The Statistical Analysis of Ecophysiological Response Curves Obtained from Experiments Involving Repeated Measures

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THE STATISTICAL ANALYSIS OF ECOPHYSIOLOGICAL RESPONSE CURVES OBTAINED FROM EXPERIMENTS INVOLVING REPEATED MEASURES[1]

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Abstract. Physiological ecologists often analyze the responses of physiological or biochemical traits to environmental factors such as temperature, irradiance, water potential, or the concentrations of CO_2, O_2, and inorganic nutrients. The data for such a response curve typically are gathered by sequential sampling of the same plant or animal, and their analysis should explicitly allow for this repeated-measures design. Unfortunately, the statistical analysis of response curves in ecology generally has either been ignored or incorrectly done.

In an effort to encourage rigorous analysis of response data, we address statistical treatment of response curves and illustrate the correct alternatives that are available. Four different statistical methods for analyzing response curves are considered: analysis of variance with repeated measures (ANOVAR), multivariate analysis of variance with repeated measures (MANOVAR), a nonparametric split-plot analysis (NP split-plot) and parametric comparison of models fitted to the data by nonlinear regression. Analyses of the CO_2 dependence of photosynthesis in the C_4 grass *Echinochloa crus-galli* following chilling are used as examples of these different methods.

ANOVAR, potentially the most powerful analysis, makes stringent assumptions about the variance–covariance structure of the data. Within limits these assumptions can be relaxed and a corrected significance level used. When the variance–covariance structure badly violates the ANOVAR assumptions, MANOVAR or NP split-plot are viable alternatives. In physiological ecology, however, the use of MANOVAR frequently is limited by small sample sizes and the tendency for the number of levels of the treatment factor to exceed the sample size. Greater attention to experimental design can avoid this problem. The NP split-plot is based on simple assumptions and could be widely used. The comparison of curves fitted by nonlinear regression is also distribution free and provides an interesting alternative when the responses are amenable to fitting. For any of these analyses to be viable the thoughtful choice of experimental protocols and design is essential.

Key words: analysis of variance; Huynh-Feldt correction; model fitting; nonlinear regression; nonparametric tests; physiological ecology; repeated-measures multivariate analysis; response curves; split-plot and repeated-measurement designs; statistical methods.

INTRODUCTION

Physiological and biochemical processes in living systems respond to variations in environmental factors such as temperature, irradiance, ambient concentrations of gases, soil water potential, and the concentrations of various mineral nutrients, salts, and toxic ions. Physiological ecologists study responses as diverse as changes in animal body mass, gaseous exchanges in plants, and the kinetic properties of enzymes. For example, the photosynthetic rate of an individual plant might be assayed at photon flux rates of 100, 200, 300, 400, 600, and 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to determine its photosynthetic light response (Leverenz 1987). Thermo-

regulation might be assessed by measuring the body temperature of individual animals in response to variation in ambient temperature (Lovegrove 1987). The effect of exercising can be investigated by tracking blood constituents of individual fishes over a time period (Milligan and Wood 1987). Similarly, insight into the temperature dependency of biochemical processes might be sought by analyzing K_m variation of enzymes at 20°, 30°, and 40°C (Simon et al. 1984). The results of such ecophysiological investigations are typically summarized as response curves, graphs of the response rate against an environmental factor that affects the response. The importance of response curves in physiological ecology reflects the discipline's roots in comparative physiology, where a strong tradition of laboratory stimulus–response investigations exists. Perhaps

because these same laboratory traditions depend on repeatability more than inferential statistics to decide the generality and validity of experimental results, physiological ecologists have not emphasized the statistical comparison of response curves.

In an effort to encourage more rigorous analysis of response data, we address the statistical treatment of response curves obtained by measuring the response of *each* experimental unit (plant, animal, tissue preparation) to *all* the investigated levels of the environmental factor governing the response. Such an experimental design, typical in ecophysiological research, would be referred to as a repeated-measure design in the statistical literature (Winer 1971, Timm 1980). Repeated-measure designs have the advantage of increasing the precision of the treatment analysis by accounting for inter-individual variation (Cook and Ware 1983). Recent reviews have dealt with the analysis of longitudinal data (Cook and Ware 1983, Ware 1985) or of growth curves (Koziol 1986, Kokoska and Johnson 1987), which also arise from repeated-measure designs. Unfortunately, ecologists appear largely to ignore the appropriate treatment of response curve data (but see Kowal et al. 1976, Gurevitch and Chester 1986). Almost none of the recent papers in *Ecology, Oecologia* (Berlin), *Physiological Zoology,* and *Plant, Cell, and Environment* that compared response curves used appropriate statistical techniques.

In this paper we compare different statistical methods that can be used to analyze response curves derived by repeated measurements. We consider both multivariate and univariate parametric methods, as well as nonparametric methods of statistical inference, together with a complementary model-fitting approach. We summarize the advantages and drawbacks of each method, and offer guidance on the choice of the best method for the analysis of particular response curves. Objective criteria that can be used to select the most appropriate analytical method are given as a dichotomous key to the choice of a technique based on differences in experimental design and data structure. Our aim is not to elaborate new statistical methods, but, rather, to bring to the attention of ecologists information that is at present largely confined to the statistical literature. The use of each technique is illustrated by a didactic biological example, and is explicitly related to procedures in widely available statistical programs. In the example, seven photosynthetic rates corresponding to seven CO_2 concentrations were measured on each plant. Although we have used the comparison of these CO_2 response curves as an illustration, our approach pertains to any response curves obtained from repeated measurements.

MATERIALS AND METHODS

The biological example to illustrate the different statistical methods is part of a larger study aimed at analyzing the cold tolerance of a C_4 grass species, *Echi-*

nochloa crus-galli (Potvin et al. 1986). Northern and southern plants of that species were subjected to daytime chilling, and the consequences of chilling, as well as the population difference, on the CO_2 dependence of photosynthesis was determined by comparison of the CO_2 response curves for photosynthesis.

Seeds of *Echinochloa crus-galli* var. *crus-galli* (L.) Beauv. from Québec and Mississippi were grown under a cycle of 14 h at 26°C, 10 h at 20°, in growth chambers at the Duke University Phytotron (Durham, North Carolina, USA). Photosynthetic photon flux density (PPFD) was 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, air humidity was 70%, and CO_2 concentration 350 $\mu L/L$. Four-week-old plants were divided in two groups: control plants that remained at 26° and chilled plants that were subjected to a full day (14 h) of chilling at 7°. Each control plant was paired at random to a plant to be chilled, since plant growth prior to chilling was very uniform. Pairing was recommended by Koch (1970) to enable analysis of interaction effects in nonparametric tests. All other conditions were unchanged. Following one night (10 h) of recovery at 20°, CO_2 curves of carbon uptake were generated for chilled and control plants. Net photosynthesis was measured at seven concentrations of CO_2 (100, 175, 250, 350, 500, 675, 1000 $\mu L/L$) using the absolute IRGA system (Horiba, PIR-2000) described in Potvin et al. (1986). Measurement temperature in the single-leaf cuvette was 26°, PPFD was 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and water-vapor pressure deficit was held constant at 1 kPa. A CO_2 response curve was generated for three plants from each population and chilling treatment combination. Each plant was subjected to the seven CO_2 levels in increasing, consecutive order, which is the normal protocol in these types of experiments. The photosynthetic response to a CO_2 level was recorded after photosynthesis had remained unchanged for 5 min. Raw data are presented in Table 1.

Statistical analyses

Data were analyzed using four different statistical procedures and three statistical packages: SPSS (version 9.0, Norusis 1986), BMDP (Dixon 1983), and SAS (SAS Institute 1985). In our summary of the procedures we assume a sufficient knowledge of the SAS, BMDP, and SPSS programs to implement the procedures. Information on the specific commands used to program a typical ANOVAR can be found in the Appendix.

Repeated-measures analysis of variance.—ANOVAR was run from SAS procedure GLM (profile analysis in SPSS or BMDP 2V). Mauchly's criterion test for the compound symmetry of the variance–covariance matrix was obtained automatically, together with corrected significance levels in case of the rejection of the symmetry assumption. A mixed model was assumed; the repeated subject [plant, $S_{k(ij)}$] was random while the remaining effects were fixed. The model used is similar to the split-plot design:

TABLE 1. Photosynthetic rates, measured as CO_2 uptake, obtained from (A) Québec and (B) Mississippi plants of the C_4 grass *Echinochloa crus-galli*. Control and chilled plants were paired a priori as follows: Québec: 1 with 6, 2 with 4, 3 with 5; Mississippi: 1 with 6, 2 with 5, 3 with 4.

Plant	Treatment	CO_2 concentrations (μL/L)						
		95	175	250	350	500	675	1000
		CO_2 uptake rate (μmol·m^{-2}·s^{-1})						
A) Québec plants								
1	Control	16.0	30.4	34.8	37.2	35.3	39.2	39.7
2	Control	13.6	27.3	37.1	41.8	40.6	41.4	44.3
3	Control	16.2	32.4	40.3	42.1	42.9	43.9	45.5
4	Chilled	14.2	24.1	30.3	34.6	32.5	35.4	38.7
5	Chilled	9.3	27.3	35.0	38.8	38.6	37.5	42.4
6	Chilled	15.1	21.0	38.1	34.0	38.9	39.6	41.4
B) Mississippi plants								
1	Control	10.6	19.2	26.2	30.0	30.9	32.4	35.5
2	Control	12.0	22.0	30.6	31.8	32.4	31.1	31.5
3	Control	11.3	19.4	25.8	27.9	28.5	28.1	27.8
4	Chilled	10.5	14.9	18.1	18.9	19.5	22.2	21.9
5	Chilled	7.7	11.4	12.3	13.0	12.5	13.7	14.4
6	Chilled	10.6	18.0	17.9	17.9	17.9	18.9	19.9

$$X_{ijklm} = \mu + P_i + T_j + PT_{ij} + S_{k(ij)} + C_l$$
$$+ PC_{il} + TC_{jl} + PTC_{ijl}$$
$$+ SC_{k(ij)l} + \epsilon_{ijklm},$$

where X_{ijklm} is the response of the kth plant from the ith population and subjected to the jth treatment, μ is the population mean for the response, P_i is the effect of population, T_j is the effect of the jth treatment, $S_{k(ij)}$ is the subject effect (due to individual plants), C_l is the CO_2-level main effect, ϵ_{ijklm} is the random deviation. In the present case: $i = 1, 2; j = 1, 2; k = 1, 3$ and $l = 1, \ldots, 7$. In this model, $S_{k(ij)}$ is nested under the population and treatment main effects, and is the appropriate error term for P_i, T_j, and PT_{ij}, which will be referred to as between-subject effects (Hand and Taylor 1987). The effects involving the C_l term will be called within-subject effects, and the appropriate error term for these is $SC_{k(ij)l}$ (Winer 1971:561).

Multiple comparisons also were made a posteriori using Helmert contrasts (SAS procedure GLM), which allow a closer analysis of the CO_2 influence on the main effects. This contrast compares each level of a factor to the mean of the subsequent levels. Canonical coefficients are then computed for the contrasts generated. A small coefficient indicates that the carbon uptake at that level has reached the saturated rate. Because CO_2 curves are asymptotic, this contrast is especially useful in interpreting differences in the shapes of the response curves. For example, a large negative value for the sixth CO_2 contrast under the treatment effect would indicate that the difference between control and chilled plants is larger when measured at 1000 μmol of CO_2.

Repeated-measures multivariate analysis of variance. —MANOVAR (Timm 1980, Hand and Taylor 1987) was performed on the data (SAS procedure GLM; profile analysis in SPSS MANOVA and BMDP 2V). Prior to the analysis, the homogeneity of the variance–covariance matrices was tested by the univariate Coch-

rane test and by the multivariate Box M test (SPSS MANOVA). Much as ANOVAR, MANOVAR distinguishes between-subject effects and within-subject effects, but unlike ANOVAR always assumes fixed-effect models. In MANOVAR the within-subject effects in the preceding model are tested by transforming the data in $p - 1$ contrasts (where p = the number of parameters), a transformation that is automatic in SAS procedure GLM.

Nonparametric, split-plot analysis. —Data were compared using a split-plot nonparametric analogue. The analysis was based on recommendations by Koch et al. (1980) regarding the nonparametric treatment of analysis for repeated measurements. The among-plants effects, P_i, T_j, and PT_{ij}, were tested using the Kruskal-Wallis rank analysis as presented in BMDP 3S. Since in this case both P_i and T_j have only two levels each, the Kruskal-Wallis test reduces to the Wilcoxon rank test. The within-subject effects, C_l, PC_{il}, and TC_{jl} were tested by means of the Friedman test (BMDP 3S).

Prior to the analysis, however, data had to be summed in a variety of ways to allow the test of the various effects. Summation proceeded as indicated by Koch (1970), and is summarized in Tables 2 and 3. As in ANOVAR and MANOVAR, the P_i and T_j between-subject effects are analyzed as the photosynthetic mean over all CO_2 concentrations (Table 2A). To obtain a test for a $P_i \times T_j$ interaction, differences in mean photosynthesis between chilled and control plants paired at random were computed (Table 2B). For example, using data from Table 1, the computed difference in CO_2 uptake rate is between 33.2 and 33.4 μmol·m^{-2}·s^{-1}, the mean rates corresponding to Québec plants 1 and 6, which had been previously paired. These random pairwise differences were then grouped as populations, and the Kruskal-Wallis test performed in BMDP 3S. The CO_2 within-subject main effect was analyzed across the seven levels for each of the treatment-by-population com-

TABLE 2. CO_2 uptake rate values (in micromoles per square metre per second) and associated ranks to test for the effects of (A) population (P_i) and treatment (T_j) and (B) the population × treatment interaction (PT_{ij}) in the nonparametric split-plot analysis. Pairing was done according to Table 1.

Treatment	Québec Uptake	Québec (Rank)	Mississippi Uptake	Mississippi (Rank)
A) Mean photosynthesis and associated ranks				
Control	33.2	(9)	26.4	(5)
	35.2	(11)	27.3	(6)
	37.6	(12)	24.1	(4)
Chilled	33.4	(10)	18.0	(3)
	32.7	(8)	12.1	(1)
	30.0	(7)	17.3	(2)
B) Differences in photosynthesis between a priori paired control and chilled plants and associated ranks				
	0.2	(1)	9.1	(5)
	5.2	(3)	15.2	(6)
	4.9	(2)	6.1	(4)

binations. The PC_{il} interaction was tested for each CO_2 concentration as the mean photosynthetic difference between control and chilled plants for each population (Table 3A). For example, at 100 µL/L CO_2 for Québec: (16.0 + 13.6 + 16.2) − (15.1 + 9.3 + 14.2) = 7.2, and this value was then used in the test. The TC_{jl} effect was similarly computed for each CO_2 level as the population mean difference for chilled and control plants. This is illustrated by the chilled plants at 100 µL/L CO_2: (15.1 + 9.3 + 14.2) − (7.7 + 10.5 + 10.6) = 9.8. Consequently, to run the analysis in the statistical program, six different data files had to be created by summing or subtracting appropriate rows and columns in the full data matrix.

Comparison of fitted curves.—Curves were fitted by nonlinear regression to the four combinations of population and treatment, each of which will hereafter be referred to as a group. The four groups compared are Québec control, Québec chilled, Mississippi control,

and Mississippi chilled. The model fitted by the iterative least-square method available in BMDP AR was:

$$y = A[1 - e^{-k(x-x_0)}]. \tag{1}$$

This model is well-suited to our CO_2-response data, but other responses may require a different model. Initial parameters for the regression were found after solving an equation that linearized the data:

$$\ln(1 - y/a) = -kx + kx_0. \tag{2}$$

The four fitted regressions were then compared following Mead and Curnow (1983). The total sum of squares (SS), which is the residual SS prior to grouping, was obtained by fitting the regression equation to the complete data set. The F ratios were computed as follows:

$$F = ss_o/(p_i + g - p - 1) \quad \text{and}$$
$$F = ss_w/(N - g - p_i),$$

where p_i is the number of parameters in the ith group, p is the number of parameters, g is the number of groups, N is the number of cases of all groups combined ($N = \Sigma n$), ss_w is residual SS of the groups, and ss_o is total SS $- ss_w$. In this example, the test for an overall group effect was obtained as follows: $ss_w = 82.2 + 116.0 + 173.4 + 172.5 = 544$, with df $= 84 - 4 - 12 = 68$, and therefore $ms_w = 7.99$. On the other hand, $ss_o = ss - ss_w = 5717 - 544 = 5173.1$, with df $= 12 + 4 - 3 - 1 = 12$, and the $ms_o = 431.1$. The F ratio is thus $431.1/7.99 = 53.88$, which, compared to $F_{12,68} = 2.46$, is highly significant. Following the suggestion of Kokoska and Johnson (1987), we eliminated pairwise comparisons for groups that appeared to have a similar response curve. This was the case for the two Québec groups ($F_{4,34} = 3.01$). Consequently, only two differences were computed, namely Mississippi control vs. Québec combined and Mississippi chilled vs. Québec combined. This method has the advantage of decreasing the number of simultaneous tests and consequently the probability of Type 1 error.

TABLE 3. CO_2 uptake rate values (in micromoles per square metre per second) and associated ranks to test for the effects of (A) the population × CO_2 level interaction (PC_{il}) and (B) the treatment × CO_2 level interaction (TC_{jl}).

A) Differences in mean photosynthesis between control and chilled values, and associated ranks.

	Ambient CO_2 levels (µL/L)							
	100		175		250		350	
Population	Uptake	(Rank)	Uptake	(Rank)	Uptake	(Rank)	Uptake	(Rank)
Québec	7.2	(2)	17.7	(2)	8.8	(1)	7.1	(1)
Mississippi	5.1	(1)	16.3	(1)	34.3	(2)	39.3	(2)

B) Differences in photosynthesis between the two populations, and associated ranks.

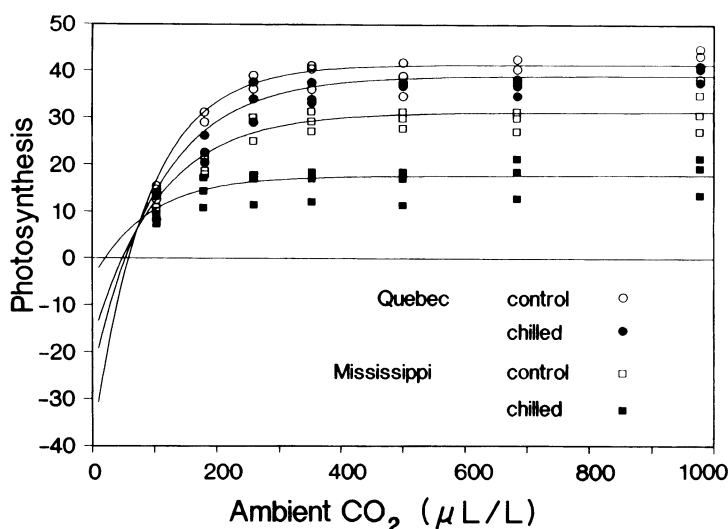|	Ambient CO_2 levels (µL/L)							
	100		175		250		350	
Treatments	Uptake	(Rank)	Uptake	(Rank)	Uptake	(Rank)	Uptake	(Rank)
Control	11.9	(2)	29.5	(2)	29.6	(1)	31.4	(1)
Chilled	9.8	(1)	28.1	(1)	55.1	(2)	64.2	(2)

FIG. 1. CO_2 uptake photosynthetic response curves for Québec plants of the C_4 grass *Echinochloa crus-galli*, control and chilled, and Mississippi plants, control and chilled. The data were fitted with Eq. 1: $y = A[1 - e^{-k(x - x_0)}]$, and the estimated values of the equation parameters are given in Table 9A.

RESULTS

Visual comparison of the CO_2 curves for each combination of population and chilling (Fig. 1) suggests that the CO_2-saturated photosynthetic CO_2 uptake is higher for plants from Québec than from Mississippi. CO_2 uptake values on the order of 40 μmol·m^{-2}·s^{-1} are seen for northern plants, compared to 30 μmol·m^{-2}·s^{-1} for southern ones. The curves have the typical shape of the CO_2 response of C_4 plants. In all cases CO_2 uptake saturates at low external CO_2 concentrations. Chilling has a drastic effect on the CO_2 curves of the Mississippi plants but a lesser one on Québec plants. Photosynthesis saturated some 10 μmol·m^{-2}·s^{-1} lower for chilled Mississippi plants compared to control values.

ANOVAR

The ANOVAR and MANOVAR both assume that the data are normally distributed. However, because

of small sample sizes ($n = 3$ in each cell of our example), none of the distribution techniques available (Mardia 1980) is powerful enough to test normality. As there is no a priori reason to suspect departure from normality, we will assume it. Prior to the examination of the ANOVAR results, the hypothesis of the compound symmetry of the covariance matrix should be tested. Mauchly's criterion for our data (0.00085, $P > .004$) indicates that compound symmetry must be rejected. Nonetheless, ANOVAR may still be used under relaxed assumptions if the Huynh-Feldt conditions (Huynh and Feldt 1970) are met. The Huynh-Feldt epsilon is a measure of the strength of the violation of compound symmetry; if the Huynh-Feldt conditions are met, $\epsilon = 1$, and small values of ϵ indicate strong violations. In the present case, $\epsilon = 0.9679$, indicating that the violation is very mild. Consequently, our analysis can be based on corrected significance levels.

In the Huynh-Feldt-corrected ANOVAR, the between-subject effects of the model (population, treatment, and population × treatment) are all statistically significant (Table 4). Similarly the CO_2 effect and the interaction of this effect with all other effects are also significant. The altenative Greenhouse-Geisser correction (Greenhouse and Geisser 1959) gives comparable results for our data (Table 3), but this will not always be the case.

The canonical coefficients from Helmert contrasts (Table 5) indicate that, for both populations and treatments, photosynthesis reached a plateau between the second and the third CO_2 concentrations (i.e., 175–250 μL/L). The CO_2 concentration at which photosynthesis saturates was thus independent of the treatment and the population factors. Observed significant differences in the curves were mainly caused by difference in the saturated rates of carbon uptake (Table 5).

TABLE 3. Continued.

	Ambient CO_2 levels (μL/L)				
500		675		1000	
Uptake	(Rank)	Uptake	(Rank)	Uptake	(Rank)
8.8	(1)	12.0	(1)	7.0	(1)
41.9	(2)	36.8	(2)	39.1	(2)

	Ambient CO_2 levels (μL/L)				
500		675		1000	
Uptake	(Rank)	Uptake	(Rank)	Uptake	(Rank)
27.0	(1)	32.9	(1)	34.7	(1)
60.1	(2)	57.7	(2)	66.8	(2)

TABLE 4. Analysis of variance of CO_2 uptake rate with repeated measurements on plants. The analysis assumed a mixed model in which ambient CO_2 level is the only fixed effect.

Sources of variation	MS	F	df	P	Corrected P, Greenhouse-Geisser	Corrected P, Huynh-Feldt
Between subjects						
P_i	3441.9	92.9	1	.0001	⋯	⋯
T_j	947.4	25.6	1	.001	⋯	⋯
PT_{ij}	245.8	6.64	1	.03	⋯	⋯
Within subjects						
C_l	685.5	185.3	6	.0001	.0001	.0001
PC_{il}	65.0	17.6	6	.0001	.0001	.0001
TC_{jl}	15.2	4.10	6	.002	.02	.002
PTC_{ijl}	20.2	5.5	6	.0002	.007	.0003

MANOVAR

A multivariate test for the assumed homogeneity of the variance–covariance matrices is provided by the Box M test (Norusis 1986). In our case the test could not be performed because the cell matrices are singular. We therefore used the univariate Cochrane test, which indicated that the variance was homogeneous for each of the seven dependent variables. This univariate test is not fully satisfactory for multivariate data because it looks only at the variance of the data. Because of the untestability of the homogeneity of the variance–covariance matrices, and since the variances are homogeneous, we proceeded with the analysis.

The values of Pillai's trace (Olson 1976) are reported in Table 6. This statistic is to be preferred in MANOVA(R) when the univariate degrees of freedom (df) for the error (df_e) are <10 p, p being the number of dependent variables (Olson 1976). In the present case, $df_e = 8$, and 10 $p = 60$. Pillai's trace has also been shown to be most robust in the face of departure from the MANOVA(R) assumptions, which could not be tested here (Olson 1976). The MANOVAR results (Table 6) indicate that the effect of CO_2 is highly significant—not surprisingly, since CO_2 levels affect carbon uptake. In contrast to ANOVAR, MANOVAR indicates that the only other significant within-subject effect is the population × CO_2 interaction ($P < .05$).

TABLE 5. Canonical coefficients for a posteriori Helmert contrasts* of CO_2 uptake rates in ANOVAR.

CO_2-level contrasts	CO_2 level	Population	Treatment	Population × treatment
1 vs. 2–7	5.70	5.52	5.02	3.20
2 vs. 3–7	−0.13	−0.02	−0.47	0.71
3 vs. 4–7	−0.19	−0.51	1.07	0.37
4 vs. 5–7	−1.29	−1.41	−1.12	−1.19
5 vs. 6–7	2.37	2.59	1.21	1.09
6 vs. 7	−1.31	−0.94	−2.13	−0.47

* In which each level of a factor is compared to the mean of subsequent levels.

Nonparametric (NP) split-plot

The NP split-plot indicates that both the population and CO_2 main effects are highly significant, respectively $P < .01$ and $P < .001$ (Table 7). The only interaction found significant by this test is the population × treatment interaction ($P < .01$).

Comparison of nonlinear regressions

The four regressions fitted to the photosynthetic responses of, respectively, Québec control, Québec chilled, Mississippi control, and Mississippi chilled, are:

$$y = 41.47[1 - e^{-0.0115(x-58.27)}], \tag{3}$$
$$y = 39.10[1 - e^{-0.0093(x-53.08)}], \tag{4}$$
$$y = 31.28[1 - e^{-0.0093(x-48.35)}], \quad \text{and} \tag{5}$$
$$y = 17.78[1 - e^{-0.011(x-20.59)}]. \tag{6}$$

Normal probability plots suggested that the residuals were normally distributed; Fig. 1 illustrates the fit of the curves to the data. Significance testing indicates the existence of a significant group effect (Table 8). Subsequent pairwise comparisons showed that the combined Québec populations were significantly different from either of the Mississippi subpopulations.

SUMMARY AND DISCUSSION

Not all apparent response curves are correctly analyzed as repeated-measures designs, and widely used statistical techniques like the analysis of variance can only be used to analyze a few particular types of response curves that are *not* based on repeated measures. The statistical problems inherent in comparing response curves in ecology are related to the use of the same experimental unit over a range of treatment levels. Data gathered following such a design are most often called repeated-measures data (Winer 1971) or, if the response to a treatment is followed over time, longitudinal data (Ware 1985). From a statistical viewpoint, the analysis of mass gain in an animal that was successively weighed during its life is totally different from the analysis of plant growth as obtained by dry biomass increment between successive harvests. The experi-

TABLE 6. Multivariate analysis of variance of CO_2 uptake rate with repeated measurement on CO_2 concentrations. As in a split-plot analysis, the effects are computed either (A) between subjects or (B) within subjects.

A) Between subjects

Sources	MS	F	df	P
P_i	3441.9	92.9	1	.001
T_j	947.4	25.6	1	.001
PT_{ij}	245.8	6.64	1	.03

B) Within subjects

Sources	Pillai's trace	F	df	P
C_l	0.996	137.0	6, 3	.001
PC_{il}	0.972	17.3	6, 3	.02
TC_{jl}	0.872	3.4	6, 3	.17
PTC_{ijl}	0.699	1.2	6, 3	.49

mental designs typically used to determine plant growth curves (Hunt 1982) are based on repeated harvests, not repeated measures; each point defining the response curve is derived from a single individual, and the appropriate statistical analyses (Causton and Venus 1981) differ from those used to analyze an animal growth curve derived from repeated measures. In the type of true repeated-measures designs considered in this paper, the dependent variables under consideration (here photosynthetic rates at each CO_2 level) are correlated. This peculiar characteristic of the data explains why the two most widespread statistical methods in the ecological literature, namely ANOVA and MANOVA, were not included in the analysis. These techniques fail to take into account not only the correlation existing among the data but also the predictable shapes of many types of response curves. The use of ANOVA or MANOVA to analyze response curves based on repeated measures would thus be incorrect. In the following discussion we consider various aspects of only the correct analyses that are available for the analysis of ecophysiological response curves.

To allow for the repeated-measures design, the model used in ANOVAR and MANOVAR makes a crucial distinction separating the between- and the within-sub-

TABLE 7. Nonparametric split-plot analysis, equivalent to the parametric ANOVAR, using Kruskal-Wallis rank analysis to test the among-experimental-units sources of variation and the Friedman statistic for the within-experimental-unit variation.

Sources	Statistics	P
Between subjects		
P_i	$H = 8.36$	<.01
T_j	$H = 1.68$	NS
PT_{ij}	3.97	<.05
Within subjects		
C_l	$\chi^2 = 61.2$	<.001
PC_{il}	$\chi^2 = 1.29$	NS
TC_{jl}	$\chi^2 = 1.29$	NS
PTC_{ijl}	Not computed	

TABLE 8. F values for the nonlinear regression fitting Eq. 1 to the CO_2 uptake rate data.

Sources	F	df	P
Among groups	53.88	12, 68	<.001
Between Québec	3.01	4, 34	>.05
Pairwise comparisons			
Québec combined vs. Miss. chilled	70.70	8, 51	<.001
Québec combined vs. Miss. control	17.07	8, 51	<.001

ject effects. In both analyses, the between-subject effects are tested on the sum of the p variables (Morrison 1976), and in our example, as shown in Tables 4 and 6, the results from both procedures are identical. ANOVAR and MANOVAR do not attempt to account for any correlation among the dependent variables, but rather absorb any correlational structure by using the $S_{k(ij)}$ mean square (MS) as the proper error term for the between-subject effects. This allows a simple derivation of the mean squares (Winer 1971). To allow an exact test of the within-subject effects, however, ANOVAR does assume that all covariances in the pooled matrix are equivalent, and that the correlations among levels of the within-subject variables are constant over all combinations of levels. This very stringent assumption is referred to by Winer (1971:561) as "compound symmetry." A paper by Huynh and Feldt (1970) relaxed the symmetry assumption. These authors have shown that the sufficient condition for ANOVAR is the equality of variances of differences of all pairs of treatment measures that are assumed to be correlated. This assumption may be met more readily than that of compound symmetry. Greenhouse and Geisser (1959) have proposed an alternative test, G-G, which can be applied when the covariance matrices differ from group to group. Both the Huynh-Feldt (H-F) and the G-G tests allow a more general use of ANOVAR. Greenhouse and Geisser (1959) argued that ANOVAR with corrected significance level was the only solution when the ratio of p to n precluded a multivariate analysis. It has been suggested (Langlet 1985) that when an approximate test needs to be used, H-F and G-G should be compared and the hypotheses of interest accepted or rejected only if the two tests are in agreement.

Since >1 dependent variable is used to characterize a group, repeated measurement also can be analyzed by a multivariate technique. Such methods have been advised when a correlation occurs among the dependent variables. The literature can be confusing when it comes to the multivariate analysis of repeated measurements. One method is reported under two different names: MANOVAR (Cole and Grizzle 1966) and profile analysis (Morrison 1976, Johnson and Wichern 1982). The computations involved in both analyses

are identical; however, the naming of the techniques differs among disciplines and with the nature of the dependent variables. "Profile analysis" is a term generally applied in the social sciences, where no relationship is assumed between the dependent variables, nor is any necessary correspondence between treatment interventions and the dependent variables considered. In MANOVAR, by tradition, the dependent variables are taken as measures of the same individuals or items across occasions. Analysis of photosynthetic curves is an example of a typical MANOVAR problem, while profile analysis would be used to analyze answers to a questionnaire. When testing the within-subject effects, MANOVAR/profile analysis has the considerable advantage over ANOVAR of making no assumption about the form of the covariance matrix. Instead, in our example, six dependent, transformed variables, rather than the seven original photosynthetic rates, were considered; these transformed variables are the difference in carbon uptake between two adjacent CO_2 concentrations.

MANOVAR also has some inherent disadvantages. As Cole and Grizzle (1966) and later Koziol (1986) pointed out, MANOVAR is less powerful than its univariate counterpart. This is readily seen when comparing the denominator df $= [ab(n - 1) - p + 1]$ (Johnson and Wichern 1982), in MANOVAR with the df_e for the within-subject effects, df $= ab(n - 1)(r - 1)$ (Winer 1971), in ANOVAR; a and b are levels for factors A and B, n is the sample size, and p is the number of dependent variables. In our example, the df $= 2[2(3 - 1)] - 6 + 1 = 3$ for MANOVAR compared to $2[2(3 - 1)(7 - 1)] = 48$ for the within-subject effects of ANOVAR. The comparison of Tables 4 and 6 illustrates the biological implications of the lesser power of MANOVAR. While significant differences are found by ANOVAR at all levels of the within-subject effects, MANOVAR shows only a significant CO_2 effect. The inappropriate use of MANOVAR in the present case would result in a failure to detect biologically important differences among the treatment effects (Type II error). Here the failure to detect the significant TC_{jl} interaction, which indicates that the photosynthetic response to varying CO_2 levels is affected by chilling, would have weakened our interpretation of these data. This finding, which amplifies the previous results showing modification of chilling tolerance for high-CO_2-grown plants (Sionit et al. 1981, Potvin et al. 1986), warrants further investigation. It is worth noting that the incorrect use of ANOVA or MANOVA certainly would also have missed this biologically interesting interaction, since these analyses do not even provide a test for within-subject effects. When the data structure precludes ANOVAR, it should be noted that even very modest increases in replication at low levels will dramatically increase the power of MANOVAR to detect differences (Vonesh and Schork 1986).

MANOVAR also is sensitive to the ratio of p to n, which again is a problem when analyzing certain types of response curves in which the sample size is small. Comparison of the df clearly illustrates the problem: if the number of dependent variables is large compared to other terms, the df decreases significantly and, consequently reduces the power of the test. Consequently, an unfavorably low ratio of sample size to p again may lead to frequent type II error. An unfavorable balance between n and p can also lead to the untestability of the homogeneity of the variance–covariance matrices (Tabachnick and Fidell 1983), as in the present case. In a multifactor MANOVAR, when $p > N - K$, N being the total sample size and K the number of groups being compared, the error matrix E becomes singular (Greenhouse and Geisser 1959, Winer 1971, Kowal et al. 1976). By definition a singular matrix cannot be inverted. The consequences of the singularity of E are fatal to the analysis, because all the criteria in MANOVA are based on the characteristic root of HE^{-1} where E is the error matrix and H the hypothesis matrix (Morrison 1976). Therefore the criterion cannot be computed if E is singular and MANOVAR cannot be performed.

The CO_2 curves in our example thus represent a frequent problem associated with the analysis of response curves. In the present case the total number of observations, $N = 12$, is sufficient to allow the analysis ($12 - 4 = 8 > p = 6$), but the modest sample size exerts a prohibitive effect on the power of the analysis. A warning against the use of multivariate technique when the number of dependent variables is large compared to the sample size has often been issued (Koch et al. 1980, Langlet 1985, Ware 1985), and should be heeded more often in the design of ecophysiological experiments. In cases when the shape of the response curve is complex or poorly known there may be no alternative to investing more heavily in measurements defining the response of an individual (high p) than in characterizing variation in response among individuals (low n). More often, as in the present example, the investigator knows the general shape of the response a priori. An experimental design emphasizing measurements at a few thoughtfully selected cardinal points (low p) and sampling more individuals (high n) opens the way to the effective use of the MANOVAR analysis. In our example, the 21 measurements feasible for each of the four treatments were allocated to seven CO_2 levels on each of three plants. Although it would have required a break with tradition in physiological ecology, we could have achieved a more powerful analysis of treatment effects had we instead sampled seven plants at 95, 350, and 1000 $\mu L/L$ CO_2. When interest lies more on the comparison of responses across treatments than on the definition of the shape of responses, available resources are better invested in replication (n) than in too many levels (p) of the independent variable stimulating the response. This type of design, which strengthens the MANOVAR analysis, may prove crit-

ical to the variance–covariance structure if the data preclude use of the preferred ANOVAR analysis.

In the case of experiments where sample size is necessarily very small, nonparametric methods may prove excellent alternatives to the traditional parametric methods such as ANOVAR and MANOVAR. Nonparametric (NP) techniques make few assumptions about the populations of origin of the data and do not require that the data be normally distributed (Hollander and Wolfe 1973). In the case of the analysis of repeated measurements, the NP split-plot also involves no assumption about the structure of the covariance matrix, and consequently it is applicable under far more general assumptions than those associated with ANOVAR or MANOVAR. The design of an experiment for which data are to be compared by NP split-plot, however, should include a random pairing of the subjects a priori. This random pairing is necessary to allow a test of the between-subject interaction effect, and any a posteriori pairing could lead to an invalid analysis. For example, if high rates are paired with high rates, the observed difference is minimized. Conversely, if high rates are paired with low rates, any treatment effect is maximized. Random pairing a priori avoids this pitfall. Since this requirement for random pairing of the observations does not affect sample sizes in the design, it is readily accommodated, and should not be ignored. The notable price for the greater flexibility of the nonparametric model compared to ANOVAR and MANOVAR is a loss in sensitivity of the analysis (Koch et al. 1980). Nevertheless NP split-plot analysis sometimes may be the only adequate inferential technique available to analyze response curves because of logistical limitations on sample sizes.

The attitude that nonparametric methods are, at best, a second choice is a remnant of opinions prevailing in the 1960s (Noether, in Conover and Iman 1981) and is changing. For physiological ecologists often forced to deal with small sample sizes, nonparametric techniques are advantageous. It is worth noting that an analogue to the nonparametric method followed here, known as a "rank transformation" (Kepner and Robinson 1988), can be used. In the rank transformation approach, which is clearly detailed in Conover and Iman (1981), the data are analyzed by a parametric technique such as ANOVAR after being transformed to ranks. This procedure is useful when available statistical packages do not include the nonparametric Friedman or the Kruskal-Wallis tests. However, we would follow the advice given by Noether in the commentaries to Conover and Iman (1981) that, when available, these direct nonparametric tests are preferable to the rank order transformation.

The last approach to the analysis of response curves taken in this paper is qualitatively different from the previous three methods. A model was fitted to the data and subsequently used to compare the curves. This approach was inspired by the widespread use of model fitting to analyze growth curves (Zeger and Harlow 1987, Diggle 1988). Curve fitting compares advantageously to some of the methods cited above by being distribution free. Another advantage of this approach is that when an appropriate model is used the parameters of the fitted equations have a biological meaning. In our example with CO_2 response curves, we chose to use the Mitscherlich response function often used in the agriculture literature to relate a plant's response to fertilization. The biological rationale for choosing Eq. 1, $y = A[1 - e^{-k(x - x_0)}]$, is that atmospheric CO_2 concentration is indeed a "fertilizer" for photosynthesis. In the present case:

A, the asymptotic level, represents the predicted saturated rate of CO_2 uptake;

k, the slope of the linearized relationship (Eq. 2), is the rate of increase in photosynthesis with enhanced CO_2;

x_0, the x intercept, is the CO_2 compensation point. Consequently, the analysis of response curves using model fitting adds a dimension of interpretability that is missing in the strictly inferential methods. Furthermore, with available statistical packages, such as BMDP AR or SAS procedure NLIN, nonlinear models are no more difficult to fit than linear ones. Mead and Curnow (1983) pointed out that the strictly linear models involved in the analysis of variance are often inappropriate to describe biological relations. There are, however, also some difficulties inherent in this model-fitting approach. Currie (1982) presented an analysis of the effect of the design matrix, i.e., the values given to the independent variable, and of fitting techniques on the values of the estimated parameters from the Michaelis-Menten equations. His simulations indicate that both factors may alter the values of the coefficients. That his data found the regression relatively insensitive, within a certain range, to the initial values of the parameters is of only some comfort.

Once an adequate model is fitted to the data, the responses can be analyzed in terms of the parameters of the fitted function, or the regression equations can be compared. These methods represent different approaches to model fitting. Analyzing the fitted parameters amounts to reducing the curves to "cardinal" points bearing an important biological meaning (Cook and Ware 1983, Brown and Donnelly 1988). When the equations are compared, these points, rather than the coefficients, are regarded as representing the responses (Kokoska and Johnson 1987). In this paper we have chosen the second method, to reduce the potential danger associated with the absence of objective criteria for selecting the most appropriate model. The problems involved in choosing among competing models have been illustrated in the case of the predator–prey relationship (Trexler et al. 1988). When several competing models were fitted to the data, an unambiguous choice of a "best" model could never be decided. The authors suggest that the possibility of discriminating between

TABLE 9. Estimated values for the parameters when (A) Eq. 1 and (B) Eq. 7 were fitted to the data.

Population	Treatment	A	k	x_0	Residual ss
A) Eq. 1: $y = A[1 - e^{-k(x - x_0)}]$					
Québec	Control	41.47	−.0115	58.27	115.97
Québec	Chilled	39.10	−.0093	53.08	173.39
Mississippi	Control	31.28	−.0093	48.35	82.17
Mississippi	Chilled	17.78	−.0110	20.59	172.45
B) Eq. 7: $1/y = 1/A[1 + 1/-k(x - x_0)]$					
Québec	Control	44.86	−.0234	33.35	127.04
Québec	Chilled	43.35	−.0158	5.86	191.85
Mississippi	Control	34.24	−.0176	11.19	98.71
Mississippi	Chilled	18.84	−.0304	29.21	166.81

different models increases when the variance in the response is low. Brown and Donnelly (1988) also indicate that when the variation among subjects is high, analysis of fitted parameters may prove inadequate.

To illustrate better the problem of choosing the model to fit, we have fitted our data with a second model,

$$1/y = 1/A[1 + 1/-k(x - x_0)]. \qquad (7)$$

This relationship describes a rectangular hyperbola, and the biological meaning of the three parameters can be taken as the same as in Eq. 1. Eq. 7 has repeatedly been used to model the light dependence of photosynthesis (Prioul and Chartier 1977, Leverenz 1987) and is but one of the six possible models used to describe light-response curves (Thornley 1976). Since CO_2 dependence curves have a shape similar to the modelled light curves, the consideration of this alternative model is highly relevant. On the basis of the residual ss, we were unable to discriminate between the two models (Table 9). However, estimated values of the parameters were very different, and could easily lead to biologically different interpretations (Table 9). In our case, the values of the parameters were highly dependent upon the model chosen; this illustrates the potential danger in restricting the analysis to the coefficients, and emphasizes the advantage of comparing the overall curves.

Mosteller and Tukey (1977) in their text on regression indicated that regression has several purposes, amongst others: summarizing information and predictions. In the analysis of response curves, we do not

TABLE 10. A dichotomous key for selecting the preferred method for the analysis of response curves. Symbols and tests are explained in the text (see Summary and Discussion section). The qualitatively different alternative of comparing models fitted to the response curves also exists and is discussed in the text.

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1. *a.* If Mauchly's criterion is nonsignificant: use ANOVAR
   b. If Mauchly's criterion is significant, go to 2

2. *a.* If Huynh-Feldt $\epsilon \approx 1$ *and* H-F- and G-G-corrected significance levels agree: use ANOVAR, with H-F-corrected significance levels
   b. If not, go to 3

3. *a.* If $N - K > p$ and $n > p$: use MANOVAR
   b. If not, use nonparametric (NP) split-plot analysis

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attempt to use regression as a predictive tool. We want to use the equations as a good summary of the information obtained on the individuals. Therefore, we want a good fit of the data, "without regard to whether it is the best possible fit" (Mosteller and Tukey 1977). In this perspective, comparison of fitted equations becomes far more comfortable. If the fit is good, then comparing the curves should be consistent regardless of the model used. This is illustrated in the present example where the observed group effect was respectively $F = 53.88$ for Eq. 1 and $F = 50.07$ for Eq. 7.

Concluding remarks

From our experience with ecophysiological response curves and our review of the related statistical literature, we prefer a progressive scheme of alternative analyses to decide the best technique for a particular data set (Table 10). The question of which analysis should be adopted is mainly based on the form of the covariance matrix and on the number of replicates. When Mauchly's criterion supports the hypothesis of a sphericity pattern for the covariance matrices, ANOVAR is without doubt the most appropriate analysis, and should be used. If the assumptions of the ANOVAR are mildly violated, the ANOVAR with significance levels adjusted by the Greenhouse-Geisser (G-G) or Huynh-Feldt (H-F) corrections should be considered. If the conclusions based on these two corrections are contradictory, then ANOVAR should be avoided altogether. Timm (1980) argues that in many circumstances MANOVAR is preferable to a corrected ANOVAR, but when p is large compared to n, MANOVAR is weakened and should be avoided (Ware 1985). The alternative when small sample sizes are a necessity is the NP split-plot analysis (Koch 1970). In general, when going from a more structured model (such as ANOVAR) to a less structured one (MANOVAR, then NP split-plot), the power of the tests is reduced while the assumptions underlying the analyses are relaxed. Finally, as a qualitatively distinct approach to the analysis of response curves, the comparison of a model fitted to the responses has a lot to offer: the model-fitting approach is distribution free, takes into account the shape of a response, and, if done well, relates the results to biologically meaningful parameters.

We end by emphasizing the relationship between experimental design and the viable possibilities for the statistical comparison of response curves. On the one hand, it should be clear that a poorly planned or haphazard experimental design can undercut the analysis of the data. For example, the power of MANOVAR is compromised by a poor p/n ratio, and NP split-plot should only be used if random pairs have been assigned a priori. On the other hand, serious problems can inadvertently be introduced into the analysis of even a well-designed experiment. First, in determining a response curve, measurements usually are taken in rapid succession on the same individual, allowing only time to attain a quasi-equilibrium response to each level of the controlling factor. It is widely assumed that the order of measurements on an individual does not influence the shape of the resulting response curve; there is some experimental evidence that this widespread assumption holds provided assays do not extend over more than a few hours (Leverenz 1988). If there is any carry-over effect between assays, this will introduce a misleading sequential effect into the correlation matrix. When feasible, experimental protocols that assay the response to the levels of the stimulus in random order are to be preferred. Second, the analysis of response curve data will be immensely more complicated if the data are either unbalanced or incomplete (Crépeau et al. 1985, Jennrich and Schluchter 1986, Davis and Wei 1988). In physiological ecology, imbalance is most likely to occur as uneven numbers of replicates within the experimental groups. This typically might occur when some plants in an experiment die or become obviously aberrant (e.g., diseased, damaged in handling). For example, if the numbers of chilled and unchilled plants from either Mississippi or Québec in our example had not always been equal, the design would have been unbalanced. Incomplete data are most likely to occur in physiological ecology as missing points on measured response curves, perhaps through instrument malfunction or recording errors. In planning and executing experiments every effort, including allowance for extra plants and readings, should be made to avoid imbalanced or incomplete data. If either problem arises, it is likely that the analysis in available program packages will be possible (Berk 1987) but substantially more difficult. Careful attention to these considerations in designing and executing experiments will pay dividends in the ease of correctly analyzing response curves (Federer 1986).

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APPENDIX

Annotated example of commands for ANOVAR in two different statistical packages.

A. SAS procedure GLM

1) **proc GLM**; calls for the general linear model procedure.
2) **class pop tre**; indicates that the two classificaton variables are, in the present example, population and treatment.
3) **model P$_{s1}$–P$_{s7}$ = tre pop pop*tre/nouni**; specifies the model describing the data. **P$_{s1}$–P$_{s7}$** indicates that there are seven dependent variables. The between-subjects effects (see Materials and Methods: Repeated-measures Analysis of Variance) are then listed. **Nouni** prevents the computation of single ANOVAs corresponding to each of the seven dependent variables.
4) **repeated CO$_2$ 7/printe**; generates hypothesis tests for the dependent variables that are referred to as within-subject effects. In our case, the main dependent factor, CO_2, has seven levels. The **printe** command provides a sphericity test, known as Mauchly's criterion (see Results: ANOVAR). This test determines whether the probabilities associated with ordinary F tests are correct, or if approximate tests such as that of Huynh and Feldt (1970) should be considered. These statements will generate MANOVAR as well as ANOVAR.

B. BMDP 2V

1) **/ design groupings are pop tre**. Indicates the main between-subject effects.
2) **dependent are 3 to 9**. Specifies that the dependent variables appear as the 3rd to the 9th variables.
3) **level is 7. name is CO$_2$**. Generates an ANOVAR with seven levels for the repeated within-subject factor named CO_2.