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#### THE NEED FOR THE ANALYSIS OF TREATMENT × PERIOD INTERACTION IN ANIMAL EXPERIMENTS

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#### Abstract

Many growth experiments, in which weights are taken at different times on the same animals, involve the comparison of factorial main effects and interactions but exclude time (period) as an effect. The objective of this paper is to show that more information can be obtained by analysing the data as a repeated measures design. As an example, feedlot cattle being prepared for market are often on growth implants and provided different diets depending on the stage of growth and maturity. Growth promoting implants, either single or double, may be slow or fast acting. During the growing period, a diet with less grain and medium energy is fed but during the finisher period the grain component is increased. Responses to implant and diet may be dependent on the Any model designed to analyze the length of time between measurements. responses within time, will be limited as it will not include all treatment x time interactions, which can be very important. A repeated measures or split plot in time can detect these treatment x time interactions, but criteria such as the sphericity of the covariance matrix should be satisfied, so that the within subject effects can be correctly tested. The paper describes four statistical models appropriate for such data using  $SAS^R/STAT$ software.

Key words: repeated split-plot variance treatment period interaction.

#### 1. Introduction

Many experiments in animal science involve the comparison of main effects and interactions over time. This is particularly so with growth type studies where body weights are taken at different times on the same animals and gains calculated as a difference in weight over time or period. Very often period or time is not considered a main effect in the model, and analyses of variance are performed within periods (Hidiroglou et al. 1980; Price et al. 1983; Beacom et al. 1988; Bailey 1989), such as the first 28 d, grower and finisher periods etc. The basis for these is because the correlation between growth rates get smaller as the period or interval widens. There are however, citations in the literature that have used a repeated measures/split plot approach, especially when blood parameters or hormone profiles are analyzed over time (Buckley et al. 1986; Bush 1991).

The objective of this paper is to show how important period or time can be in making meaningful conclusions from an experiment, where the response variable is a measure of growth, subject to treatments that affect the response differently over period or time.

In general, when the response to two or more treatments is uniform across time, a factorial design is appropriate (fig 1a & 1b). However, when the response to treatment is not uniform across period (fig 2a & 2b), then a factorial design (which does not include time as an effect), cannot detect a treatment x period interaction and valuable information can be lost. Hence, a repeated measures/split plot design which includes period as an effect in the model would be more appropriate.

#### 2. The Repeated Measures ANOVA

In experiments where multiple measurements are made on the same subject over time, the repeated measures analysis is appropriate. The treatments are applied to random groups of animals and data collected over time. In the output, the main effects and interactions appear as the between subject effects while, the effect of period or time and interactions of treatment and period appear as within subject effects. Thus, the analysis has two parts and two error terms for testing the null hypothesis of no effect. Although a number of software packages are available for repeated measures, we will confine ourselves to the  $SAS^R/STAT$  system. There are certain limitations in that all animals need to have complete observations. However, the SAS<sup>R</sup> code and other procedures that handle unbalanced designs are provided by Milliken and Johnson (1984), Schluchter (1988) and Entsuah and Williams (1991). Α second limitation is that the period and interaction of period and treatment least square means or means cannot be directly obtained in a repeated measures analysis, using the REPEATED statement.

A repeated measures design can be analyzed as a split plot in time provided certain criteria are met. In a classical split plot design, the sub plots (split) receiving the treatments are randomised. However, when the sub plot is time, it becomes a fixed effect, as repeated measurements are made on animals subject to treatments at fixed times. Due to this non-random assignment with respect to time, the first criterion to be satisfied is that of compound symmetry. The partial correlation coefficients in the covariance matrix for the response should be of similar magnitude (auto-correlation). There is a tendency in animal growth data, for correlations to decrease with the increase in the time interval. It is therefore imperative that when there are a large number of periods, that they be meaningfully collapsed to a few, so as not to violate compound symmetry of the correlation matrix. The second criterion that needs to be satisfied is that of sphericity of the covariance matrix. Sphericity requires a set of orthogonal contrasts to have equal variances and zero covariance (Mauchley 1940; Pendergast and Littell 1988).

In the event that these criteria are violated, the usual F test (variance ratio) becomes too liberal for period (time) or period x treatment interactions and a type I error might result, where the null hypothesis is rejected when in fact is true. With this background we will work through a hypothetical example using  $SAS^R/STAT$  software and point out similarities, differences, strengths and weaknesses of each design.

#### 3. Hypothetical Animal Example

Let us assume that it is our objective to determine the effects of two growth implants (I) {A, B & C=control} and two diets (D) {P & Q=standard}. The response variable is average daily gain (ADG) in steers. The experiment is set up with 5 steers per implant/diet combination and n=30. Body weights were taken at 4 times (periods) of the trial and ADG calculated for three periods and overall. The hypotheses to be tested are that of no implant effect  $h_0$  I, no diet effect  $h_0$  D and no interaction effect  $h_0$  ID. There are four possible methods of analysing the data using the general analysis of variance (ANOVA) approach.

#### 3.1 Method I

Analysis of the ADG data within period and overall with the main effects implant (I), diet (D) and I x D interaction. This can be accomplished using the following  $SAS^R$  code:

DATA METHOD1; INPUT NUMBER IMPLANT \$ DIET \$ WEIGHT1 WEIGHT2 WEIGHT3 WEIGHT4; PERIOD1=100; PERIOD2=90; PERIOD3=100; ADG1=(WEIGHT2-WEIGHT1)/100; ADG2=(WEIGHT3-WEIGHT2)/90; ADG3=(WEIGHT4-WEIGHT3)/100; ADGOVLL=(WEIGHT4-WEIGHT1)/290;

CARDS; 1 A P 200 300 350 400 2 A P 210 305 342 402 • . • • • . . . • . . 30 C 202 293 340 400 Q PROC ANOVA;....OR GLM\* CLASSES IMPLANT DIET; MODEL ADG1-ADG3 ADGOVLL=IMPLANT DIET IMPLANT\*DIET/SS3; MEANS IMPLANT DIET IMPLANT\*DIET/SNK ETYPE=3; LSMEANS IMPLANT DIET IMPLANT\*DIET/STDERR ETYPE=3; RUN;

## \* PROC GLM should be used for unbalanced data and where contrasts are to be made. @ LSMEANS is only available with PROC GLM.

The output will show a univariate analysis of variance with main effects of implant, diet and I x D for ADG1-ADG3 and ADGOVLL within each period. The F statistic will accept or reject  $h_0$  I,  $h_0$  D and  $h_0$  ID. A Student-Newman-Keuls' (SNK) test will separate means.

The results of the analysis of variance and means for treatments are shown in tables 1 and 2. Note that only the relevant information is presented from the  $SAS^R$  printout. During the first 100 d, (ADG1) animals on implant A showed significantly higher gains, in the second period (ADG2) those on implant B showed higher gains and in the last period (ADG3), there were no differences between implanted and the control animals. When ADG overall was considered, animals on implant B had higher gains, probably due to a carry over. Although implant A appears fast acting compared to B, its effect was no different to the control during the third period. When ADG's were plotted by treatment and period (figure 3) there is a possible interaction effect, which cannot be tested through a factorial design as the response is analyzed within period. As such, an alternate approach is required to study the data more thoroughly.

#### 3.2 Method II

Using the same format for the data as before (method I), a repeated measures analysis can be performed, using  $SAS^P/STAT$  software. The null hypotheses  $(h_0)$  to be tested are, is there no implant effect  $h_0$  I, no diet effect  $h_0$  D, no interaction between implant and diet  $h_0$  ID (between subjects), no period effect  $h_0$  P, no period by implant interaction  $h_0$  PI, period by diet interaction  $h_0$  PD or period by implant by diet interaction  $h_0$  PID (within subject) effect. In this analysis the usual F tests are valid for the four within subject effects only if the sphericity criterion is **not** violated.

The repeated measures can be used in a multivariate mode to determine the effect of implant, diet and interaction on ADG1-ADG3 jointly. The multivariate approach takes into account the correlation between ADG's. The Wilks' Lambda, Pillai's Trace, Hotelling-Lawley Trace and Roy's greatest root are the test statistics available, which are equivalent to the F test in a univariate analysis. The following SAS<sup>R</sup> code will perform the required analysis:

PROC GLM DATA=METHOD1; CLASSES IMPLANT DIET; MODEL ADG1 ADG2 ADG3=IMPLANT DIET IMPLANT\*DIET; REPEATED PERIOD 3/SHORT PRINTM PRINTH PRINTE SUMMARY; LSMEANS IMPLANT DIET IMPLANT\*DIET/STDERR ETYPE=3; MEANS IMPLANT DIET IMPLANT\*DIET/SNK ETYPE=3; RUN; Having the three response variables, ADG1, ADG2 and ADG3 on the left of the equals sign and using the REPEATED statement, tells  $SAS^R$  to perform the multivariate analysis as well. By default  $SAS^R$  will give both Univariate and Multivariate tests which can be suppressed either by using NOU or NOM statements respectively, after the slash (/) in the REPEATED statement. The term PERIOD 3 in the REPEATED statement before the slash identifies three periods corresponding to ADG1, ADG2 and ADG3. The term SHORT after the / instructs SAS to give the multivariate tests in a condensed form. The terms PRINTH and PRINTM requests  $SAS^R$  to give the hypotheses and error matrices for each effect that is being tested. The PRINTE term requests  $SAS^R$  to give the error matrix for all within subject factors and partial correlation coefficients for ADG1-ADG3 and PERIOD1-PERIOD3. In addition the PRINTE option provides the sphericity test for each set of transformed variables and for a set of orthogonal contrasts ( SAS User guide 1986).

In the particular example the partial correlations from the error and cross products matrix for ADG as given in the  $SAS^R$  output were as follows:

#### Partial correlations for responses

DF=23	ADG1	ADG2	ADG3
ADG1	1.0	-0.72	0.11
ADG2	-0.72	1.0	-0.50
ADG3	0.11	-0.50	1.0

In this example, the assumption of auto correlation has been violated. The test for sphericity of Mauchley's criterion was 0.62, Chisquare=10.98 and the probability  $>X^2$  was 0.004. In other words, one would have to reject a null hypothesis of sphericity (ie the correlation matrix is not spherical). The sphericity condition being violated, all F tests for the within subject effects are now too liberal. The SAS<sup>R</sup> system provides a more conservative test such as, the Greenhouse Giesser (G-G) and a mid range test the Hunh-Felt (H-F) epsilon.

The analysis of variance from a repeated measures design is shown in table 3. The probabilities for the between subject effects are very similar to the P values for ADG overall in the factorial analysis of variance for implant, diet and I  $\times$  D. The same conclusions can be made in that overall, the probability of rejecting a null hypothesis of no difference for implant is high and significant, whereas, accepting a null hypothesis of no effects from a repeated measures analysis, the null hypothesis of no effect for period and I  $\times$  P should be rejected. The usual F test, G-G and H-F tests all lead us to the same conclusion. The usual F test becomes too liberal when probabilities are marginal, at which time acceptance of the F test will lead to a type I error. Thus, our conclusions (under failed sphericity) would be that differences between periods is significant and that the response in ADG for implant is dependent on period.

Unfortunately, when the REPEATED statement is used, the SAS<sup>R</sup> system only handles the data where all repeated observations have a numerical value. In unbalanced designs, a RANDOM statement in GLM with the TEST option to construct synthetic denominator mean square with Satterthwaite approximations are needed (Wolfinger et al. 1991). A further disadvantage is that you cannot obtain means or least square means for period and any of the period by treatment interactions when a REPEATED statement is used. A split plot analysis can be used to get the means and least square means.

#### 3.3 Method III

Under conditions where the sphericity criterion is not violated, a univariate split-plot and the repeated measures analysis can be used to test main effects and interactions inter-changeably. The advantage (as pointed out earlier) is that means and least square means can be obtained for balanced and unbalanced data using the split plot approach. However, in doing so, the data has to be restructured, more  $SAS^R$  code is required and appropriate error terms have to be selected for testing main effects and interactions, using a TEST statement as the model is now mixed. The following  $SAS^R$  code will restructure the data so as to be able to perform a split-plot analysis on the  $SAS^R$  system.

DATA METHOD3; INPUT NUMBER IMPLANT \$ DIET \$ WEIGHT1 WEIGHT2 WEIGHT3 WEIGHT4; PERIOD1=100; PERIOD2=90; PERIOD3=100; ADG1=(WEIGHT2-WEIGHT1)/PERIOD1; ADG2=(WEIGHT3-WEIGHT2)/PERIOD2; ADG3=(WEIGHT4-WEIGHT3)/PERIOD3; PERIOD='1';ADG=ADG1;OUTPUT; PERIOD='2';ADG=ADG2;OUTPUT; PERIOD='3';ADG=ADG3;OUTPUT; DROP ADG1 ADG2 ADG3;

#### CARDS;

The above code should output a SAS<sup>R</sup> data set that looks like this:

1	А	Р	1	1.00
1	А	Ρ	2	0.55
1	Α	Ρ	3	0.50
2	А	Ρ	1	0.95
2	А	Ρ	2	0.41
2	Α	Ρ	3	0.60
•	•	٠	٠	•
•	•	•	•	•
•	•	•	•	•
30	C	Q	1	
30 30		• • • Q Q	1 2	0.91 0.52

The following  $SAS^R$  program (code) will perform a split-plot ANOVA on the restructured data.

PROC GLM;

CLASSES NUMBER IMPLANT DIET PERIOD; MODEL ADG=IMPLANT DIET IMPLANT\*DIET NUMBER(IMPLANT\*DIET) PERIOD PERIOD\*IMPLANT PERIOD\*DIET PERIOD\*IMPLANT\*DIET/SS3; TEST H=IMPLANT DIET IMPLANT\*DIET E=NUMBER(IMPLANT\*DIET)/HTYPE=3 ETYPE=3; LSMEANS IMPLANT DIET IMPLANT\*DIET/STDERR E=NUMBER(IMPLANT\*DIET) ETYPE=3; LSMEANS PERIOD PERIOD\*IMPLANT PERIOD\*DIET PERIOD\*IMPLANT\*DIET/STDERR ETYPE=3;

RUN;

When the data are analyzed as a split plot in time, the null hypotheses,  $h_0$  I,  $h_0$  D,  $h_0$  ID,  $h_0$  P,  $h_0$  PI,  $h_0$  PD and  $h_0$  PID can be tested by the appropriate analyses with either the usual or the more conservative F tests, depending on the violation of the sphericity criterion. As such another dimension (effect) is now added to the analysis to test whether the response (ADG) to implanting or diet or the interaction, is different as you go across periods. This could not have been detected by a factorial analysis of variance. The ANOVA for the response variable ADG and means separated with the SNK test are shown in tables 3 and 4.

#### 3.4 Method IV

The independent, or explanatory, variables in our hypothetical experiment are of two types: DIET and IMPLANT are nominal or qualitative variables, but PERIOD is quantitative. These conditions imply that an area of interest in the design could be estimating the rate of change in ADG, over time and conditioned upon the types of diet and implants. The interaction of IMPLANT\*PERIOD, demonstrated by Methods II and III, will also be detected by placing the problem in a regression context , providing evidence for heterogeneity among the three slope coefficients representing each implant over time. Littell et al. (1991) provide an example for using dummy variables in SAS to analyze models containing both qualitative and quantitative variables (see also, Draper and Smith 1981).

Data should be structured as in Method III. The following code defines the model and invokes SAS, via the SOLUTION command, to create three dummy variables: one for DIET and two for IMPLANT.

```
DATA METHOD4;

PROC GLM;

CLASSES IMPLANT DIET PERIOD;

MODEL ADG = IMPLANT DIET PERIOD IMPLANT*DIET

IMPLANT*PERIOD DIET*PERIOD/SOLUTION;

RUN;
```

SAS creates a dummy variable for DIET such that when P "dummy1"=1 and O, otherwise. Similarly, for IMPLANT when A "dummy2"=1 and O otherwise, when B, then "dummy3"= 1, otherwise O. By default, the last level or treatment, becomes the baseline equation so in this example, it is diet P and implant C (based upon alphabetic order). Intercepts for implants A and B must be redefined: for IMPLANT A,  $a_A' = a_A - a_C'$ , for B,  $a_B' = a_B - a_C$ . Likewise, regression coefficients become: for A,  $b_A' = b_A - b_C$ , and for B,  $b_B' = b_B - b_C$ . The regression equations can then be expressed as:

Table 5 contains the output of parametric estimates for the full model; the IMPLANT\*PERIOD interaction was again highly significant, as previously shown in table 3. Other output has been omitted for discussion purposes.

Substituting the appropriate estimates from table 5 into the above equations yields these results:

IMPLANT	EQUATION
A	ADG = $(0.85 + 0.40) + (-0.10 - 0.19)$ PERIOD = $1.25 - 0.29$ (PERIOD)
В	ADG = $(0.85 + 0.10) + (-0.10 - 0.02)$ PERIOD = $0.95 - 0.12$ (PERIOD)
С	ADG = 0.85 - 0.10(PERIOD)

These three functions are graphically displayed in Figure 4. The faster rate of action by implant A is obvious, as are the near equal delayed actions by B and C. While this approach correctly identifies the IMPLANT\*PERIOD interaction and provides estimates of the average rate of change in ADG among periods, it fails, due to averaging, to demonstrate the more subtle variability among the three implants shown in Figure 3, i.e. the rapid loss of activity in period 2 by implant A and the opposite effect of implant B (the intermediate effect of C is also obvious).

While this approach (Method IV) may fail to detect the temporal differences demonstrated in figure 3, simple effects coefficients or cell means comparisons can be constructed in  $SAS^R$  using the ESTIMATE or CONTRAST statements (see Littell et al. 1991; pp 91-98). The following code placed after the MODEL statement (above) will compare implant A vs B and C within each PERIOD (note:the order of the independent variable names in the CLASS statement determines the order of cell coefficients).

CLASSES IMPLANT DIET PERIOD; (MODEL,ABOVE)/SS1; ESTIMATE 'IMPLANT A vs B,C in Period 1' IMPLANT 1 -.5 -.5 IMPLANT\*PERIOD 1 0 0 -.5 0 0 -.5 0 0; ESTIMATE 'IMPLANT A vs B,C in PERIOD 2' IMPLANT 1 -.5 -.5 IMPLANT\*PERIOD 0 1 0 0 -.5 0 0 -.5 0; ESTIMATE 'IMPLANT A vs B,C in PERIOD 3' IMPLANT 1 -.5 -.5 IMPLANT\*PERIOD 0 0 1 0 0 -.5 0 0 -.5; RUN:

Comparisons among cell means would be accomplished by simply replacing the ESTIMATE with CONTRAST statement.

The overall resolution of this approach (Method IV) would seem to fall between analysis of ADG by averaging over all time periods and the split plot in time method. Its advantage mainly lies in identifying the correct temporal interaction and providing estimates of relative differences in rates of action of implants upon ADG over time. It can be strengthened by adding specific comparisons among simple effects or means at each level of time.

#### 4.Discussion

The results from the analysis of variance (table 3) for ADG are very similar to the factorial design analysis for ADGOVLL shown in table 1. Both analyses reject a null hypothesis of no implant effect and accept the null hypothesis of no diet and implant x diet interaction. The means for the effects are also similar in the two analyses (tables 2 and 4). However, there is a period effect and a period x implant effect that is highly significant (table 3), which was not tested for when the data were analyzed as a factorial. If conclusions are based on a factorial analysis alone, it would appear that implant B elicits a better growth response overall compared to A and C and as such, one could recommend its use in an experiment, such as this, which is 290 days in duration. The analysis does not however, recognise that the responses to implanting with A and B are highly dependent on time. The conclusions based on the split plot analysis would be that, although implant B shows the better response overall, implant A is preferred over a shorter period due to its quick action and implant B is better during the second period due to its delayed action. One cannot make the same conclusion based on a factorial as the period x implant interactions were not tested. Both analyses show that the effect of the implants A and B were no different than the control during the third period. Sometimes when two diets are compared with one having palatability problems in the presence of a slow and fast acting implant, the interaction between implant, diet and period may become significant. Such an effect can only be tested by a split plot type of analysis. In our hypothetical example, the sphericity criterion was violated as the covariance matrix was not spherical. This was due to a differential response of ADG to the implant treatments in each period. Thus, the split plot or repeated measures analysis provides more factual (hypotheses tested) information about the data than does the factorial analysis and is more suitable for the analysis of these types of data.

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Table 1. ANOVA (Factorial design) separating out main effects for the response variables.

### Dependent variable ADG1

Source	df	Type III SS	Mean Square	F value	Pr> F
Implant	2	0.872	0.463	54.56	0.0001
Diet	1	0.004	0.004	0.48	0.4942
Implant x Diet	2	0.019	0.009	1.20	0.3179
Dependent vari	able ADG2	2			
Implant	2	1.752	0.876	43.39	0.0001
Diet	1	0.004	0.004	0.20	0.6621
Implant x Diet	2	0.001	0.000	0.01	0.9852
Dependent vari	able ADG	3			
Implant	2	0.042	0.021	3.11	0.0631
Diet	1	0.001	0.001	0.18	0.6780
Implant x Diet	2	0.000	0.000	0.00	0.9980
Dependent vari	able ADGO	OVLL			
Implant	2	0.012	0.006	8.75	0.0014
Diet	1	0.000	0.000	0.28	0.6037
Implant x Diet	2	0.002	0.001	1.62	0.2189

	Average Daily Gain					
Effect	level	ADG1	ADG2	ADG3	ADGOVLL	
Implant	A	1.05a	0.47a	0.46a	0.67a	
Implant	B	0.66b	1.06b	0.42a	0.70b	
Control	C	0.72b	0.74c	0.51a	0.65a	
Diet	P	0.82m	0.75m	0.50m	0.68m	
Standard	Q	0.80m	0.77m	0.46m	0.67m	

# Table 2. Means for main effects Implant and Diet and significance tests, factorial ANOVA

a,b,c Separates means for Implant effect m.. no difference between Diets Units of response Kg  $d^{-1}$ 

New Prairie Press https://newprairiepress.org/agstatconference/1992/proceedings/24 Table 3. ANOVA (Split plot in time) separating out main effects and interactions for thr response variable ADG.

### Dependent variable ADG

Source	DF	Type III SS	Mean Sq.	F value	Pr>F
Implant	2	0.060	0.030	13.57	0.001
Diet	1	0.001	0.001	0.17	0.681
Implant*Diet	2	0.006	0.003	1.43	0.259
Number(Implant*Diet)	24	0.053	0.002	0.14	1.000
Period	2	2.115	1.057	64.53	0.000
Period*Implant	4	2.607	0.652	39.76	0.000
Period*Diet	2	0.009	0.004	0.26	0.769
Period*Implant*Diet	4	0.013	0.003	0.21	0.934
Residual	48	0.787	0.016		

Table 4. Means for main effects of Implant, Diet, Period and selected interactions and significance tests for Average Daily Gain from a Split plot ANOVA

			Effects				
I	mplant		Diet		Pei	riod	
A 0.66a	B 0.72b	C – – – – – – – – – – – – – – – – – – –	P 0.68m	Q 0.67m	1 0.81x	2 0.76x	3 0.46y

		Implant	
Period	A	В	С
1 2 3	1.05 0.47 0.46	0.66 1.06 0.42	0.72 0.74 0.51
	Di	Let	
Period	P	Q	
1 2 3	0.82 0.75 0.50	0.80 0.77 0.46	

a,b.. Separates means for Implant, m..for Diet and x,y..for Period The non-significant interactions are not shown Units for Average Daily Gain kg  $d^{-1}$ 

Parameter	Estimate	Parameter=0	Pr>T	S.E. of Estimate
Intercept	0.85	7.19	0.0001	0.12
IMPLANT A	0.40	2.67	0.009	0.15
В	0.10	0.68	0.50	0.15
С	0.00	•	۵	۰
DIET P	0.04	0.28	0.78	0.13
Q	0.00	•	٠	•
@IMPLT*DIET A P	-0.04	-0.39	0.70	0.11
AQ	0.00	•	•	•
ΒP	-0.03	-0.24	0.81	0.11
ВQ	0.00	•	•	•
CP	0.00	٠	•	•
СО	0.00	٠	•	•
PERIOD (PER)	-0.10	-1.95	0.05	0.05
PER*IMPLT A	-0.19	-2.89	0.005	0.06
В	-0.02	-0.25	0.81	0.06
С	0.00	•	•	•
PER*DIET P	-0.005	-0.09	٠	•
Q	0.00	•	ø	•

Table 5. Estimates of coefficients using dummy variables.

@ IMPLT=IMPLANT



