computations are available in some of the more popular statistical software packages, such as SPSS-X (SPSS Inc. 1988) and BMDP (Dixon 1988), and hence, their application does not appear difficult.

Finally, we must point out that this method does not consider the controversy on the use of regression for predictive versus descriptive purposes. It appears that standard predictive regression should be used only when regression is used to predict the dependent variable from the independent one (Sokal and Rohlf 1969). When both variables are random, the geometric mean regression and more recently the major axis and the bivariate structural relationship have been proposed for descriptive purposes (see Jolicoeur 1975, 1990; Jolicoeur and Ducharme 1992 and references therein), although this has not yet been generally applied (Bagenal and Braum 1978; Bolger and Connolly 1989).

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