EDITORIAL

The use of MIXED models in the analysis of animal experiments with repeated measures data

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Wang, Z. and Goonewardene, L. A. 2004. **The use of MIXED models in the analysis of animal experiments with repeated measures data.** Can. J. Anim. Sci. **84**: 1–11. The analysis of data containing repeated observations measured on animals (experimental unit) allocated to different treatments over time is a common design in animal science. Conventionally, repeated measures data were either analyzed as a univariate (split-plot in time) or a multivariate ANOVA (analysis of contrasts), both being handled by the General Linear Model procedure of SAS. In recent times, the mixed model has become more appealing for analyzing repeated data. The objective of this paper is to provide a background understanding of mixed model methodology in a repeated measures analysis and to use balanced steer data from a growth study to illustrate the use of PROC MIXED in the SAS system using five covariance structures. The split-plot in time approach assumes a constant variance and equal correlations (covariance) between repeated measures or compound symmetry, regardless of their proximity in time, and often these assumptions are not true. Recognizing this limitation, the analysis of contrasts was proposed. If there are missing measurements, or some of the data are measured at different times, such data were excluded resulting in inadequate data for a meaningful analysis. The mixed model uses the generalized least squares method, which is generally better than the ordinary least squares used by GLM, if the appropriate covariance structure is adopted. The presence of unequally spaced and/or missing data does not pose a problem for the mixed model. In the example analyzed, the first order ante dependence [ANTE(1)] covariance model had the lowest value for the Akaike and Schwarz's Bayesian information criteria fit statistics and is therefore the model that provided the best fit to our data. Hence, *F* values, least square estimates and standard errors based on the ANTE (1) were considered the most appropriate from among the five models demonstrated. It is recommended that the mixed model be used for the analysis of repeated measures designs in animal studies.

Key words: Repeated measures, General Linear Model, Mixed Model, split-plot, covariance structure

Wang, Z. et Goonewardene, L. A. 2004. **Recours aux modèles mixtes pour l'analyse des expériences sur les animaux avec mesures répétitives**. Can. J. Anim. Sci. **84**: 1–11. En zootechnie, on analyse couramment les données venant d'observations répétées sur des animaux (l'unité expérimentale) répartis entre divers traitements dans le temps. Par convention, les données issues de mesures répétitives sont prises soit comme variable unique (dispositif en tiroir dans le temps), soit comme variable multiple avec analyse de la variance (des valeurs contrastantes), le modèle linéaire général du SAS acceptant les deux méthodes. Depuis peu, on fait de plus en plus souvent appel au modèle mixte pour analyser les données répétitives. Le présent article donne des explications générales sur l'application de cette méthode à l'analyse des mesures répétitives et illustre la fonction PROC MIXED du SAS au moyen d'un ensemble équilibré de données sur des bouvillons tiré d'une étude de croissance pour cinq structures de la covariance. L'approche en tiroir dans le temps suppose une variance constante et des corrélations (covariances) identiques entre les mesures répétitives ou une symétrie composée, peu importe la proximité des données dans le temps. Or, ces hypothèses tiennent rarement la route. Face à cette limite, on suggère plutôt l'analyse des valeurs contrastantes. Si certaines mesures manquent ou si certaines mesures sont prises à des moments différents, on a tendance à les exclure, de sorte qu'une analyse valable devient impossible. Le modèle mixte recourt à la méthode des moindres carrés généralisés qui est souvent préférable à celle des moindres carrés ordinaires utilisée par le modèle linéaire général, quand on opte pour la bonne structure de la covariance. L'existence de données à écarts inégaux ou l'absence de certaines données ne soulèvent aucune difficulté dans le modèle mixte. Dans notre exemple, le modèle de covariance du premier degré avant dépendance [ANTE (1)] donne la valeur la plus faible pour les statistiques

Starting with Vol. 84 (2004), the Canadian Journal of Animal Science will not normally accept papers reporting the use of the GLM procedure to analyze data-sets that include random effects or repeated measurements on the same experimental unit where the data show heterogeneous variances and/or unequal within-subject time-dependent correlations. This Editorial paper addresses the reasoning behind this descision. For further details see the Operations Manual (http://pubs.nrc-cnrc.gc.ca/aic-journals/apssubmit.html).

Abbreviations: **AIC**, Akaike information criterion; **ANOVA**, analysis of variance; **ANTE(1)**, first order ante dependence; **AR(1)**, first order autoregressive; **BIC**, Bayesian information criterion; **BW**, body weight; **CS**, compound symmetry; **GG**, Greenhouse Geiser; **GLM**, general linear model; **GLS**, generalized least squares; **FNWT**, final body weight; **HF**, Huynh-Feldt; **INWT**, initial body weight; **MANOVA**, multivariate analysis of variance; **OLS**, ordinary least squares; **SAS**, Statistical Analysis System; **UN**, unstructured

ajustées aux critères d'information bayésiens d'Akaike et Schwarz. Il procure donc le meilleur ajustement. On estime que les valeurs *F*, la valeur estimative des moindres carrés et l'erreur-type reposant sur le modèle ANTE (1) sont celles qui conviennent le mieux parmi les cinq modèles examinés. Les auteurs préconisent le modèle mixte pour analyser les mesures répétitives dans les études sur les animaux.

Mots clés: Mesures répétitives, modèle linéaire général, modèle mixte, dispositif en tiroir, structure de la covariance

The repeated measures experiment is a common design in animal science research (Goonewardene et al. 2000; ZoBell et al. 2003), and the analysis refers to multiple measurements made on the same experimental unit, observed either over time or space. In repeated measures designs, the usual practice is to apply treatments to experimental units in a completely randomized design and measurements are made sequentially over time. With this type of experimental design, there are basically two fixed effects (treatment and time) and two sources of random variation (between and within animals). Some of the more common designs in animal sciences include repeated measurements of such things as weight, gain, blood parameters, and products of metabolism and digestibility of nutrients. Such measurements are commonly taken on subjects which have been randomly allocated to fixed treatment effects such as feeds, drugs, hormones, etc., with pens or blocks considered as random effects in the design (Silvia et al. 1995; Wells and Preston 1998; Goonewardene et al. 2000; Platter et al. 2003). Sometimes a repeated measures analysis can be combined with a Latin square design and analyzed as a split-plot with multiple error terms (SAS/STAT 1990; Yandell 1997).

Often, measurements made on the same animal are more likely to be correlated than two measurements taken on different animals, and two measurements taken closer in time on the same animal are likely to be more correlated than measurements taken further apart in time. The basic objectives for repeated measures data are to examine simple factor effects (main effects) and the interaction effects between them. The distinguishing characteristic of the repeated measurements analysis model from other models is the assumption about the error variance and covariance structure (Wolfinger 1996; Littell et al. 1996; 1998; Templeman et al. 2002). With the repeated model, the usual assumptions about error variances being independent and homogeneous are no longer valid (Wolfinger 1996; Littell et al. 2000; SAS Institute, Inc. 2002). The analysis of repeated measures data therefore requires an appropriate accounting for correlations between the observations made on the same subject and possible heterogeneous variances among observations on the same subject over time.

Objective and Focus
A scan of Volume 82 (2002) of the Canadian Journal of Animal Science shows that repeated measures analyses have been used in 25 papers. Many authors used the GLM procedure in SAS to analyze their data although some used the MIXED model with a repeated statement. While the GLM procedure may have been appropriate to use then, the MIXED procedure of SAS is a more robust and flexible procedure for the analysis of such data (Cnaan et al. 1997; SAS Institute, Inc.1997; Littell et al. 1998; Templeman et al. 2002). However, it appears that unfa-miliarity with mixed model methodology and PROC MIXED limits its use. The objective of this paper is to provide a minimal background understanding of the mixed model in repeated measures analysis in animal science by using steer data from a growth study, and to demonstrate the use of PROC MIXED of the SAS system. The focus of this paper is on how to use PROC MIXED to analyze balanced repeated measures data from the user's point of view. The use of PROC MIXED to analyze unbalanced repeated measures data will be discussed in another paper. Detailed theoretical treatments and descriptions about mixed model methodology are provided by other authors (Wolfinger 1996; Cnaan et al. 1997; SAS Institute, Inc.1997; Littell et al. 2000). The general approach discussed in this paper is divided into two parts; the first part attempts to estimate the variance and covariance, the second part attempts to substitute variance and covariance estimates into the mixed model and use generalized least squares methodology to assess significance for fixed effects.

Data for Illustration
Data from a steer growth trial published by Mir et al. (1998) are used to demonstrate mixed model methodology and analyze repeated measures data. In this experiment, six diets (1–6) were randomly assigned to 60 steers, with 10 steers per diet, over 105 d. Body weights were measured every 21 d (3 wk) at 0 (INWT = 237 ± 17 kg), 21, 42, 63, 84 and 105 d (end). These will be referred to as time 0, 1, 2, 3, 4, and 5 throughout this paper. These data were analyzed by using PROC MIXED with start body weight measured at 0-d as a covariate, and the subsequent weights as repeated measures in the analyses. Five commonly used covariance models, SIMPLE, CS, AR(1), ANTE(1) and UN, were fitted to the steer growth data. However, there are many covariance structures that are available in the MIXED procedure, and a list of the available covariance structures can be found on the SAS online documentation for Version 8.1.

STATISTICAL METHODOLOGY
The approaches used in repeated measures analyses can be categorized as conventional and modern. The conventional approach uses PROC GLM in the SAS system and the analyses are done using either a univariate analysis of variance (ANOVA) or a multivariate analysis of variance (MANOVA), and are often referred as the split-plot in time and the analysis of contrasts respectively (Littell et al. 1991; Wolfinger 1992). The mixed model approach uses mixed model methodology or PROC MIXED in the SAS system. The advantages, limitations, strengths and weaknesses of conventional analyses as well as the mixed model method are summarized in Fig. 1. Some of these attributes will be discussed further in subsequent sections.
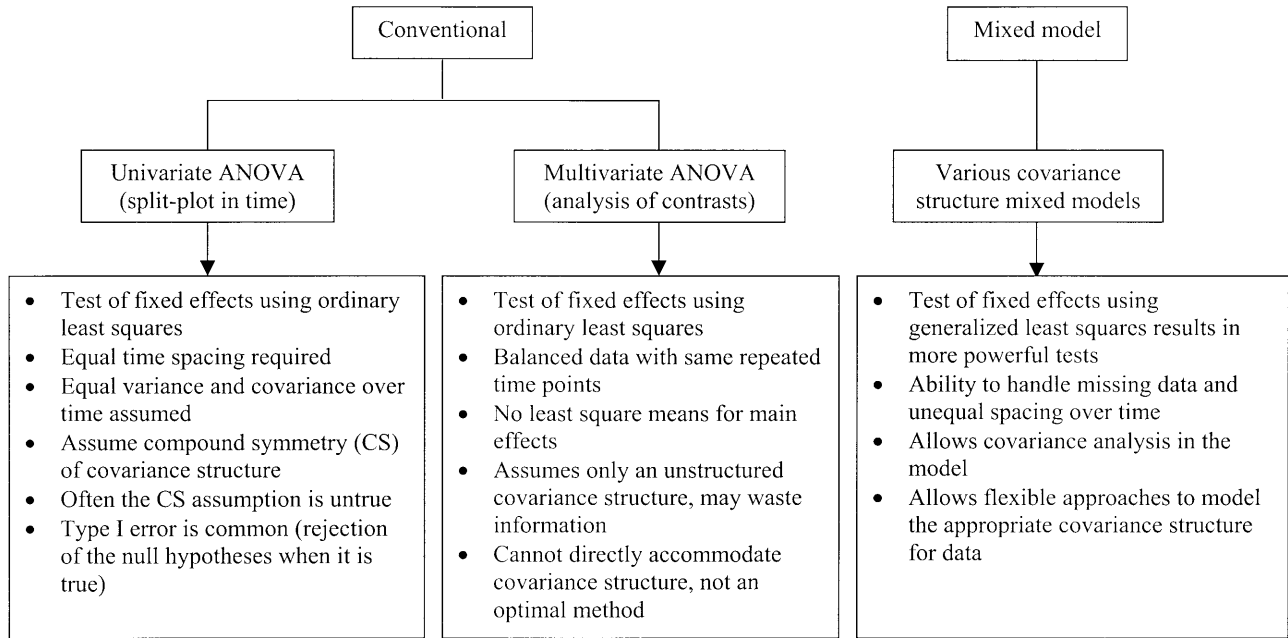
Fig. 1. A summary of the advantages and limitations of the conventional and mixed model approaches for the analysis of repeated data.

Conventional Approaches

Univariate Analysis of Variance

The univariate analysis of variance of the repeated measures data can be performed using PROC GLM with the following SAS code. Assume that the repeated data set was named STEERS associated with the libname IN. The first 10 observations of STEERS are provided in Table 1. There are many ways to bring data sets into the SAS system from other database applications for statistical analysis and details can either be found in the SAS online documentation or other sources (Cody and Smith 1991; Littell et al. 1998).

```
PROC GLM DATA=IN.STEERS;
CLASS DIET TIME ANIM;
MODEL FNWT=DIET ANIM(DIET) TIME DIET*TIME;
RANDOM ANIM(DIET)/TEST;
RUN;
QUIT;
```

This type of analysis has been used commonly in animal research experiments for many years (Goonewardene 1990; Milliken and Johnson 1992). The analysis basically treats the repeated measures data as split-plot design, where the experimental units regarding treatments are considered as the whole-plot units, while the experimental units at a specific time are considered as the sub-plot or split-plot units. It is worth noting that the animal assigned to a diet is specified as a RANDOM effect in the above code in order to carry out a correct *F*-test. The /TEST option in the RANDOM statement performs hypothesis tests for the effects specified in the model statement with appropriate error terms as determined by the expected mean squares. The statistical model used for this analysis is defined in Eq. 1:

Table 1. The first 10 observations of the example data set STEERS

DIET	ANIM	TIME	INWT[z]	FNWT[y]
4	61	1	266	285
4	61	2	266	313
4	61	3	266	330
4	61	4	266	344
4	61	5	266	372
1	62	1	212	235
1	62	2	212	251
1	62	3	212	260
1	62	4	212	272
1	62	5	212	282

[z]Initial body weight at the beginning of the trial.
[y]Final body weight at the end of the trial.

$$y_{ijt} = \mu + \alpha_i + d_{j(i)} + \gamma_t + (\alpha\gamma)_{it} + e_{ijt} \qquad (1)$$

where:

y_{ijt} is the live body weight measured at time *t* on the *j*th steer assigned to the *i*th diet,

μ is the overall mean effect,

α_i is the *i*th fixed diet effect,

$d_{j(i)}$ is the random effect of the *j*th steer within the *i*th diet, $d_{j(i)} \sim NID(0, \sigma_d^2)$ (approximately normally independently distributed with mean of 0 and variance of σ_d^2),

γ^t is the fixed *t*th time effect when the measurement was taken,

$(\gamma\alpha)_{it}$ is the fixed interaction effect between diet and time,

e_{ijt} is the random error associated with the *j*th steer assigned to the *i*th diet at time *t*, $e_{ijt} \sim NID(0, \sigma_e^2)$ (approximately normally independently distributed with mean of 0 and variance of σ_e^2).

Assuming $d_{j(i)}$ and e_{ijt} are independent.

$$E(y_{ijt}) = \mu + \alpha_i + \gamma_t + (\alpha\gamma)_{it}$$

Table 2. The first eight observations of the example data set MULT

ANIM	DIET	BW1[z]	BW2[z]	BW3[z]	BW4[z]	BW5[z]	BW6[z]
62	1	212	235	251	260	272	282
63	1	227	247	272	286	293	311
65	1	255	277	290	307	317	339
69	2	231	243	256	277	291	302
74	2	221	242	248	266	278	294
86	2	259	277	295	314	325	360
71	3	257	282	318	342	360	384
75	3	239	264	276	291	300	319

[z]BW1–BW6 are the body weight measurements (kg) corresponding to day 0, day 21 … day 105.

$$Var\ (y_{ijt}) = Var\ (d_{j(i)} + e_{ijt}) = \sigma_d^2 + \sigma_e^2\ Cov\ (y_{ijr}, y_{ijk}) =$$
$$Cov\ (d_{j(i)} + e_{ijr}, d_{j(i)} + e_{ijk}) = \sigma_d^2 .... for.. t \neq k \quad (2)$$

From Eq. 2 we know that measurements observed at each time have a constant variance of $(\sigma_d^2 + \sigma_e^2)$, the covariance between two observations made at different times on the same subject is the same for all pairs of measurements σ_d^2 regardless of their proximity in time. These assumptions are most likely not true in many animal experiments because measurements made on the same animal are likely to be more correlated than two measurements taken on different animals. Furthermore, two measures taken closer in time on the same animal are likely to be more correlated than measures taken far apart in time. However, when these assumptions hold, the univariate ANOVA method (split-plot in time) is valid for analysis of repeated data. The PROC GLM is a fixed effect procedure and it cannot directly accommodate the random animal effect in the model although a RANDOM statement was used in the code to specify the animal within a diet (Littell et al. 1998; Templeman et al. 2002). This RANDOM statement was used to carry out a correct *F*-test provided the model assumptions are met. If the model assumptions are wrong, then the RANDOM statement provides no help for the *F*-test.

Multivariate Analysis of Variance

The multivariate analysis of variance is used where the assumptions imposed by the univariate ANOVA (measures at each time have equal variances and the correlations between any two measures are the same) do not hold. When this occurs, the MANOVA with a repeated statement in PROC GLM can be used. The analysis also allows a covariate (BW1) to be used in the MODEL statement. The following SAS code performs the multivariate analysis, assuming the data set was named MULT associated with libname IN. The appropriate format for the data is provided in Table 2.

```
PROC GLM DATA=IN.MULT;
CLASS DIET;
MODEL BW2-BW6=DIET BW1/SS3;
REPEATED TIME/PRINTE SUMMARY;
RUN;
QUIT;
```

The common feature of using the repeated statement is that it applies to differences between measures on the same sub-ject. Therefore, the repeated statement in this MANOVA allows one to examine trends over time and it can produce many meaningful statistics that are essentially univariate tests. The MANOVA analysis requires balanced data with the same repeated time points for all subjects. If one repeated measure is missing from a subject, then all the data for this subject will be eliminated from the analysis. For unbalanced data, this procedure may not be suitable to arrive at a meaningful analysis. The MANOVA is a method that avoids the covariance problems raised in repeated measures analyses. However, the method cannot directly accommodate the covariance structure as in the mixed model. In fact, it is based on an unstructured within-subject covariance matrix and therefore, it is not an optimal method (SAS Institute, Inc. 2002).

Comparison of the ANOVA and MANOVA Methods

The ANOVA method ignores the time-dependent correlations in the repeated measures data. Therefore, risks exist in using incorrect standard errors for the comparison of means at different times (Littell et al. 1998; Templeman et al. 2002). The inappropriate choice of standard errors for mean comparisons can result in an excessive Type I statistical error. Type I error is the rejection of the null hypothesis when it is true. Although the Greenhouse-Geiser (GG) and Huynh-Feldt (HF) adjustments can be applied in an attempt to account for within-subject time-dependent correlations by adjusting the denominator degrees of freedom in the MANOVA analysis, these corrections are often inadequate (SAS Institute, Inc. 2002). The MANOVA method also assumes an unstructured covariance matrix to overcome the above-mentioned problems, which is far more general than most repeated measures data require. Therefore, it wastes a great amount of information inherent in repeated measures data and results in a less powerful test (SAS Institute, Inc. 2002). More importantly, the MANOVA method cannot handle missing measures on subjects.

Mixed Model Approach

The mixed model procedure (PROC MIXED) allows a greater flexibility in modeling covariance structures for repeated measures data, and adequately accounts for the within-subject time-dependent correlations (Littell et al. 1998; Templeman et al. 2002). It also has better capabilities to handle missing observations in repeated measures

data than the MANOVA. Therefore, the mixed model approach is considered superior to the conventional approaches. In addition, with an appropriate covariance structure specified, the MIXED procedure uses GLS to estimate and test the fixed effects in the model, which is considered superior to the OLS method used by the GLM procedure (SAS Institute, Inc. 1999). The GLS procedure can account for all of the covariance parameters modeled for the data whereas OLS cannot. Because of this, the mixed model analysis is more precise and therefore recommended, although assessing an appropriate covariance structure for the data is not easy.

Statistical Model for the Example Data
The statistical model for the mixed model analysis in the example data set can be defined as:

$$y_{ijt} = \mu + \alpha_i + d_{j(i)} + \gamma_t + (\alpha\gamma)_{it} (b + \varphi_j) x_{ij} + e_{ijt} \qquad (3)$$

where:
b is the common regression coefficient of initial weight of x_{ij},
φ_i is the slope deviation of the ith diet from the common slope b,
x_{ij} is the initial body weight measure of steer j on diet i at the beginning of the study and the remaining terms are the same as in Eq. 1.

$$E(y_{it}) = \mu + \alpha_i + \gamma_t + (\alpha\gamma)_{it} + (b + \varphi_j) x_{ij} \qquad (4)$$
$$Cov(y_{ijt}, y_{i'j't'}) = V_{ij}$$

where, V_{ij} is a block diagonal covariance matrix for each subject j with diet i. The V_{ij} can take many different forms depending on the nature of the repeated measure data.

Commonly Used Covariance Structures for the Repeated Measures Model

Mixed Model Analysis with a SIMPLE Covariance Structure
The SIMPLE covariance structure assumes that all observations are independent of each other and there is no correlation (covariance) between any pair of observations, even between the repeated measures on the same subject. The SIMPLE structure has an equal variance σ^2 on the main diagonal and 0 elsewhere in the covariance matrix and is expressed as:

$$V_{ij} = \begin{bmatrix} \sigma^2 & 0 & \cdots & 0 \\ & \sigma^2 & \cdots & 0 \\ & & \ddots & \vdots \\ Symmetric & & & \sigma^2 \end{bmatrix} \qquad (5)$$

This structure is considered simple because only a single parameter estimate is required. This is the covariance structure assumed in the standard fixed model analysis of variance. However, the SIMPLE covariance structure is seldom true with repeated measures data.

Mixed Model Analysis with CS Covariance Structure
The compound symmetry refers to equal variances ($\sigma_d^2 + \sigma_e^2$) on the main diagonal and equal covariances (σ_d) on all off diagonals. This structure is the simplest correlated covariance structure for repeated measures data since it assumes a constant correlation between observations regardless of the distance between time points. This is essentially the same as the univariate ANOVA (split-plot in time) analysis, which was used for many years in the past. The CS structure requires two parameters, the between subject (σ_d^2) and the within-subject (σ_e^2) variance estimates. The covariance matrix is expressed as:

$$V_{ij} = \begin{bmatrix} \sigma_d^2 + \sigma_e^2 & \sigma_d & \cdots & \sigma_d \\ & \sigma_d^2 + \sigma_e^2 & \cdots & \sigma_d \\ & & \ddots & \vdots \\ Symmetric & & & \sigma_d^2 + \sigma_e^2 \end{bmatrix} \qquad (6)$$

The CS covariance structure is only appropriate when the so-called Huynh-Feldt condition is met, that is equal correlation between measures on the same subject (Huynh and Feldt 1970, 1976). However, the HF condition is also seldom true with repeated measures because the time-dependent correlations most likely exist with repeated measures data. If the HF condition is not met, other covariance structures should be pursued.

Mixed Model Analysis with AR(1) Covariance Structure
The first-order autoregressive covariance structure assumes the correlation between adjacent measures is ρ, regardless of the order of the adjacent pairs such as 1st and 2nd, 2nd and 3rd, and so on. It also assumes that the correlation for any pair of observations that are measured n units apart have a correlation of ρ^n. The correlation between observations is a function of distance in time. In addition, it assumes equal variances σ^2 on the main diagonal and the variance times the corresponding correlations on the off diagonals of covariance matrix, and is expressed as:

$$V_{ij} = \sigma^2 \begin{bmatrix} 1 & \rho & \rho^2 & \rho^3 \\ & 1 & \rho & \rho^2 \\ & & 1 & \rho \\ Symmetric & & & 1 \end{bmatrix} \qquad (7)$$

In the AR(1) structure, since the correlations are increasing in power as distances increase between pairs of observations, the corresponding covariances decrease. The AR(1) structure requires equally spaced times, and time must be ordered correctly and the structure needs only two parameter estimates. If unequally spaced time points are present, one should consider other covariance structures such as SP(POW).

Mixed Model Analysis with ANTE(1) Covariance Structure
The first-order ante dependence covariance structure allows unequal variances over time and unequal correlations and

covariance among different pairs of measurements. The magnitude of the covariance depends on the values of both the correlations and standard deviations associated with them. The covariance matrix is expressed as:

$$V_{ij} = \begin{bmatrix} \sigma_1^2 & \sigma_1\sigma_2\rho_1 & \sigma_1\sigma_3\rho_1\rho_2 & \sigma_1\sigma_4\rho_1\rho_2\rho_3 \\ & \sigma_2^2 & \sigma_2\sigma_3\rho_2 & \sigma_2\sigma_4\rho_2\rho_3 \\ & & \sigma_3^2 & \sigma_3\sigma_4\rho_3 \\ Symmetric & & & \sigma_4^2 \end{bmatrix} \quad (8)$$

This structure requires $t + (t - 1)$ parameter estimates where t is the number of times repeated. With this structure, time periods must be ordered correctly but equal spacing between times is not necessary.

Mixed Model Analysis with UN Structure

The unstructured covariance structure allows unequal variances over time and unequal covariances for each time combination. This is the most complex structure and $t(t - 1)/2$ parameters need to be estimated where t is the same as defined in Eq. 8, and expressed as:

$$V_{ij} = \begin{bmatrix} \sigma_1^2 & \sigma_{12} & \sigma_{13} & \sigma_{14} \\ & \sigma_2^2 & \sigma_{23} & \sigma_{24} \\ & & \sigma_3^2 & \sigma_{34} \\ Symmetric & & & \sigma_4^2 \end{bmatrix} \quad (9)$$

This covariance structure is modeled by the MANOVA repeated measures analysis. Because of the complexity of the UN structure, it is generally more difficult to fit. A smaller data set with many repeated observations on one subject may fail to converge. As the UN structure estimates many parameters (over parameterization), it may waste a considerable amount of information in the data and result in less powerful tests.

MODEL DEVELOPMENT

Littell et al. (2000) recommended the following steps for selecting an appropriate covariance structure in the analysis of repeated measures data using PROC MIXED:

(a) Model the mean structure (the model expectation) by specifying the fixed effects in the model to fit the repeated measures data. At this step, try to fit all possible fixed effects in the mean model, test for the significance and then reduce the model to obtain a desirable mean model using PROC GLM.

(b) Specify a closer covariance structure for between and within-subject effects. Usually, the covariance for the within-subject measurements can be complex for repeated measures data, but generally assumes that the repeated measures within subjects are correlated and between subjects are independent. Therefore, most of the time, the within-subject covariance is a block diagonal matrix with one block per subject. Then specify an appropriate covariance structure in each block. The guidelines on how to specify an appropriate

covariance structure are discussed in the next section. The details of available covariance structures can be found in SAS help Version 8 under PROC MIXED: REPEATED Statement – TYPE = option.

(c) Use the covariance structure identified in step b and fit the mean model using the MIXED procedure in SAS. This accounts for the covariance in the data, which the preceding GLM analysis in step a did not. In this step, one may be able to further reduce the mean model to obtain a more parsimonious model based on the tests for the fixed effects and the model fit statistics information criteria. The model fit statistics will be discussed subsequently.

(d) Based on the results obtained in step c, make statistical inferences and draw conclusions about the analysis based on the objectives of the study.

Specification of an Appropriate Covariance Structure

A model with an appropriate covariance structure for the within-subject correlation is essential to arrive at an accurate conclusion in a repeated measures analysis. Ignoring the important within-subject correlation by using a model that is too simple will increase the Type I error rate for fixed effect tests in the analysis, while too complicated a model will lead to a sacrifice in test power and the efficiency of tests for the fixed effects. It has been shown that inference in the repeated measures analysis can be severely compromised by a poor choice of covariance models (Wolfinger 1996; Guerin et al. 2000). Although the true covariance structure for a particular dataset is seldom known, an approximately correct covariance model must be specified in order to obtain a valid analysis.

The first thing one should do is to exclude the covariance structures that clearly make no sense to the data before starting to select a covariance structure. In general, the SIMPLE covariance structure may not be an appropriate choice for repeated measures data; equal time spacing covariance structures, such as AR(1), should not be considered for unequally time-spaced data; homogeneous covariance structures, such as AR(1), should be ruled out if the data show heterogeneous variances over time. After ruling out some of covariance structures that clearly make no sense to the data, these steps should be followed to select an appropriate covariance structure for the data.

(a) Try to run the UN covariance structure first to examine the pattern of the covariance matrix of the data. The pattern often suggests a simpler covariance structure that may fit the data better. The UN is the most complex structure and it may fail to converge for smaller data sets with many repeated measures on a single subject. When the UN model fails to converge, then analyze the data using the MANOVA procedure and then look at the correlation matrix provided by the MANOVA analysis. The pattern of the correlation matrix could provide a hint for a simpler more appropriate covariance structure that fits the data.

(b) Based on the pattern obtained from the above analysis, the biology of the study and experimental knowledge about

your data, a few covariance structures that appear to be reasonable for the data may be tried.

(c) Compare the model fitting statistics information from the above runs of different models, and then select the model that best fits the data. In general, a simpler covariance structure that best fits to the data should be chosen.

Model Comparison

A comparison of candidate models can be achieved by running the PROC MIXED procedure with various covariance structures. The information criteria provided by PROC MIXED can be used as a statistical tool to assist in model selection. Three information criteria are provided in SAS version 8.1 and they are the Akaike Information Criteria (AIC), the finite-sample corrected Akaike Information Criteria (AICC) and the Schwarz's Bayesian Information Criteria (BIC). The value of information criteria closest to zero indicates a better model fit to the data (SAS Institute, Inc. 1999). As its name implies, AICC is a finite-sample corrected AIC for small samples, and it reduces the bias that results from AIC. With large samples, the AICC converges to the AIC. In general, AICC is preferred to AIC. The BIC tends to choose a simpler model than AIC because it increases the penalties, as the number of parameters required in the model increases. As suggested above, when the model becomes too simple, it tends to inflate the Type I error rate. Therefore, when Type I error control is critical in the study, AICC should be used. On the other hand, if a test power is the major concern, then BIC may be preferable. In general, for repeated measure analysis, among plausible within-subject covariance models for a particular study, the model that minimizes either AICC or BIC is preferable. When AICC and BIC values are close, then the simpler covariance model is generally preferred.

ANALYZING THE EXAMPLE DATA USING THE MIXED MODEL

The Data Profiles

The individual body weights were plotted against time, and for clarity, data from only 17 of the steers are shown in Fig. 2. A close examination of Fig. 2 reveals (a) a fanning shape over time, which provides evidence that variation in body weight is increasing as weight increases. As such a constant variance over time is no longer a valid assumption, and heterogeneous variance is the reality, (b) within the study period, as each steer's growth profile approximates a linear relationship over time, it indicates that a linear model is likely to be appropriate, and (c) larger or smaller steers tend to remain larger or smaller during the entire trial, and this indicates a clear subject-to-subject variability in steer growth over time. Also, measures on the same animal are likely to be more closely correlated than measures taken from different animals. Two measures taken on the same individual are positively correlated because they possess common effects from the same animal. Hence, a linear mixed model with an appropriate covariance structure can be expected to accommodate these features exhibited by the data.

The diet means for body weight over the trial period are shown in Fig 3. The diet means for body weights are similar at the start of the study, but the magnitude of differences among the diets begins to differentiate over time. For example, the magnitude of the difference between diets 1 and 3 is small at the beginning (day 0) but large at the end (day 105), whereas, between 1 and 4, the largest difference appears at time 2 and the difference at time 5 and 6 is virtually absent. Figure 3 indicates that a general conclusion about diet means cannot be made independent of time. In order to make a comparison of the diets, appropriate estimates of the differences between treatment means at different times and of the differences between means for the same treatment at different times would be interesting and should therefore be considered.

Examination of the Covariance Structure

A preliminary analysis showed that the within diet, regression of INWT on FNWT was not significant ($P > 0.05$), therefore a common slope for diets was used in the subsequent analysis. Table 3 was obtained by fitting Eq. 3 with a UN covariance structure to the example data. The variances are on the diagonal, correlations and covariances between repeated measures are shown above and below the diagonal, respectively (Table 3). It appears that the variances increase over time (heterogeneous) and the correlations within the same subject decrease over time. It can also be seen that covariances or correlations between adjacent measurements on the same steer are more correlated at later time periods than at earlier times. For example, the covariance and correlation between adjacent measurements of time 4 and 5 are 162.87 and 0.92, respectively, which are much larger than their corresponding covariance and correlation between adjacent measurements of 30.02 and 0.58 at times 1 and 2. Therefore, the conventional ANOVA method of analysis for repeated data is not justified. Hence, a mixed linear model with heterogeneous and correlated covariance structure should be considered for these data.

Fitting Different Covariance Models

In order to illustrate the advantages of using mixed model analysis over the conventional approaches, the same data set was fitted to five different covariance structures [SIMPLE, CS, AR(1), ANTE(1) and UN]. The SAS code given below is for PROC MIXED with a simple covariance structure. The SIMPLE covariance structure is the default option for TYPE = options in the MIXED procedure. Also, SIMPLE is the covariance structure assumed in the standard fixed model ANOVA. Other covariance models [CS, AR(1), ANTE(1) and UN] can be invoked by simply replacing the SIMPLE in the 'TYPE =' options with CS, AR(1) ANTE(1), or UN covariance structures. To use the covariance structures available in the SAS system other than those listed above, one just needs to replace the SIMPLE in the 'TYPE =' option with your choice of 31 available covariance structures that can be found in the online documentation of SAS version 8.1.

```
/* MIXED MODEL APPROACH - ANALYSIS THE STEER
GROWTH DATA */
PROC MIXED DATA=IN STEERS COVTEST;
```
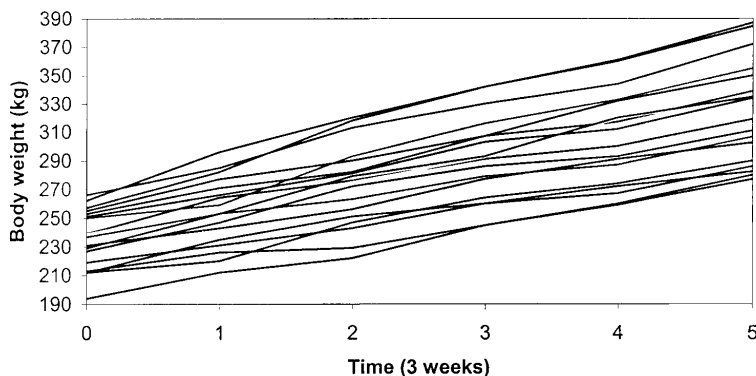
Fig. 2. Tri-weekly body weight profiles of 17 steers during a 105-d growth study. (Only part of the data are shown.)
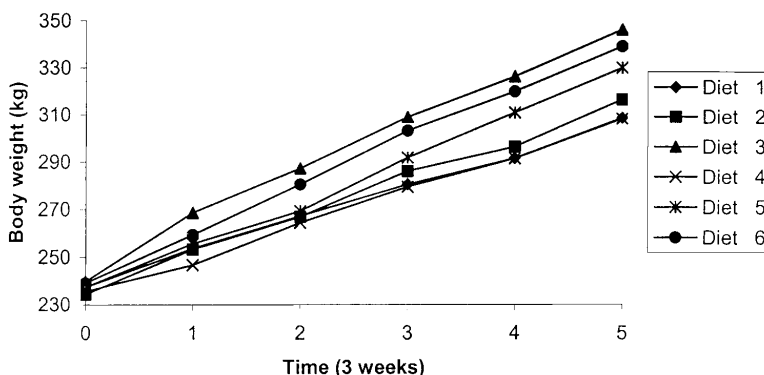

Fig. 3. Tri-weekly average body weight profiles of steers on six diets during a 105-d growth trial.

Table 3. The covariance and correlation matrix[z] obtained by fitting the UN structure for the example data set STEERS

TIME	1	2	3	4	5
1	**34.30**	0.58	0.53	0.49	0.38
2	30.02	**77.27**	0.86	0.81	0.75
3	34.06	82.41	**120.15**	0.90	0.85
4	34.90	86.14	118.72	**146.36**	0.92
5	32.67	95.66	136.40	162.87	**213.19**

[z]Variances along the diagonal, covariances are in the lower triangle and correlations are in the upper triangle.

```
CLASS ANIM DIET TIME;
MODEL FNWT = DIET | TIME INWT / DDFM=KR;
REPEATED TIME / SUBJECT=ANIM (DIET) TYPE = SIMPLE
R RCORR;
 RUN;
QUIT;
```

The fit statistics for the five models are presented in Table 4. The AICC and BIC values for the ANTE(1) covariance model are both smaller than the corresponding AICC and BIC for the rest of the covariance models. We can conclude that the ANTE(1) covariance model provides the best fit to the data among the five models selected and is therefore the model of choice.

Test of the Fixed Effects for the Five-covariance Models

One may choose to request tests for fixed effects and specific interactions. In this example, DIET, TIME and interac-

Table 4. Model fit statistics[z] with five different covariance structures for the example data set STEERS

Fit statistics[x]	Covariance structures[y]				
	SIMPLE	CS	AR(1)	ANTE(1)	UN
-2 Log likelihood	2103.7	1940.4	1852.6	**1798.4**	1791.0
AIC	2105.7	1944.4	1856.6	**1816.4**	1821.0
AICC	2105.7	1944.4	1856.6	**1817.1**	1822.9
BIC	2107.8	1948.6	1860.8	**1835.2**	1852.5

[z]A smaller model fit statistic value indicates a better fit to the data.
[y]SIMPLE = simple, CS = compound symmetry, AR(1) = first order autoregressive, ANTE(1) = ante dependence, UN = unstructured.
[x]AIC = Akaike information criterion, AICC = finite sample corrected Akaike information criterion, BIC = Schwarz's Bayesian information criterion.

tion of DIET × TIME were requested. Table 5 presents the value of F-tests of the fixed effects computed by the MIXED procedure for the five-selected covariance models. The values of the F-test are similar for the ANTE(1) and UN models, which happen to fit the data better than SIMPLE, CS and AR(1) (Table 4). On the other hand, the F values differ substantially for SIMPLE, CS and AR(1) models, which did not provide a good fit to the data. The failure of SIMPLE model to recognize between steer variations resulted in excessively large F values for DIET and INWT, and these are both animal effects. The CS model gives essentially the same result that would be obtained with the univariate ANOVA. This model produces excessively large F values

Table 5. *F* test of fixed effects for five covariance structures[z] for the example data set STEERS

Effect	SIMPLE	CS	AR(1)	ANTE(1)	UN
DIET	**43.9****	11.91**	11.2**	11.37**	11.34**
TIME	402.91**	**1181.76****	**604.96****	399.79**	417.98**
DIET*TIME	1.88 *	**5.51****	3.25**	2.91**	3.22**
INWT	**1174.12****	318.54**	317.97**	477.5**	444.54**

[z]SIMPLE = simple, CS = compound symmetry, AR(1) = first order autoregressive, ANTE(1) = ante dependence, UN = unstructured.
* *F*-test significant at the 0.05 level.
** *F*-test significant at the 0.01 level.

Table 6. Least square mean estimates (EST) and standard errors (SE) of six diets from five covariance models[z] for the example data set STEERS

	SIMPLE		CS		AR(1)		ANTE(1)		UN	
Diet	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE
1	280.23	1.4706	280.23	2.8235	280.23	2.9076	280.23	2.9824	280.23	2.9792
2	287.23	1.4741	287.23	2.8302	287.26	2.9142	286.68	2.9853	286.73	2.9823
3	304.33	1.4733	304.33	2.8286	304.31	2.9126	304.81	2.9846	304.77	2.9816
4	279.88	1.4717	279.88	2.8255	279.89	2.9096	279.57	2.9833	279.6	2.9802
5	291.29	1.4706	291.29	2.8235	291.29	2.9076	291.31	2.9825	291.31	2.9792
6	298.09	1.4720	298.09	2.8261	298.08	2.9102	298.44	2.9836	298.41	2.9805

[z]SIMPLE = simple, CS = compound symmetry, AR(1) = first order autoregressive, ANTE(1) = ante dependence, UN = unstructured. All estimates (EST) are significant at the 0.01 level.

for TIME and DIET by TIME interaction effects. This is a common problem when applying the CS model to analyze repeated measures data, as the HF condition is not met. The *F* value for TIME that results from AR(1) model is large due to the fact that AR(1) model failed to account for the correlations between measures observed on the same animal further apart in time and the heterogeneous variances over time. The *F* values based on the ANTE(1) and UN models are similar because both models are adequately modeling the covariance of the data and therefore result in valid tests. However, the ANTE(1) has the lowest AICC and BIC fit statistics and hence is the model of choice from among the five models tested.

Effect of Covariance Structures on Least Square Means for Fixed Effects

Least square means can be obtained in the same way as in the PROC GLM procedure by adding the following LSMEANS statement in the PROC MIXED code as shown previously. The /PDIFF in the LSMEANS statement invokes SAS to provide the difference between all pairs of means and the standard error of the difference.

```
LSMEANS DIET DIET*TIME/PDIFF;
```

Table 6 provides least square mean estimates for the six diets for the five covariance structures. Least square means are similar for each diet regardless of the covariance structure, because the example data are balanced. With unbalanced data, the least square mean estimates would be different (Cnaan et al. 1997). The standard errors of the least square estimates differ for the different covariance models because they are adjusted for the covariance parameters in the mixed model (Littell et al. 1998). However, the least square means are the same in GLM and MIXED as they are both calculated in the same way.

Effect of the Covariance Structures on Linear Combinations of Fixed Effects

Let us now examine the different covariance structures on comparisons for diet 1 and diet 3 at five different time periods as shown in Table 7. The values in Table 7 can be obtained by adding the ESTIMATE statement with appropriate linear contrasts in the SAS code as follows:

```
ESTIMATE 'D1-D3 at T1' DIET 1 0 -1 0 0 0 DIET*TIME
1 0 0 0 0 0 0 0 0 0 0 -1 0 0 0 0;
ESTIMATE 'D1-D3 at T2' DIET 1 0 -1 0 0 0 DIET*TIME
0 1 0 0 0 0 0 0 0 0 0 0 -1 0 0 0;
ESTIMATE 'D1-D3 at T3' DIET 1 0 -1 0 0 0 DIET*TIME
0 0 1 0 0 0 0 0 0 0 0 0 0 -1 0 0;
ESTIMATE 'D1-D3 at T4' DIET 1 0 -1 0 0 0 DIET*TIME
0 0 0 1 0 0 0 0 0 0 0 0 0 0 -1 0;
ESTIMATE 'D1-D3 at T5' DIET 1 0 -1 0 0 0 DIET*TIME
0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 -1;
```

The estimates of the difference between diet 1 and diet 3 are similar across models, but the standard errors of the estimates are similar and constant over time for SIMPLE, CS and AR(1) models because these models assume a constant variance over time as previously shown. On the other hand, standard errors of the estimates with ANTE(1) and UN models are similar within the same time period, and increase over time. This is because both ANTE(1) and UN models are able to recognize heterogeneous variation over time as observed in these data. The results in Table 7 clearly indicate that the inappropriate choice of the SIMPLE, CS and AR(1) models resulted in a high risk for Type I statistical error in the earlier time periods (1 and 2) and a high risk for Type II error (failing to reject the null hypothesis when the alternate hypothesis is true) in later time periods (4 and 5) for the above estimates compared with ANTE(1) and UN model. Therefore, ANTE(1) and UN models adequately describe the example data while the Simple, CS and AR(1) models do not.

Table 7. Least square mean estimates (EST) and standard errors (SE) of differences between diets 1 and 3 at different time periods from five covari-ance[z] models for the data set STEERS

Difference[y]	SIMPLE		CS		AR(1)		ANTE(1)		UN	
	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE
D1–D3 at T1	–11.99	4.65	–11.99	4.68	–11.97	4.72	–12.47	2.63	–12.43	2.62
D1–D3 at T2	–16.99	4.65	–16.99	4.68	–16.97	4.72	–17.47	3.96	–17.43	3.93
D1–D3 at T3	–25.49	4.65	–25.49	4.68	–25.47	4.72	–25.97	4.95	–25.93	4.90
D1–D3 at T4	–31.69	4.65	–31.69	4.68	–31.67	4.72	–32.17	5.46	–32.13	5.41
D1–D3 at T5	–34.34	4.65	–34.34	4.68	–34.32	4.72	–34.82	6.60	–34.78	6.53

[z]SIMPLE = simple, CS = compound symmetry, AR(1) = first order autoregressive, ANTE(1) = ante dependence, UN = unstructured. All estimates (EST) are significant at the 0.01 level.
[y]D1–D3 at T1 = difference between diet 1 and 3 at time 1. D1–D3 at T2 = difference between diet 1 and 3 at time 2. D1–D3 at T3 = difference between diet 1 and 3 at time 3. D1–D3 at T4 = difference between diet 1 and 3 at time 4. D1–D3 at T5 = difference between diet 1 and 3 at time 5.

CONCLUSIONS

The univariate ANOVA method often does not handle the time-dependent correlations adequately in repeated measures data and may often result in a Type I statistical error, that is rejection the null hypothesis when it is true. The MANOVA method assumes an unstructured covariance matrix that is far more general than required by most repeated measures data. It also wastes large amounts of information thereby reducing the power of the test, and cannot handle missing data effectively. The mixed model allows a flexible approach to model appropriate covariance structures that would adequately account for within-subject correlations over time. The mixed model uses a generalized least squares method to estimate and test the fixed effects, which is generally superior to the ordinary least squares used by GLM. Mixed model methodology has the ability to handle missing data and unequal spacing, and allows a covariate in the model. It is recommended that the mixed model be used for the analysis of repeated measures designs in animal studies. This is demonstrated by our example in which the ANTE(1) covariance structure fitted the data best among the five models selected and provided the most appropriate F tests for fixed effects, estimates of least square means and standard errors.

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