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Appropriate Covariance Structure for Linear Mixed Model in the Animal Experiments with Repeated Measurement Data

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Abstract

Working on repeated measurement analysis in animal experimental data, the linear model become less appropriate, because by assuming the error variances being independent and homogeneous might be violated. Thus, this study intends to develop linear mixed model for animal experiments with repeated measurement data. The work of this study was aimed to determine the covariance structure for linear mixed model using animal experiments with repeated measurement data. The study presented general form of variance-covariance structure in case of repeated measures data and general form of parameter estimation. Data from comparing effects of treatment (diet) on cumulative gas production in case of equal time spacing, unequal time spacing and unbalance data were used to demonstrate mixed model methodology and analyze repeated measurement data. The results of the estimates of correlation coefficients and variance-covariance matrix for all three cases revealed that the variances and correlations within the same animal were different over time. This structure was satisfied with ANTE(1) model. By comparing seven covariance structures (SIMPLE, CS, AR(1), UN, CHS, ARH(1) and ANTE(1)) using MIXED procedure, the ANTE(1) covariance structure from fitted the data was the best choice based on four fit indices criteria (−2RLL, AIC, AICC and BIC) and F-test of fixed effects. Therefore, the ANTE(1) structure was an appropriate covariance structure to describe repeated measurement data from the animal experiments in case of equal time spacing, unequal time spacing and unbalance data under heteroscedasticity and correlated data over time.

Keywords: Linear mixed model, covariance structure, repeated measurement.

1. Introduction

In some cases of animal experiment, treatments are assigned to particular experimental units and measured repeatedly over time in order to test effect of treatment. Therefore, there are two fixed effects (treatment and time) and two sources of random variation (between and within experimental unit) (Wang and Goonewardene 2004). The problem with repeated measurements on the same subject is that there is correlation between repeated measurements which are not independent. Thus, assumptions about the error variances being independent and homogeneous are no longer valid. Moreover, the risk of using incorrect standard errors for the comparison of means can result in an excessive type I error. Therefore, it is necessary to define appropriated experimental error for testing hypotheses (Kaps and Lamberson 2004).

In the present, the mixed model is used instead of general linear model (GLM) procedure with a repeated statement. The conventional approaches using GLM for the analysis of repeated data are Univariate analysis of variance (split-plot in time) and Multivariate Analysis of Variance (analysis of contrasts). Univariate analysis of variance requires equal time spacing, equal variance and covariance over time and assume compound symmetric (CS) of covariance structure while multivariate analysis of variance requires balanced data with same repeated time points for all experimental units and an unstructured covariance structure that it wastes a great amount of information inherent in repeated measures data and results in a less powerful test (SAS Institute, Inc. 2002 as cited in Wang and Goonewardene 2004). However, the mixed model analysis allows a various variance-covariance structures to be explicitly modeled. The linear mixed models (LMM) procedure is an extension of the general linear model so that the data are permitted to exhibit correlated and non-constant variability like multilevel models, hierarchical linear models, random coefficient models and longitudinal data analysis with continuous repeated measures (Laird and Ware 1982). The MIXED procedure uses generalized least squares (GLS) to estimate and test the fixed effects in the model which is superior to the ordinary least squares (OLS) method used by GLM procedure because it can account for all of the covariance parameters modeled for the data (SAS Institute, Inc. 1999 as cited in Wang and Goonewardene 2004). Another advantage of mixed models is their ability to handle uneven spacing and missing observations of repeated measurements. In addition, mixed models can also be extended as generalized mixed models to non-normal data distribution. Therefore, this study intends to develop LMM for animal experiments with repeated measurement data by determining the best covariance structure for these data sets. There are several kinds of covariance structure in LMM. It is useful for applying, if appropriated structure is known for animal experiment with repeated measurement data. Thus, the work of this study was aimed to determine the appropriate covariance structure for LMM using animal experiments with repeated measurement data in case of equal time spacing, unequal time spacing and unbalance data. In addition, it must be fitted for influencing factors from violation of constant variance and uncorrelated data over time.

2. Method

The effect of collection time was included in LMM for this study and accounted for the model by defining time effect as values of a categorical independent variable. The study assumed an experiment with a treatments and b animals for each treatment (replication) with each animal measured k times. Therefore, the model is (Kaps and Lamberson 2004):

$$y_{ijt} = \mu + \tau_i + \alpha_{j(i)} + \gamma_t + (\tau\gamma)_{it} + e_{ijt}, i = 1,...,a;\ j = 1,...,b;\ t = 1,...,k, \quad (1)$$

where y_{ijt} is the observation ijt, μ is the general mean, τ_i is the fixed effect of treatment i, $\alpha_{j(i)}$ is the random effect of the j^{th} animal within the i^{th} treatment, $\alpha_{j(i)} \sim NID(0, \sigma_\alpha^2)$, σ_α^2 is a variance between animals (subjects) within treatment and it is equal to the covariance between repeated measurements within animals. γ_t is the fixed effect of time t, $(\tau\gamma)_{it}$ is the fixed interaction effect between treatment i and time t, e_{ijt} is the random error with the mean 0 and variance σ^2 and σ^2 is the variance between measurements within animals.

3. Parameter Estimation of Linear Mixed Model

The model (1) was shown as, in matrix form:

$$\underline{y} = \underline{X}\underline{\beta} + \underline{Z}\underline{\alpha} + \underline{e},$$

where $\underline{y}$ is the $n\times 1$ response vector for observations which n is the number of observations, $\underline{X}$ is the $n\times(a+k+ak)$ model matrix for the fixed effects, $\underline{\beta}$ is the $(a+k+ak)\times 1$ vector of fixed-effect coefficients, $\underline{Z}$ is the $n\times b$ model matrix for the random effects, $\underline{\alpha}$ is the $b\times 1$ vector of random-effect coefficients, and $\underline{e}$ is the $n\times 1$ vector of errors for observations.

Moreover, α and y are assumed to be jointly Gaussian distributed as

$$\begin{bmatrix}\alpha\\ y\end{bmatrix} \sim N\left(\begin{bmatrix}0\\ X\beta\end{bmatrix}, \sigma^2\begin{bmatrix}G & GZ'\\ ZG & V\end{bmatrix}\right),$$

where G contains the variance components for each random effect factor.

Thus, y has the marginal probability density function $N(X\beta,\sigma^2 V),$ where $V = ZGZ' + \Sigma$ with G and Σ assumed known. The MIXED procedure uses generalized least squares (GLS) to estimate parameter and test the fixed effects in the model. Gumedze and Dunne (2011) showed the solution for β and α that are given by

$$\hat{\beta} = (X'V^{-1}X)^{-1}X'V^{-1}y, \text{ and } \tilde{\alpha} = GZ'V^{-1}(y - X\hat{\beta}).$$

According to example data in animal experiment for three cases, the study defined

$$y_{n\times 1} = \begin{bmatrix} y_{111}\\ y_{112}\\ y_{113}\\ \vdots\\ y_{abk}\end{bmatrix}_{n\times 1}, \quad n = a\times b\times k,$$

where y_{ijt} is observation of treatment i animal j at collection time t

$$X_{n\times(m+1)} = \begin{bmatrix} j & | & \underline{X}_1 & \underline{X}_2 & \underline{X}_3\end{bmatrix}, \text{ (set } m = a+k+ak\text{)}$$

where j is the $n\times 1$ matrix of 1s, $\underline{X}_1$ is the $n\times a$ design matrix for treatment (τ) effects of $\tau_1 - \tau_a$, $\underline{X}_2$ is the $n\times k$ design matrix for collection time (γ) effects of $\gamma_1 - \gamma_k$, $\underline{X}_3$ is the $n\times(ak)$ design matrix for the interaction effect between τ and γ,

$$\underline{\beta}_{(m+1)\times 1} = \begin{bmatrix}\mu\\ \underline{\beta}_1\\ \underline{\beta}_2\\ \underline{\beta}_3\end{bmatrix},$$

where $\underline{\beta}_1$ is the $a\times 1$ vector of treatment (τ) effect coefficients, $\underline{\beta}_2$ is the $k\times 1$ vector of collection time (γ) effect coefficients, $\underline{\beta}_3$ is the $ak\times 1$ vector of interaction effect between τ and γ, Z_i represent the column vector of the random effects of design matrix $\underline{Z}$. Thus,

$$Z_{n\times b} = \begin{bmatrix}\underline{Z}_1 & \underline{Z}_2 & \dots & \underline{Z}_b\end{bmatrix},$$

where $\underline{Z}_1$ is the $n \times 1$ design matrix for 1st animal effect within the i^{th} treatment, $\underline{Z}_2$ is the $n \times 1$ design matrix for 2nd animal effect within the i^{th} treatment, and $\underline{Z}_b$ is the $n \times 1$ design matrix for b^{th} animal effect within the i^{th} treatment,

$$\underline{\alpha}_{b\times 1} = \begin{bmatrix} \underline{\alpha}_1 \\ \underline{\alpha}_2 \\ \vdots \\ \underline{\alpha}_b \end{bmatrix},$$

where $\underline{\alpha}_1$ is the 1×1 vector of 1st animal effect within the i^{th} $(i = 1, 2, ..., a)$ treatment coefficients, $\underline{\alpha}_2$ is the 1×1 vector of *2*nd animal effect within the i^{th} $(i = 1, 2, ..., a)$ treatment coefficients, $\underline{\alpha}_b$ is the 1×1 vector of b^{th} animal effect within the i^{th} $(i = 1, 2, ..., a)$ treatment coefficients, and

$$e = \begin{bmatrix} e_{111} \\ e_{112} \\ e_{113} \\ \vdots \\ e_{abk} \end{bmatrix}_{n\times 1}.$$

The first step was estimation variance of y, $V = ZGZ' + \Sigma$, V can be modelled by setting up the random-effects design matrix Z and by specifying covariance structures for G and Σ. For G, it contained variance components in a diagonal structure. For this study, the j^{th} animal within the i^{th} treatment was treated as random effect, the estimation of covariance structure was

$$G = \begin{bmatrix} \sigma^2_{\gamma_1} & & & & & & \\ & \sigma^2_{\gamma_2} & & & & & \\ & & \sigma^2_{\gamma_3} & & & & \\ & & & \cdots & & & \\ & & & & \cdots & & \\ & & & & & \sigma^2_{\gamma_{b-1}} & \\ & & & & & & \sigma^2_{\gamma_b} \end{bmatrix}_{b\times b},$$

where blanks denote zeros.

The second estimation was the variance-covariance structure (Σ). In case of repeated measures data, there was no constant correlation between repeated measures, no constant variance and covariance. The variance-covariance (Σ) was of the form

$$\begin{bmatrix} \sigma_1^2 & & & & \\ \sigma_{21} & \sigma_2^2 & & & \\ \sigma_{31} & \sigma_{32} & \cdots & & \\ \vdots & \vdots & \cdots & \sigma^2_{k-1} & \\ \sigma_{k1} & \sigma_{k2} & \cdots & \sigma_{k,k-1} & \sigma_k^2 \end{bmatrix},$$

where k by k block corresponds to a single animal.

As $V = ZGZ' + \Sigma,$ the full dimension of the matrix V is $k \times ab = n$ by $n,$ the form of V (for example: animal 1) was

$$\begin{bmatrix} \sigma_{\gamma_1}^2 + \sigma_1^2 & & & & \\ \sigma_{\gamma_1}^2 + \sigma_{21} & \sigma_{\gamma_1}^2 + \sigma_2^2 & & & \\ \sigma_{\gamma_1}^2 + \sigma_{31} & \sigma_{\gamma_1}^2 + \sigma_{32} & \dots & & \\ \vdots & \vdots & \dots & \sigma_{\gamma_1}^2 + \sigma_{k-1}^2 & \\ \sigma_{\gamma_1}^2 + \sigma_{k1} & \sigma_{\gamma_1}^2 + \sigma_{k2} & \dots & \sigma_{\gamma_1}^2 + \sigma_{k,k-1} & \sigma_{\gamma_1}^2 + \sigma_k^2 \end{bmatrix}.$$

The next was estimation fixed effects parameters β estimated by GLS, $\hat{\beta} = (X'V^{-1}X)^{-1} X'V^{-1} y$ which is the best linear unbiased estimator (BLUE) of $\beta.$ From matrix V as shown above, let

$$V^{-1} = \begin{bmatrix} a_{11} & a_{12} & a_{13} & \dots & \dots & a_{1,n-1} & a_{1,n} \\ a_{21} & a_{22} & a_{23} & \dots & \dots & a_{2,n-1} & a_{2,n} \\ a_{31} & a_{32} & a_{33} & \dots & \dots & a_{3,n-1} & a_{3,n} \\ \vdots & \vdots & \vdots & \dots & \dots & \vdots & \vdots \\ \vdots & \vdots & \vdots & \dots & \dots & \vdots & \vdots \\ a_{n-1,1} & a_{n-1,2} & a_{n-1,3} & \dots & \dots & a_{n-1,n-1} & a_{n-1,n} \\ a_{n,1} & a_{n,2} & a_{n,3} & \dots & \dots & a_{n,n-1} & a_{n,n} \end{bmatrix}_{n \times n}.$$

Thus, $X'V^{-1}X$ was

$$\begin{bmatrix} \left[\sum_{i=1}^{n} a_{i1} + \sum_{i=1}^{n} a_{i2} + \dots + \sum_{i=1}^{n} a_{in}\right] & \left[\sum_{i=1}^{n} a_{i1} + \sum_{i=1}^{n} a_{i2} + \dots + \sum_{i=1}^{n} a_{i,bk}\right] & \dots & \left[\sum_{i=1}^{n} a_{i,n-b+1} + \dots + \sum_{i=1}^{n} a_{i,n}\right] \\ \left[\sum_{i=1}^{bk} a_{i1} + \sum_{i=1}^{bk} a_{i2} + \dots + \sum_{i=1}^{bk} a_{in}\right] & \left[\sum_{i=1}^{bk} a_{i1} + \sum_{i=1}^{bk} a_{i2} + \dots + \sum_{i=1}^{bk} a_{i,bk}\right] & \dots & \left[\sum_{i=1}^{bk} a_{i,n-b+1} + \dots + \sum_{i=1}^{bk} a_{i,n}\right] \\ \vdots & \vdots & \dots & \vdots \\ \left[\sum_{i=n-2b+1}^{n-b} a_{i1} + \sum_{i=n-2b+1}^{n-b} a_{i2} + \dots + \sum_{i=n-2b+1}^{n-b} a_{in}\right] & \left[\sum_{i=n-2b+1}^{n-b} a_{i1} + \sum_{i=n-2b+1}^{n-b} a_{i2} + \dots + \sum_{i=n-2b+1}^{n-b} a_{i,bk}\right] & \dots & \left[\sum_{i=n-2b+1}^{n-b} a_{i,n-b+1} + \dots + \sum_{i=n-2b+1}^{n-b} a_{i,n}\right] \\ \left[\sum_{i=n-b+1}^{n} a_{i1} + \sum_{i=n-b+1}^{n} a_{i2} + \dots + \sum_{i=n-b+1}^{n} a_{in}\right] & \left[\sum_{i=n-b+1}^{n} a_{i1} + \sum_{i=n-b+1}^{n} a_{i2} + \dots + \sum_{i=n-b+1}^{n} a_{i,bk}\right] & \dots & \left[\sum_{i=n-b+1}^{n} a_{i,n-b+1} + \dots + \sum_{i=n-b+1}^{n} a_{i,n}\right] \end{bmatrix}_{(m+1)\times(m+1)}.$$

Since, the conditional inverse of X is $X^c,$ if $XX^cX = X$ that conditional inverse always exists. If X is $m \times n$ then X^c is $n \times m.$ For this study, as $(X'V^{-1}X)$ was $(m+1)\times(m+1),$ so $(X'V^{-1}X)^c$ was $(m+1)\times(m+1).$ Let

$$(X'V^{-1}X)^c = \begin{bmatrix} b_{11} & b_{12} & b_{13} & .. & .. & b_{1,m} & b_{1,m+1} \\ b_{21} & b_{22} & b_{23} & .. & .. & b_{2,m} & b_{2,m+1} \\ b_{31} & b_{32} & b_{33} & .. & .. & b_{3,m} & b_{3,m+1} \\ : & : & : & .. & .. & : & : \\ : & : & : & .. & .. & : & : \\ b_{m,1} & b_{m,2} & b_{m,3} & .. & .. & b_{m,m} & b_{m,m+1} \\ b_{m+1,1} & b_{m+1,2} & b_{m+1,3} & .. & .. & b_{m+1,m} & b_{m+1,m+1} \end{bmatrix}_{(m+1)\times(m+1)},$$

where $(X'V^{-1}X)(X'V^{-1}X)^c(X'V^{-1}X) = (X'V^{-1}X).$

Since $X'V^{-1}y = \begin{bmatrix} y_{111}\sum_{i=1}^{n} a_{i1} + y_{121}\sum_{i=1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=1}^{n} a_{in} \\ y_{111}\sum_{i=1}^{bk} a_{i1} + y_{121}\sum_{i=1}^{bk} a_{i2} + \ldots + y_{abk}\sum_{i=1}^{bk} a_{in} \\ \vdots \\ \vdots \\ \vdots \\ y_{111}\sum_{i=n-2b+1}^{n-b} a_{i1} + y_{121}\sum_{i=n-2b+1}^{n-b} a_{i2} + \ldots + y_{abk}\sum_{i=n-2b+1}^{n-b} a_{in} \\ y_{111}\sum_{i=n-b+1}^{n} a_{i1} + y_{121}\sum_{i=n-b+1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=n-b+1}^{n} a_{in} \end{bmatrix}_{(m+1)\times 1}$,

therefore, $\hat{\beta} = (X'V^{-1}X)^{c} X'V^{-1}y$ was

$$\begin{bmatrix} b_{11}\left[y_{111}\sum_{i=1}^{n} a_{i1} + y_{121}\sum_{i=1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=1}^{n} a_{in}\right] + \ldots + b_{1,m+1}\left[y_{111}\sum_{i=n-b+1}^{n} a_{i1} + y_{121}\sum_{i=n-b+1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=n-b+1}^{n} a_{in}\right] \\ b_{21}\left[y_{111}\sum_{i=1}^{n} a_{i1} + y_{121}\sum_{i=1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=1}^{n} a_{in}\right] + \ldots + b_{2,m+1}\left[y_{111}\sum_{i=n-b+1}^{n} a_{i1} + y_{121}\sum_{i=n-b+1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=n-b+1}^{n} a_{in}\right] \\ b_{31}\left[y_{111}\sum_{i=1}^{n} a_{i1} + y_{121}\sum_{i=1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=1}^{n} a_{in}\right] + \ldots + b_{3,m+1}\left[y_{111}\sum_{i=n-b+1}^{n} a_{i1} + y_{121}\sum_{i=n-b+1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=n-b+1}^{n} a_{in}\right] \\ \vdots \\ \vdots \\ b_{m,1}\left[y_{111}\sum_{i=1}^{n} a_{i1} + y_{121}\sum_{i=1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=1}^{n} a_{in}\right] + \ldots + b_{m,m+1}\left[y_{111}\sum_{i=n-b+1}^{n} a_{i1} + y_{121}\sum_{i=n-b+1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=n-b+1}^{n} a_{in}\right] \\ b_{m+1,1}\left[y_{111}\sum_{i=1}^{n} a_{i1} + y_{121}\sum_{i=1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=1}^{n} a_{in}\right] + \ldots + b_{m+1,m+1}\left[y_{111}\sum_{i=n-b+1}^{n} a_{i1} + y_{121}\sum_{i=n-b+1}^{n} a_{i2} + \ldots + y_{abk}\sum_{i=n-b+1}^{n} a_{in}\right] \end{bmatrix}_{(m+1)\times 1}.$$

The resulting solution for β is not unique and is no longer unbiased. However, $X\hat{\beta}$ is unique and unbiased for $X\beta$. Therefore, the study considered $X\hat{\beta}$ as general solution for β, Let

$\underline{\hat{\beta}} = \begin{bmatrix} \mu \\ \hat{\beta}_{\tau_1} \\ \hat{\beta}_{\tau_{21}} \\ \hat{\beta}_{\tau_3} \\ \vdots \\ \vdots \\ \hat{\beta}_{\tau_{(a-1)}\gamma_{(k-1)}} \\ \hat{\beta}_{\tau_a\gamma_k} \end{bmatrix}_{(m+1)\times 1}$. Therefore, $X\hat{\beta} = \begin{bmatrix} \mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1} \\ \mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1} \\ \mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1} \\ \vdots \\ \vdots \\ \mu + \hat{\beta}_{\tau_a} + \ldots + \hat{\beta}_{\tau_a\gamma_k} \\ \mu + \hat{\beta}_{\tau_a} + \ldots + \hat{\beta}_{\tau_a\gamma_k} \end{bmatrix}_{n\times 1}$.

Hence, the parameter estimation of β for repeated measurement data that there was no constant correlation between repeated measures, no constant variance and constant covariance was considered in term of $X\hat{\beta}$ that it was estimable.

Then, the study considered the solutions for α given by $\tilde{\alpha} = GZ'V^{-1}(y - X\hat{\beta})$. Consider $GZ'V^{-1}$,

$$G = \begin{bmatrix} \sigma^2_{\gamma_1} & & & & & & \\ & \sigma^2_{\gamma_2} & & & & & \\ & & \sigma^2_{\gamma_3} & & & & \\ & & & \cdots & & & \\ & & & & \cdots & & \\ & & & & & \sigma^2_{\gamma_{b-1}} & \\ & & & & & & \sigma^2_{\gamma_b} \end{bmatrix}_{b\times b}.$$

Thus,

$$GZ'V^{-1} = \begin{bmatrix} \left[\sigma^2_{\gamma_1} a_{11} + \sigma^2_{\gamma_1} a_{b+1,1} + .. + \sigma^2_{\gamma_1} a_{(ak-1)b+1,1}\right] & \cdots & \left[\sigma^2_{\gamma_1} a_{1n} + \sigma^2_{\gamma_1} a_{b+1,n} + .. + \sigma^2_{\gamma_1} a_{(ak-1)b+1,n}\right] \\ \left[\sigma^2_{\gamma_2} a_{21} + \sigma^2_{\gamma_2} a_{b+2,1} + .. + \sigma^2_{\gamma_2} a_{(ak-1)b+2,1}\right] & \cdots & \left[\sigma^2_{\gamma_2} a_{2n} + \sigma^2_{\gamma_2} a_{b+2,n} + .. + \sigma^2_{\gamma_2} a_{(ak-1)b+2,n}\right] \\ \vdots & \vdots & \vdots \\ \left[\sigma^2_{\gamma_b} a_{b1} + \sigma^2_{\gamma_b} a_{2b,1} + .. + \sigma^2_{\gamma_b} a_{akb,1}\right] & \cdots & \left[\sigma^2_{\gamma_b} a_{bn} + \sigma^2_{\gamma_b} a_{2b,n} + .. + \sigma^2_{\gamma_b} a_{akb,n}\right] \end{bmatrix}_{b\times n},$$

$$y - X\hat{\beta} = \begin{bmatrix} y_{111} - [\mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1}] \\ y_{112} - [\mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1}] \\ y_{113} - [\mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1}] \\ \vdots \\ \vdots \\ \vdots \\ y_{abk} - [\mu + \hat{\beta}_{\tau_a} + \ldots + \hat{\beta}_{\tau_a\gamma_k}] \end{bmatrix}_{n\times 1}.$$

Thus, $\tilde{\alpha} = GZ'V^{-1}(y - X\hat{\beta})$ was

$$= \begin{bmatrix} \left[\left(\sigma^2_{\gamma_1} a_{11} + \sigma^2_{\gamma_1} a_{b+1,1} + .. + \sigma^2_{\gamma_1} a_{(ak-1)b+1,1}\right) \times \left(y_{111} - [\mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1}]\right)\right] + \ldots + \left[\left(\sigma^2_{\gamma_1} a_{1n} + \sigma^2_{\gamma_1} a_{b+1,n} + .. + \sigma^2_{\gamma_1} a_{(ak-1)b+1,n}\right) \times \left(y_{abk} - [\mu + \hat{\beta}_{\tau_a} + \ldots + \hat{\beta}_{\tau_a\gamma_k}]\right)\right] \\ \left[\left(\sigma^2_{\gamma_2} a_{21} + \sigma^2_{\gamma_2} a_{b+2,1} + .. + \sigma^2_{\gamma_2} a_{(ak-1)b+2,1}\right) \times \left(y_{111} - [\mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1}]\right)\right] + \ldots + \left[\left(\sigma^2_{\gamma_2} a_{2n} + \sigma^2_{\gamma_2} a_{b+2,n} + .. + \sigma^2_{\gamma_2} a_{(ak-1)b+2,n}\right) \times \left(y_{abk} - [\mu + \hat{\beta}_{\tau_a} + \ldots + \hat{\beta}_{\tau_a\gamma_k}]\right)\right] \\ : \\ \left[\left(\sigma^2_{\gamma_b} a_{b1} + \sigma^2_{\gamma_b} a_{2b,1} + .. + \sigma^2_{\gamma_b} a_{akb,1}\right) \times y_{111} - [\mu + \hat{\beta}_{\tau_1} + \ldots + \hat{\beta}_{\tau_1\gamma_1}]\right] + \ldots + \left[\left(\sigma^2_{\gamma_b} a_{bn} + \sigma^2_{\gamma_b} a_{2b,n} + .. + \sigma^2_{\gamma_b} a_{akb,n}\right) \times \left(y_{abk} - [\mu + \hat{\beta}_{\tau_a} + \ldots + \hat{\beta}_{\tau_a\gamma_k}]\right)\right] \end{bmatrix}_{b\times 1}.$$

4. Covariance Structure for the Repeated Measures Model

The Σ matrix is the covariance matrix for errors. Wang and Goonewardene (2004) described some kinds of covariance structures in case of a structure for four measurements in one subject as shown in Table 1.

Table 1 The description of covariance structure

Type	Description	Matrix
SIMPLE	All observations are assumed that they 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.	$\sigma^2\begin{bmatrix}1&0&0&0\\0&1&0&0\\0&0&1&0\\0&0&0&1\end{bmatrix}$
Compound symmetry (CS)	The CS refers to equal variances $(\sigma^2+\sigma_\alpha^2)$ on the main diagonal and equal covariances (σ_α^2) on all off diagonals. It is assumed that the correlation is constant between observations regardless of the distance between time points.	$\begin{bmatrix}\sigma^2+\sigma_\alpha^2&\sigma_\alpha^2&\sigma_\alpha^2&\sigma_\alpha^2\\\sigma_\alpha^2&\sigma^2+\sigma_\alpha^2&\sigma_\alpha^2&\sigma_\alpha^2\\\sigma_\alpha^2&\sigma_\alpha^2&\sigma^2+\sigma_\alpha^2&\sigma_\alpha^2\\\sigma_\alpha^2&\sigma_\alpha^2&\sigma_\alpha^2&\sigma^2+\sigma_\alpha^2\end{bmatrix}$
Heterogeneous compound symmetry (CSH)	The CS covariance structure defines heterogeneous variance.	$\begin{bmatrix}\sigma_1^2&\sigma_1\sigma_2&\sigma_1\sigma_3&\sigma_1\sigma_4\\\sigma_2\sigma_1&\sigma_2^2&\sigma_2\sigma_3&\sigma_2\sigma_4\\\sigma_3\sigma_1&\sigma_3\sigma_2&\sigma_3^2&\sigma_3\sigma_4\\\sigma_4\sigma_1&\sigma_4\sigma_2&\sigma_4\sigma_3&\sigma_4^2\end{bmatrix}$
The first-order autoregressive (AR(1))	The AR(1) covariance structure assumes that with greater distance between periods, correlations (ρ) are smaller. 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.	$\sigma^2\begin{bmatrix}1&\rho&\rho^2&\rho^3\\\rho&1&\rho&\rho^2\\\rho^2&\rho&1&\rho\\\rho^3&\rho^2&\rho&1\end{bmatrix}$
Heterogeneous first-order autoregressive (ARH(1))	The AR(1) covariance structure defines heterogeneous variance.	$\begin{bmatrix}\sigma_1^2&\rho\sigma_1\sigma_2&\rho^2\sigma_1\sigma_3&\rho^3\sigma_1\sigma_4\\\rho\sigma_2\sigma_1&\sigma_2^2&\rho\sigma_2\sigma_3&\rho^2\sigma_2\sigma_4\\\rho^2\sigma_3\sigma_1&\rho\sigma_3\sigma_2&\sigma_3^2&\rho\sigma_3\sigma_4\\\rho^3\sigma_4\sigma_1&\rho^2\sigma_4\sigma_2&\rho\sigma_4\sigma_3&\sigma_4^2\end{bmatrix}$
Unstructured (UN)	The UN covariance structure defines different variances over time and different covariances for each time combination.	$\begin{bmatrix}\sigma_1^2&\sigma_{12}&\sigma_{13}&\sigma_{14}\\\sigma_{21}&\sigma_2^2&\sigma_{23}&\sigma_{24}\\\sigma_{31}&\sigma_{32}&\sigma_3^2&\sigma_{34}\\\sigma_{41}&\sigma_{42}&\sigma_{43}&\sigma_4^2\end{bmatrix}$

Table 1 (Continued)

Type	Description	Matrix
The first-order ante dependence (ANTE(1))	The ANTE(1) covariance structure defines different variances over time and different 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.	$\begin{bmatrix} \sigma_1^2 & \rho_1\sigma_1\sigma_2 & \rho_1\rho_2\sigma_1\sigma_3 & \rho_1\rho_2\rho_3\sigma_1\sigma_4 \\ \rho_1\sigma_2\sigma_1 & \sigma_2^2 & \rho_2\sigma_2\sigma_3 & \rho_2\rho_3\sigma_2\sigma_4 \\ \rho_1\rho_2\sigma_3\sigma_1 & \rho_2\sigma_3\sigma_2 & \sigma_3^2 & \rho_3\sigma_3\sigma_4 \\ \rho_1\rho_2\rho_3\sigma_4\sigma_1 & \rho_2\rho_3\sigma_4\sigma_2 & \rho_3\sigma_4\sigma_3 & \sigma_4^2 \end{bmatrix}$

5. Data for Illustration

In orders to determine the appropriate variance-covariance structure in LMM for repeated measurement data, three data sets including equal time spacing, unequal time spacing and unbalance data were fitted to different covariance structures. Data from animal experiment published by Santhong (2013) were used to demonstrate mixed model methodology and analyze repeated measurement data in case of unequal time spacing. The data was about effects of treatment (diet) on cumulative gas production, the study used CRD for experimental design. Data set for case of unequal time spacing, there were three replications for each treatment and repeated measurement at 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th},12^{th}, 24^{th}, 48^{th} hour. The observation informed that mean cumulative gas production values over 48 hours respectively were 2.29, 6.13, 9.48, 12.36, 15.48, 16.56, 22.73 and 28.75. Data set for case of unbalance data (Thiputen and Sommart 2012), there were eleven replications for each treatment and repeated measurement at 3^{rd}, 9^{th}, 15^{th}, 21^{th}, 30^{th} hour. The observation informed that mean cumulative gas production values over 30 hours respectively were 3.27, 6.12, 12.42, 21.70 and 26.61. Data set for case of equal time spacing (Thiputen and Sommart 2012), there were eight replications for each treatment and repeated measurement at 1^{st} - 10^{th} hour. The observation informed that mean cumulative gas production values over 10 hours respectively were 6.76, 12.42, 16.74, 19.84, 24.42, 28.68, 32.69, 36.42, 40.87 and 45.02.

6. Estimation of Variance-Covariance Matrix

The estimation of variance-covariance matrix of the data for three cases (equal time spacing, unequal time spacing and unbalance data) were obtained by fitting LMM as Equation (1). The variances of measures in time t were on the diagonal, correlations and covariances between repeated measures in time t_i and t_j $(i \neq j)$ (i≠j) were shown above and below the diagonal, respectively (Tables 2-4). The results in all three cases appeared that the variances were clearly unequal over time indicating heterogeneous variances and the correlations within the same animal were different over time as well. Hence, a mixed linear model with heterogeneous and correlated covariance structure should be considered for all three cases.

Table 2 Covariance and correlation matrix^z obtained by fitting the UN structure in case of equal time spacing

Time	1	2	3	4	5	6	7	8	9	10
1	10.6366	0.9844	0.9599	0.9147	0.9283	0.9319	0.9218	0.9150	0.9041	0.8941
2	14.9837	21.7818	0.9859	0.9543	0.9648	0.9658	0.9561	0.9504	0.9402	0.9295
3	17.1872	25.2613	30.1407	0.9782	0.9787	0.9744	0.9608	0.9536	0.9424	0.9276
4	18.0895	27.0085	32.5663	36.7714	0.9856	0.9749	0.9635	0.9563	0.9444	0.9272
5	19.9693	29.6988	35.4380	39.4184	43.5030	0.9960	0.9891	0.9804	0.9728	0.9617
6	21.8748	32.4407	38.5003	42.5476	47.2829	51.8000	0.9947	0.9879	0.9815	0.9728
7	24.6110	36.5264	43.1784	47.8293	53.4042	58.6062	67.0102	0.9967	0.9936	0.9877
8	28.3270	42.1058	49.6940	55.0471	61.3780	67.4910	77.4484	90.1032	0.9979	0.9933
9	32.3708	48.1772	56.8029	62.8750	70.4432	77.5568	89.2946	103.99	120.53	0.9979
10	35.2415	52.4283	61.5457	67.9454	76.6558	84.6141	97.7132	113.95	132.40	146.05

Note: ^{z} Variances along the diagonal, covariances are in the lower triangle and correlations are in the upper triangle.

Table 3 Covariance and correlation matrix^z obtained by fitting the UN structure in case of unequal time spacing

Time	1	2	3	4	5	6	7	8
1	0.3333	0.2415	0.2882	0.2750	0.2037	0.1862	0.2040	0.2768
2	0.1667	1.4286	0.8910	0.8745	0.2131	0.8545	0.7278	0.8320
3	0.2381	1.5238	2.0476	0.9709	0.3287	0.9692	0.8042	0.9432
4	0.2857	1.8810	2.5000	3.2381	0.4092	0.9738	0.8409	0.9671
5	0.1429	0.3095	0.5714	0.8810	1.4762	0.4336	0.5220	0.3946
6	0.2381	2.2619	3.0714	3.8810	1.1667	4.9048	0.8756	0.9542
7	0.3095	2.2857	3.0238	3.9762	1.667	5.0952	6.9048	0.8583
8	0.4286	2.6667	3.6190	4.6667	1.2857	5.6667	6.0476	7.1905

Note: ^{z} Variances along the diagonal, covariances are in the lower triangle and correlations are in the upper triangle.

Table 4 Covariance and correlation matrix^z obtained by fitting the UN structure in case of unbalance data

Time	1	2	3	4	5
1	0.7477	0.8929	0.7123	0.6012	0.5667
2	1.2042	2.4327	0.8804	0.7608	0.7287
3	2.9993	6.6866	23.7138	0.9583	0.9343
4	5.1150	11.6749	45.9178	96.8110	0.9934
5	6.3449	14.7172	58.9083	126.5600	167.6500

Note: ^{z} Variances along the diagonal, covariances are in the lower triangle and correlations are in the upper triangle.

7. Comparison of Variance-Covariance Structures

Seven structures of the variance-covariance matrix were fitted for model (1) by MIXED function in R program to compare the performance of the model including SIMPLE, compound symmetry (CS), first-order autoregressive (AR(1)), unstructured (UN), heterogeneous compound symmetry (CSH), heterogeneous first-order autoregressive (ARH(1)) and first-order ante-dependence

(ANTE(1)). Akaike information criterion (AIC), finite sample corrected Akaike information criterion (AICC), Bayesian information criterion (BIC) cohesion criteria and −2 Res. Log Likelihood value were used to determine the appropriate variance-covariance structure (Tables 5-7).

Table 5 Comparison of model fit statistics with different covariance structures in case of equal time spacing

Covariance Structure	−2 Res. Log Likelihood	AIC	AICC	BIC
SIMPLE	2,032.6	2,034.6	2,034.6	2,036.1
CS	1,622.7	1,626.7	1,626.7	1,629.6
AR(1)	1,143.9	1,147.9	1,147.9	1,150.8
UN	784.8	894.8	922.3	975.4
CSH	1,201.1	1,223.1	1,224.1	1,239.2
ARH(1)	939.1	961.1	962.1	977.2
ANTE(1)	864.9	902.9	905.8	930.7

Table 6 Comparison of model fit statistics with different covariance structures in case of unequal time spacing

Covariance Structure	−2 Res. Log Likelihood	AIC	AICC	BIC
SIMPLE	517.8	519.8	519.8	520.8
CS	446.1	450.1	450.2	452.1
AR(1)	442.7	446.7	446.8	448.8
UN	292.5	364.5	400.1	402.1
CSH	401.1	419.1	420.8	428.5
ARH(1)	413.5	431.5	433.3	440.9
ANTE(1)	370.4	400.4	405.4	416.1

Table 7 Comparison of model fit statistics with different covariance structures in case of unbalance data

Covariance Structure	−2 Res. Log Likelihood	AIC	AICC	BIC
SIMPLE	1,316.7	1,318.7	1,318.7	1,320.5
CS	1,260.5	1,264.5	1,264.6	1,268.1
AR(1)	1,123.9	1,127.9	1,128.0	1,131.5
UN	675.0	705.0	707.8	731.8
CSH	864.8	876.8	877.3	887.6
ARH(1)	761.8	773.8	774.2	784.5
ANTE(1)	711.2	729.2	730.2	745.2

According to cohesion criteria results of variance-covariance structures in Tables 5-7, the unstructured covariance model (UN) has the smallest AIC, AICC, BIC and the −2 Log Likelihood scores in all three cases. However, the UN covariance model wastes a great amount of information inherent in repeated measures data and results in a less powerful test (SAS Institute, Inc**.** 2002 as cited in Wang and Goonewardene 2004). Thus the ANTE(1) covariance was another choice because it was the second cohesion criteria rank in all three cases.

Moreover, the results showed that CSH and ARH(1) which are the heterogeneous variance-covariance models were better than CS and AR(1) for the repeated measure data regarding the experimental data for all cases. Since generalizations of CS and AR(1), that are, CSH and ARH (1) respectively, which have unique value of variance each time (Pusponegoro et al. 2017). The SIMPLE and CS covariance structure were found to be the worst covariance structures in modeling. Since the SIMPLE covariance structure assumes that all observations are independent of each other and there is no correlation (covariance) between the repeated measures on the same animal that it was not corresponding with the values of the covariance and correlation matrix of these data in all three cases. For the CS model, the Huynh-Feldt (HF) condition is not met when it is applied to analyze repeated measures data (Huynh and Feldt 1970, 1976). Since the CS model assumes that all variances over time are constant, with constant correlation over time. Therefore, the CS model failed to account for the correlations between measures observed on the same animal and the heterogeneous variances over time.

Furthermore, fixed effects and interaction effect were tested to determine the performance of the models. Table 8 presented the value of F-tests of the fixed effects for the seven-selected covariance models for all three cases.

Table 8 F test of fixed effects for seven covariance structures

EFFECT	SIMPLE	CS	AR(1)	UN	CSH	ARH(1)	ANTE(1)
equal time spacing							
trt	39.22^{**}	4.56^{**}	3.29^{*}	4.56^{**}	4.60^{**}	4.68^{**}	4.53^{*}
time	81.82^{**}	528.74^{**}	346.80^{**}	203.54^{**}	152.59^{**}	179.91^{**}	160.08^{**}
trt*time	0.86^{ns}	5.57^{**}	3.82^{**}	3.86^{**}	1.97^{**}	2.49^{**}	2.73^{**}
unequal time spacing							
trt	28.17^{**}	5.27^{**}	6.57^{**}	5.27^{**}	6.05^{**}	8.42^{**}	8.37^{**}
time	541.16^{**}	1429.2^{**}	433.24^{**}	1601.43^{**}	736.00^{**}	574.46^{**}	602.47^{**}
trt*time	3.07^{**}	8.10^{**}	5.97^{**}	10.09^{**}	5.85^{**}	5.69^{**}	4.29^{**}
unbalance data							
trt	14.48^{**}	5.52^{**}	3.20^{*}	5.32^{**}	5.86^{**}	5.89^{**}	5.24^{**}
time	77.05^{**}	143.50^{**}	137.52^{**}	177.48^{**}	104.14^{**}	127.41^{**}	126.16^{**}
trt*time	0.83^{ns}	1.63^{ns}	2.17^{*}	42.66^{**}	13.19^{**}	31.09^{**}	24.65^{**}

Note: ^{ns} F-test non-significant at the 0.05 level, *F-test significant at the 0.05 level and **F-test significant at the 0.01 level.

Significance of the fixed effects were shown in Table 8. For the case of equal time spacing, the results showed that treatment and time effect were significant in all seven covariance structures. Significant interaction effects were in every covariance structures except SIMPLE structure which was the lowest in F-value. For the case of unequal time spacing, the results showed that treatment, time and interaction effect were significant in all seven covariance structures. The case of unbalance data, the results showed that treatment and time effect were significant in every covariance structures. Significant interaction effects were in every covariance structures except SIMPLE and CS structure. The results of fixed effect and interaction effect test have seen that, for all three cases, the SIMPLE model showed the extreme high value in treatment (trt) effect, extreme low value in time (time) effect and interaction effects (trt*time). Similarly, with the results of comparing the values of cohesion criteria, the SIMPLE covariance structure was not fitted with data set in all three cases.

The F values based on the CSH, ARH(1) and ANTE(1) models were significant at the 0.01 level in both fixed and interaction effect test except ANTE(1) in case of equal time spacing (significant at the 0.05 level). Since these models are adequately modeling the covariance of the data, the results were in valid tests. Moreover, ARH(1) and CSH structure model have the constant parameter, then these can be more efficient and powerful covariance structures in terms of detecting treatment effects (Pusponegoro et al. 2017). However, the ANTE(1) covariance model provided the best fit because it had the lowest AIC, AICC, BIC and the −2 Log Likelihood fit statistics among the six model tested (exclude UN model).

Thus, it was the model of choice among the seven models tested. According to variance-covariance structure (Σ) as estimated in all three cases, it appeared that the variances and correlations within the same animal were clearly unequal over time. It can also be seen that covariances or correlations between adjacent measurements on the same animal were more correlated at later time periods than earlier times. Thus, this structure was satisfied with ANTE(1) structure because it is assumed that the magnitude of the covariance depends on the values of both the correlations and standard deviations associated with them.

Tables 9-11 provided least square mean estimates of treatments from the seven covariance structures. In case of equal time spacing and unequal time spacing in Tables 9-10, least square means were same for each treatment regardless of the covariance structure, because the data were balanced. However, with unbalance data, the least square mean estimates were different (Table 11). The standard errors of the least square estimates differ in all three cases for the different covariance models because they are adjusted for the covariance parameters in the mixed model (Littell et al. 1998).

Table 9 Least square mean estimates (EST) and standard errors (SE) of treatments from seven covariance models structures in case of equal time spacing

Treatment	SIMPLE		CS		AR(1)		UN		CSH		ARH(1)		ANTE(1)	
	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE
1	24.27	0.88	24.27	2.58	24.27	3.04	24.27	2.58	24.27	2.57	24.27	2.54	24.27	2.59
2	33.57	0.88	33.57	2.58	33.57	3.04	33.57	2.58	33.57	2.57	33.57	2.54	33.57	2.59
3	27.16	0.88	27.16	2.58	27.16	3.04	27.16	2.58	27.16	2.57	27.16	2.54	27.16	2.59
4	20.54	0.88	20.54	2.58	20.54	3.04	20.54	2.58	20.54	2.57	20.54	2.54	20.54	2.59

Note: All estimate (EST) are significant at the 0.01 level.

8. Conclusions

The linear mixed model (LMM) was applied on repeated measurement data from animal experiment with cumulative gas production as dependent variable, treatment (τ) and collection time (γ) as fixed effects and the j^{th} experimental unit (animal) within the i^{th} treatment (α) as random effect. There were three data sets considering in case of equal time spacing, unequal time spacing and unbalance data.

Table 10 Least square mean estimates (EST) and standard errors (SE) of treatments from seven covariance models structures in case of unequal time spacing

Treatment	SIMPLE		CS		AR(1)		UN		CSH		ARH(1)		ANTE(1)	
	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE
1	12.17	0.38	12.17	0.88	12.17	0.78	12.17	0.88	12.17	0.82	12.17	0.69	12.17	0.69
2	13.54	0.38	13.54	0.88	13.54	0.78	13.54	0.88	13.54	0.82	13.54	0.69	13.54	0.69
3	15.00	0.38	15.00	0.88	15.00	0.78	15.00	0.88	15.00	0.82	15.00	0.69	15.00	0.69
4	14.58	0.38	14.58	0.88	14.58	0.78	14.58	0.88	14.58	0.82	14.58	0.69	14.58	0.69
5	17.04	0.38	17.04	0.88	17.04	0.78	17.04	0.88	17.04	0.82	17.04	0.69	17.04	0.69
6	17.96	0.38	17.96	0.88	17.96	0.78	17.96	0.88	17.96	0.82	17.96	0.69	17.96	0.69
7	16.13	0.38	16.13	0.88	16.13	0.78	16.13	0.88	16.13	0.82	16.13	0.69	16.13	0.69

Note: All estimate (EST) are significant at the 0.01 level.

Table 11 Least square mean estimates (EST) and standard errors (SE) of treatments from seven covariance models structures in case of unbalance data

Treatment	SIMPLE		CS		AR(1)		UN		CSH		ARH(1)		ANTE(1)	
	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE	EST	SE
1	12.10	0.99	12.07	1.63	12.04	2.21	12.02	1.76	11.96	1.63	12.02	1.65	12.01	1.77
2	10.88	0.99	10.79	1.63	10.67	2.21	10.60	1.76	10.55	1.63	10.61	1.65	10.58	1.77
3	13.72	0.99	13.67	1.63	13.50	2.21	13.38	1.76	13.72	1.63	13.43	1.65	13.40	1.77
4	19.39	0.99	19.45	1.63	19.64	2.21	19.79	1.76	19.54	1.63	19.71	1.65	19.75	1.77

Note: All estimate (EST) are significant at the 0.01 level.

From the estimates of correlation coefficients and variance-covariance matrix for all three cases, they revealed that the variances and correlations within the same animal were different over time. Therefore, the study applied MIXED procedure to estimate parameters because the data are permitted to exhibit correlated and non-constant variability and handle unequal time spacing and unbalance data. Moreover, there are various covariance structures considering to be fitted with data.

The study modeled variance-covariance structure using seven structures namely, SIMPLE, CS and AR(1) and using non-constant variance such as UN, CSH, ARH(1) and ANTE(1) in order to compare the estimations fitted with heteroscedasticity of the data. The result showed that the ANTE(1) model perfectly described cumulative gas production correlation and variability over time for all three cases, based on four fit indices criteria (−2RLL, AIC, AICC and BIC) and F-test of fixed effects. Therefore, the ANTE(1) model was the best choice of a proper variance-covariance (Σ) structure to describe repeated measurement data from animal experiment in case of equal time spacing, unequal time spacing and unbalance data under heteroscedasticity and correlated data over time.

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