Module 4

Single Factor Analysis of Variance

Certificate in EnvIroStats (Non-Award)

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Module 4

Single Factor ANOVA

Lesson 1: Key Concepts in ANOVA

Overview

Analysis of variance, or ANOVA for short, is fundamental for much of the application of statistics in biology. In its simplest form, it provides an extension of the t-test, enabling the simultaneous comparison of two or more means. In more complex designs it enables us to consider the effects of two or more factors simultaneously without disregarding the possible interaction between them.

Analysis of variance is more than just a technique. It provides insight into the nature of variation of natural events. It represents a major conceptual advance which, once understood, will guide you in the way you plan and execute much of your research. Analysis of variance is an indispensable conceptual and practical tool for the modern biologist.

In its simplest form, the analysis is used to understand the effects of a single-factor, acting alone. We might, for example, wish to discover if ‘Method of Determination’ has an effect on the concentration of copper detected in fish tissue. The methods available to us include flame atomic absorption spectroscopy, graphite furnace atomic absorption spectroscopy, anodic stripping voltametry and inductive coupled plasma mass spectroscopy (Table 4–1).

Table 4–1. Comparison of four methods for measuring levels of copper in fish tissue.

Flame AAS	Graphite furnace AAS	Anodic stripping voltametry	Inductive coupled plasma mass spec
25	23	25	18
24	18	25	26
25	22	20	22
26	28	18	28
	17	23	17
	25	19	16
	19	26	
	16		

Analysis of variance provides a basis for deciding between whether there is good evidence of a true difference between methods, or whether the differences we observe between methods arose by chance alone. This is a basic similarity shared with all hypothesis tests.

In the context of ANOVA, ‘Method of Determination’ is the Factor, with four discrete **factor classes**, and copper concentration is the **response variable**.

Single-factor analysis of variance, or one-way ANOVA as it is sometimes called, can be considered in a practical sense to be an extension of the t-test. Had there been only two methods involved in the above comparison, a t-test would have been appropriate. Indeed, such an analysis was undertaken in Module 3. The t-test is used to test hypotheses regarding the equality of two population means, whereas the single-factor ANOVA tests hypotheses regarding two or more population means.

Two models of single-factor ANOVA are recognised—the **fixed model** and the **random model**. In the fixed model, the objective of the analysis is not only to determine whether the factor under consideration has an overall effect, but which factor classes differ significantly from which others. The factor classes are chosen specifically and are therefore under the full control of the experimenter. In the case above, we might be interested both in whether the method of determination has an overall effect on the levels of copper detected and may wish to know which method gave the highest determination. The methods are fixed in the sense that if the study were to be repeated, exactly the same methods would be chosen again. Because we are interested in the performance of the four specifically chosen methods of analysis in the example above, the model is fixed.

A significant result in the fixed model single-factor ANOVA indicates significant variation among the means over and above that expected to occur by chance alone, but it does not provide information on which factor classes differ from which others. A significant result in the ANOVA must be followed by a set of comparisons to determine where the differences lie. The appropriate procedures to follow-up a significant result in a fixed model include testing the significance of differences between pairs of means using one of several **multiple comparison procedures**. SAS provides a wide range of options for undertaking multiple comparisons following a significant result in a fixed ANOVA.

In the random model, the factor classes are selected at random from a substantial population of possible choices. Variation among the observed means is due both to sampling error in the selection of factor classes and to variation among measurements within factor classes. The factor is not considered fixed because if the experiment or study were to be repeated, there would be no reason to expect that the same specific factor classes would be chosen again. In a random model, one is not interested in comparing specific means, only in whether the factor under consideration has had a differential effect. The appropriate follow-up procedure in the random model is to estimate the added variance component due to the effect of the factor. The example given above would be a random model if we had chosen the four methods listed in Table 4–1 at random from a large pool of possible choices. Had this been the case, we would not have been interested in specific comparisons among methods, only in whether choice of method added significantly to variation in the determinations of copper concentration.

Examples of fixed and random designs in single-factor ANOVA are given as worked examples later.

Rationale

Analysis of variance arose from the study of replicated samples, that is, from the study of samples drawn from the same or identical populations. Consider by way of example, a case where an experiment is designed to investigate the effect of cropping by macro-invertebrates on algal standing crop. Seven transects were established parallel to the banks of a small stream, in what appeared to be homogeneous riffle environment. Five paving bricks were placed along each transect and left for two months to accumulate a cover of algal growth. At the end of two months, they were removed from the stream and the accumulated algae was scraped from them, dried and weighed (Table 4–2).

This design has the factor TRANSECT with seven factor classes corresponding to the seven transects. Each transect comprises a sample of five replicated paving bricks and corresponding biomass measurements (the response variable). The transects themselves are laid in a homogeneous environment. The samples each of five pavers can therefore be considered to represent identical populations, and we should not expect significant differences among transects. Bear this in mind for the discussion that follows.

Table 4–2. Algal biomass (mg/m^2) from paving bricks laid along transects in shallow stream riffle.

Transect						
1	2	3	4	5	6	7
22.4	10.3	21.9	36.4	19.6	15.7	20.2
18.7	9.5	16.8	21.9	19.5	24.0	18.5
29.2	25.6	16.0	24.7	21.4	23.2	33.0
23.2	24.0	18.8	21.2	28.8	16.8	19.9
22.9	21.0	27.9	23.9	28.3	17.7	21.6

Statistics from these data reveal an ambiguous pattern (Table 4–3, Figure 4–1). Clearly the means vary from transect to transect, but then replicated means taken from the same population would be expected to vary by chance alone, through sampling error.

Table 4–3. Mean and variance in algal biomass (mg/m^2) collected from paving bricks.

	Transect						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Mean	23.3	18.1	20.3	25.7	23.5	19.5	22.6
Variance	14.24	58.57	23.34	38.35	21.69	14.73	34.75

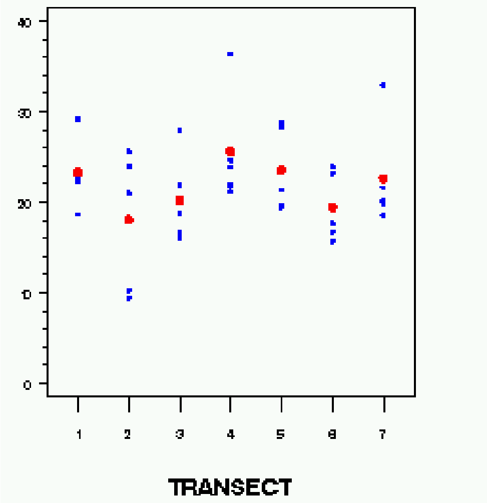


Figure 4–1. Variation in algal biomass (mg/m^2) collected from paving bricks laid along transects in shallow stream riffle. Means are shown as larger dots.

The central problem addressed by ANOVA is:

> *Do the observed differences among sample means provide evidence of true differences among the populations from which the samples were drawn, or did the observed differences arise by chance alone?*

The basis for a decision on this comes from examination of variances:

- the variances among measurements within samples (ie the transects) and
- the variance of the sample means.

It is one of the ironies of the analysis that much time is spent considering variances in order to test an hypothesis on means.

If each sample of five pavers can be considered to have been drawn from the same or identical populations, they must have the same parametric mean and variance. Consider now how we might estimate the common population variance in biomass within transects, that is, σ^2.

The obvious approach is to calculate the variance separately for each transect,

$$VAR[Y] = \frac{\sum^{n}\left(Y - \overline{Y}\right)^2}{n-1} \approx \sigma^2$$

for *n* = 5 pavers.

These are shown in Table 4–3. However, no single such estimate will give us the best estimate of the common population variance, σ^2, because each is based on only five values. Instead, a better estimate of the common population variance is obtained by averaging the a = 7 sample variances.

$$\overline{VAR[Y]} = \frac{1}{a}\sum^{a}\frac{\sum^{n}\left(Y - \overline{Y}\right)^2}{n-1}$$

$$= \frac{\sum^{a}\sum^{n}\left(Y - \overline{Y}\right)^2}{a(n-1)}$$

$$= 29.38 \approx \sigma^2$$

This estimate of the common population variance, calculated as the average within sample variance, is called **mean square within**. It is an estimate of the common population variance that is calculated solely from variation among measurements within samples (ie $Y - \overline{Y}$), in this case, within transects.

A second method for estimating the common population variance involves using information on variation among the seven replicated sample means. Statisticians have established a relationship between the variation exhibited by replicated sample means and the variation exhibited by measurements within samples. We have:

$$\sigma^2_{\overline{Y}} = \frac{\sigma^2}{n}$$

This equation is most familiar when expressed in terms of standard deviations rather than variances. It is simply a restatement of the equation that equates the standard error as the standard deviation over the square root of the sample size *n*.

This equation makes intuitive sense. The variance of the means will depend inversely on the sample size:

$\sigma^2_{\overline{Y}}$ is inversely proportional to *n* because the larger the samples, the closer will each of the sample means be to the true parametric mean and the lower will be the variance among the sample means.

Also, the variance of the means will be directly proportional to the within sample variance:

$\sigma^2_{\bar{Y}}$ is directly proportional to σ^2

because the more variable the measurements themselves, the less representative each sample mean will be of the true parametric mean, for a given sample size.

So according to the equation

$$\sigma^2_{\bar{Y}} = \frac{\sigma^2}{n}$$

the greater the variance within samples, the greater will be the variance among the means and the greater the sample size, the smaller will be the variance among the means.

This equation can be rearranged to yield

$$n.\sigma^2_{\bar{Y}} = \sigma^2$$

which provides us with a second method of estimating the common population variance, this time based on observed variation among replicated population means.

$$n.VAR\left[\bar{Y}\right] \approx \sigma^2$$

Taking the variance of the means of Table 4–3 yields 6.99 and multiplying by the sample size (x 5) yields an estimate of the common population variance of 34.97. This estimate, because it is calculated from variation among the sample means, is called **mean square among**.

Substituting the formula for $VAR\left[\bar{Y}\right]$ to provide a formula in the same form as that for $\text{MS}_{\text{within}}$ yields:

$$MS_{among} = n.\frac{\sum^{a}\left(\bar{Y} - \bar{\bar{Y}}\right)^2}{a-1}$$

which can be rewritten

$$MS_{among} = \frac{\sum^{a}\sum^{n}\left(\overline{Y} - \overline{\overline{Y}}\right)^2}{a-1} \approx \sigma^2$$

since the sum of the squared deviations of class means from the grand mean is constant for all *n*.

So now we have two independent estimates of the common population variance. One, called MS_{within}, is calculated as the average within sample variance and is independent of information on variation among sample means. The other, called MS_{among}, is calculated from the observed variation among sample means, and is independent of information on the variability of measurements within samples. Both estimate the parameter σ^2.

$$MS_{among} = n.VAR\left[\overline{Y}\right]$$

$$= \frac{\sum^{a}\sum^{n}\left(\overline{Y} - \overline{\overline{Y}}\right)^2}{a-1} \approx \sigma^2$$

$$MS_{within} = \overline{VAR\left[\overline{Y}\right]}$$

$$= \frac{\sum^{a}\sum^{n}\left(Y - \overline{Y}\right)^2}{a(n-1)} \approx \sigma^2$$

We could test to see if these two estimates of the common population variance differ significantly by performing an F-test:

$$F = \frac{MS_{among}}{MS_{within}} = \frac{34.97}{29.38} = 1.19$$

MS_{among} is a variance estimate based on *a*=7 values (the seven sample means) and so has *a*-1=6 degrees of freedom. MS_{within} is the average of *a*=7 variances, each with degrees of freedom, and so has *a*(*n*-1)=28 degrees of freedom. For reasons that will become apparent later, the F-test in ANOVA is a one-tailed test.

From tables:

$F_{0.05(1)[6,28]} = 2.45$

and the two estimates of the common population variance are not significantly different. This should come as no surprise because, for replicated transects, MS_{among} and MS_{within} independently estimate the same parameter, the common population variance σ^2. If you are unable to follow the argument at this point, refer back to hypothesis testing and the F-test in Module 3.

Consider now what happens when we apply a differential treatment to the transects. While MS_{among} and MS_{within} are independent estimates of the common population variance, they differ in one important property. If we introduce a factor that alters the mean algal biomass of some of the transects but not others, while maintaining the spread of biomass measures about the mean for each transect, then MS_{among} will be inflated by the differential treatment while MS_{within} will remain unbiased. This can be best visualised by considering Figure 4–2.

Table 4–4. Mean and variance in algal biomass (mg/m^2) collected from paving bricks laid along transects in shallow stream riffle. Means for transects 1, 2 & 3 are inflated by application of an insecticide (+ 15 mg/m^2).

	Transect						
	1	2	3	4	5	6	7
Mean	38.3	32.1	35.3	25.7	23.5	19.5	22.6
Variance	14.24	58.57	23.34	38.35	21.69	14.73	34.75

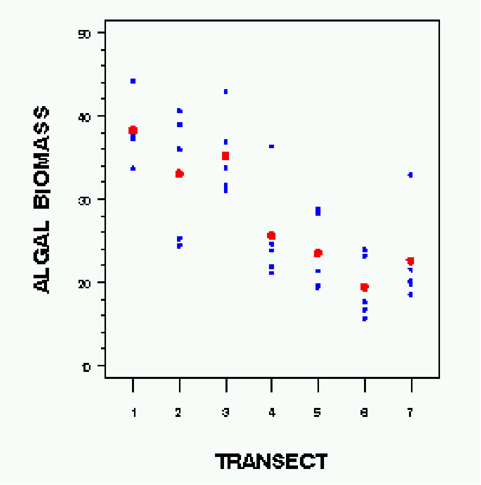


Figure 4–2. Variation in algal biomass (mg/m^2) collected from paving bricks laid along transects in shallow stream riffle. Means are shown as dots. Means for transects 1, 2 & 3 are inflated by application of an insecticide.

Table 4–4 and Figure 4–2 show the situation where a differential factor is applied to the transects. Algae reproduce and grow on each paver only to be consumed by a variety of invertebrate grazers. The biomass present at any one time reflects a balance between these two processes. Paving tiles in transects 1, 2 and 3 had the residual insecticide Pyrethrum applied to reduce grazing pressure upon them.

The obvious effect of this was to increase the standing crop of algal biomass on the treated tiles compared with the untreated tiles, by 15 mg/m^2 in each case. In terms of the calculations presented above, the effect of the differential application of insecticide was to increase the variability among the sample means. Provided this effect was additive, the relative position of the transect means would be altered, but the spread of biomass measurements about those means would remain the same.

Hence MS$_{\text{among}}$ becomes an inflated, biased estimate of the common population variance, because of the differential effect of the insecticide, and MS$_{\text{within}}$ remains an unbiased estimate of the common population variance. This bias can be tested using the F-test.

Because the variance among the means is inflated by the differential effect of the factor applied, we have:

$$VAR\left[\overline{Y}\right] \approx \sigma_{\overline{Y}}^2 + \sigma_A^2$$

where $\sigma_{\overline{Y}}^2$ is the variance among means expected by chance alone and σ_A^2 is the added variance component due to the effect of the factor applied differentially to the various transects. For MS$_{\text{among}}$ we have:

$$MS_{among} = n.VAR\left[\overline{Y}\right] \approx n.\sigma_{\overline{Y}}^2 + n.\sigma_A^2$$

$$\approx \sigma^2 + n.\sigma_A^2$$

As MS$_{\text{within}}$ continues to estimate σ^2 without bias

$$F = \frac{MS_{among}}{MS_{within}} \approx \frac{\sigma^2 + n,\sigma_A^2}{\sigma^2}$$

The null hypothesis is

$$H_0 : \sigma^2 + n.\sigma_A^2 = \sigma^2$$

or in other words

$$H_0 : \sigma_A^2 = 0$$

The alternative hypothesis is

$$H_1 : \sigma^2 + n.\sigma_A^2 > \sigma^2$$

or in other words

$$H_1 : \sigma_A^2 > 0$$

since a variance component cannot be negative. Hence, the F-test of ANOVA is a one-tailed test.

Under the null hypothesis, $\sigma_A^2 = 0$ and so MS_{among} and MS_{within} both estimate the common population variance σ^2. The F ratio would be around 1, subject only to sampling error. If the added variance component σ_A^2 is non-zero, then the F ratio will, on average, be greater than 1 by more than would be expected by chance alone, and the result will, on average, be significant.

If the factor has no effect then, barring a Type I error, the results of the ANOVA will support the null hypothesis. If the factor has an effect then, barring a Type II error, the result of the ANOVA will be significant.

For the data at hand (Table 4–4), we have:

$$MS_{among} = n.VAR\left[\overline{Y}\right] = 250.19$$

$$MS_{within} = \overline{VAR\left[Y\right]} = 29.38$$ as before.

$$F = \frac{MS_{among}}{MS_{within}} = \frac{259.19}{29.38} = 8.82$$

When compared to the tabulated F of 2.45 (see page 4-11), the result is highly significant. In fact, the probability of getting an F ratio of 16.96, or one more extreme by chance alone, is less than 0.0001. We conclude that there is an effect of the differential application of pyrethrum on algal biomass.

Hence, by comparing two estimates of the common population variance, one biased by the effect of the factor under consideration and the other remaining unbiased whether or not the factor has an effect, we are able to decide whether or not the differential treatment had an effect of the means. We come to a decision on the equality of the sample means based on a comparison of variances.

An intuitive view

The above interpretation of F in ANOVA as the ratio of two independent estimates of the common population variance may be satisfying for those of us with a sound education in statistical theory, but for the rest of us, it is a difficult concept to retain in plain language thinking.

Recall that the observed variance of the sample means is given by

$$VAR\left[\overline{Y}\right] = \frac{\sum^{a}\left(\overline{Y} - \overline{\overline{Y}}\right)^{2}}{a-1}$$

which can readily be calculated from the set of a sample means. This is how variable the means actually are.

But given the average variation within samples, MS_{within}, we would expect the means to vary by

$$\frac{MS_{within}}{n}$$

If we compare the observed with the expected in the form of an F ratio

$$F = \frac{VAR\left[\overline{Y}\right]}{MS_{within}/n} = \frac{n.VAR\left[\overline{Y}\right]}{MS_{within}} = \frac{MS_{among}}{MS_{within}}$$

which is the same F ratio that we use in the ANOVA.

Hence, the F in ANOVA can be viewed as follows.

F in single-factor ANOVA provides a comparison of how variable the sample means are with how variable they are expected to be, given observed variability within samples.

If the sample means are significantly more variable than expected by chance alone, we say that there is a significant difference among the means, or that the factor under consideration has a significant influence on the means.

Some Common Terms

At this point, it is worth reviewing the terms used so far and introducing some new terms.

In single-factor analysis of variance, two measurements are abstracted from each entity under consideration, whether the entities be points in a stream, rats in an experiment, paving bricks in a stream or water alloquots. One measurement is of a discrete variable or **factor** and the other is a continuous variate called the **response variable**. The factor takes on a set of discrete values called **factor classes**. The initial objective of ANOVA is to decide whether the factor has an effect on the response variable. Examples of factors, factor classes and response variables are given in Table 4–5.

Table 4–5. Examples of the sort of data required for single-factor ANOVA. The factor is the variable whose effect is under scrutiny, the factor classes are the discrete values taken on by that factor, the entities are the objects measured, both with respect to the factor and the response variable, and the response variable is the variable that is affected by the level of the factor.

Factor	Factor classes	Entities	Response variable
Waterbody	Grayson Pond, Beaver Lake, Rock River	Water samples	Strontium $(\mu g/l)$
Sex	Male, Female	Feral Pigs	Grain intake (kg)
Site	A-E	Water Samples	Macro-invertebrate counts
Laboratory	LAB01-25	Biological samples	Chlorophyll a
Species	E. aurifrons, E. albifrons, E. tricolor	Birds	Duration of territorial chase
Method	Interpolated mapping, minimum convex polygon, Dirichlet tessellation	Badgers	Home range size
Topographic Position	Hilltop, Valley, Sth Slope, Nth Slope	Soil sample	Phosphorus

The ANOVA Table

It is customary to present the results of an ANOVA in the form of an ANOVA Table (Table 4–6). Only four items in this table should be familiar at this point — MS_{among}, MS_{within}, F and the probability under H_o.

Table 4–6. Summary of the analysis of variance used to compare algal biomass (mg/m^2) collected from paving bricks laid along transects in a shallow stream riffle. Means for three transects are inflated by application of an insecticide.

Source	Degrees of freedom	Sums of squares	Mean square	F value	Prob under H_0
Among transects	6	1555.11	259.19	8.82	P<0.0001
Within transects	28	822.58	29.38		
Total	34	2377.69			

The sums of squares and degrees of freedom are components of the mean squares. If we consider the general form of a variance:

$$VAR[Y] = S^2 = \frac{\sum^{n}\left(Y - \overline{Y}\right)^2}{n-1}$$

It can be seen to comprise two components. The numerator is the sum of the squared deviations of the Y values from their mean, that is the **sum of squares**. The denominator is called the number of **degrees of freedom**, representing the number of independent deviations upon which the sums of squares is based.

There are several ways of gaining an appreciation of why it is *n*-1 degrees of freedom and not *n* degrees of freedom, a difficult concept to grasp without a full appreciation of the mathematical basis of statistics. Essentially, ten independent values comprise ten independent pieces of information, because knowledge of one value provides no information per se on any other value. Knowledge of the sample mean uses up one piece of information, because if you know the mean and nine of the sample values, then the tenth sample value is uniquely determined (you have 10 equations in ten unknowns). Similarly, once you specify nine deviations from the sample mean, the tenth is uniquely determined, since the sum of deviations about the mean must be zero. Only nine of the ten deviations are free to vary — there are nine degrees of freedom.

Now consider the equations for MS_{among} and MS_{within}

$$MS_{among} = \frac{\sum^{a}\sum^{n}\left(\overline{Y} - \overline{\overline{Y}}\right)^2}{a-1}$$

$$MS_{within} = \frac{\sum^{a}\sum^{n}\left(Y - \overline{Y}\right)^2}{a(n-1)}$$

From these we can define:

$$SS_{among} = \sum^{a}\sum^{n}\left(\overline{Y} - \overline{\overline{Y}}\right)^2$$

with (*a* - 1) degrees of freedom, and

$$SS_{within} = \sum^{a}\sum^{n}\left(Y - \overline{Y}\right)^2$$

with *a*(*n* - 1) degrees of freedom.

The associated degrees of freedom make intuitive sense because $\mathrm{MS}_{\mathrm{among}}$ is a variance based on a means, hence (*a*-1) degrees of freedom, whereas $\mathrm{MS}_{\mathrm{within}}$ is a variance calculated as an average of *a* sample variances each with (*n* - 1) degrees of freedom.

Partitioning the sums of squares

The statistical model developed above considers the values taken by individual measurements of the response variable to be the sum of various effects:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where Y_{ij} is the j^th value in the i^th sample, α_i is the grand mean for the combined data, μ_i is a deviation of class mean from the grand mean and e_{ij} represents the deviations of each measurement from its class mean. This equation is referred to as the ANOVA model. In plain English, it states that the value taken by each measurement of the response variable is composed of the grand mean of the population (centred), a deviation of its class mean from the grand mean, and its own deviation from its class mean.

We can rearrange this equation to yield:

$$Y_{ij} - \mu = \alpha_i + e_{ij} = (\mu_i - \mu) + (Y_{ij} - \mu_i)$$

which states that the deviations of the individual measurements from the overall mean comprise the sum of their deviations from their class mean and the deviations of the class mean from the overall mean. When we represent the parameters of this equation by their sample estimates, we have algebraic identity:

$$\left(Y_{ij} - \bar{\bar{Y}}\right) = \left(\bar{Y} - \bar{\bar{Y}}\right) + \left(Y_{ij} - \bar{Y}\right)$$

It is simple to prove, though it is not done here, that the same is true of the sums of squares.

$$\sum^{a}\sum^{n}\left(Y - \bar{\bar{Y}}\right)^2 = \sum^{a}\sum^{n}\left(\bar{Y} - \bar{\bar{Y}}\right)^2 + \sum^{a}\sum^{n}\left(Y - \bar{Y}\right)^2$$

$$SS_{total} = SS_{among} + SS_{within}$$

and for the corresponding degrees of freedom

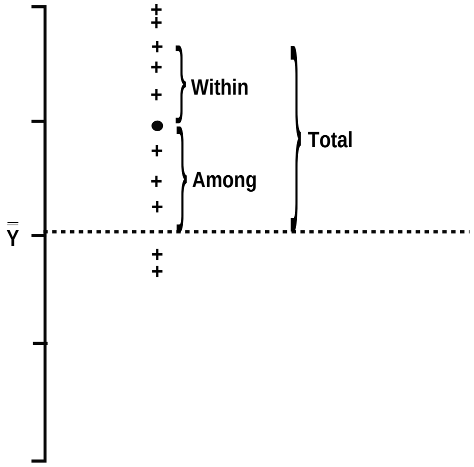
$$an - 1 = a - 1 + a(n - 1)$$

Note that the SS_{total} is calculated by pooling the data from all samples and calculating the squared deviations of the individual sample values from the overall grand mean. There are a times *n* values in all, and therefore (*an* - 1) degrees of freedom.

The above equality is what is referred to as a **partition of the sums of squares**.

When dealing with the sums of squares, it is sensible to talk of partitioning the total sums of squares into two components, one representing variation of the individual *Y* values about their own mean, and one representing variation of the sample means about the grand mean. This is shown diagrammatically in Figure 4–3.

Figure 4–3. A diagrammatic representation of the partition of sums of squares in single-factor ANOVA.



Partitioning the mean squares is not possible, since

$$\frac{\sum^{a}\sum^{n}\left(Y - \bar{\bar{Y}}\right)^2}{an - 1} \neq \frac{\sum^{a}\sum^{n}\left(\bar{Y} - \bar{\bar{Y}}\right)^2}{a - 1} + \frac{\sum^{a}\sum^{n}\left(Y - \bar{Y}\right)^2}{a(n - 1)}$$

$$MS_{total} \neq MS_{among} + MS_{within}$$

The variance obtained by pooling all the data cannot be decomposed neatly into MS_{among} and MS_{within}. It is not sensible to talk of partitioning the mean squares.

Because of their special additive properties, the sums of squares are included in the ANOVA table with the mean squares. It is also traditional to include the degrees of freedom for each (Table 4-6).

Where have we come?

In this lesson, we have covered the basics of single factor ANOVA. It is a very important lesson, because the understanding it conveys is essential for you to apply ANOVA with the flexibility you need to address the very great range of applications of the technique.

You should appreciate

- That the single factor ANOVA is an extension of the T-test, in that it enables the simultaneous comparison of two or more means.
- That there is a simple relationship between the variation within samples and the expected variation among sample means, in the absence of any treatment effect, namely

  $$\sigma_{\bar{Y}}^2 = \frac{\sigma^2}{n}$$

  and that this relationship forms the basis of the analysis.
- That ANOVA can be understood from the point of view of two estimates of the population variance common to all samples (under the null hypothesis), one biased by any effect of the treatment and one unbiased.
- That alternatively, ANOVA can be understood from the point of view of comparing observed variation among the means with that expected, given the observed level of within sample variation.
- The terminology commonly used in ANOVA, and in particular, the **factor**, with several discrete **factor levels**, that influence the **response variable**.
- That the **Mean Square** is just another name for variance.
- That a Mean Square can be decomposed into two elements, the numerator **Sums of Squares** and the denominator **degrees of freedom**.
- That unlike the mean squares, the sums of square and the degrees of freedom are additive, so it makes sense to talk of carving up the cake (partitioning the sums of squares) into components attributable to within and among sample variation.
- That there is a formal basis for reporting the results of an ANOVA, called an **ANOVA Table**.

If little of this makes sense, you will need to review the above theory, or seek alternative explanations in a text of your choice. It is essential that you understand the above concepts before moving on.

Lesson 2: Multiple Comparison Tests

Where to from here?

In the study of algal growth on submerged pavers subjected to differential application of insecticide, the analysis of variance, summarised succinctly in the ANOVA table (Table 4–6), demonstrates a significant difference among the means (F = 16.96; df = 6,28; p < 0.0005).

What the ANOVA results do not tell us is which of the means differ from which others. This is critical information, because a range of possibilities exists. All means may differ significantly from all others, only one mean may stand out significantly from all the rest, or one of a range of possibilities in between may apply. Clearly, we must carry the analysis further.

A first guess at how to tackle this problem would be to apply a series of Student's t-tests to the set of means taken two at a time. This is inappropriate for several reasons. First, each t-test would use only a fraction of the available data, being based on only two variances at a time. The power of such an approach would be greatly reduced.

Second, applying a series of t-tests in a single connected analysis results in compounding of errors. Whenever a test is applied at the = 0.05 level of significance, there is a 5% probability that a significant result will emerge when in fact there is no real difference between means (ie a Type I error). If there are seven means to compare, then 21 comparisons will be required. The probability of at least one of these comparisons being significant when there is no real difference among the means is not 0.05 but closer to:

$$1 - (1 - 0.05)^{21} = 0.66$$

The majority of statistical practitioners find this unacceptable, and prefer procedures whereby the probability of obtaining any Type I error among the totality of pairwise comparisons is 0.05.

What we need is a test that:

- uses all the data as a basis for comparisons, and
- adjusts the critical value for significance such that there is a probability of 0.05 of obtaining any Type I error at all among the pool of related pairwise comparisons.

The probability of obtaining any Type I error at all in a related set of comparisons is called the **experimentwise error rate**, horrid jargon, but difficult to avoid. A wide range of possible tests that meet these requirements have been developed, and are classed under the broad heading of **multiple comparison tests**.

Multiple comparison tests

One of the simplest multiple comparison tests to understand is Tukey's Honestly Significant Difference for equal sample sizes, generalised to the **Tukey-Kramer Method** for unequal sample sizes. It begins by taking the formula for *t* of the student's t-test

$$t = \frac{\overline{Y}_1 - \overline{Y}_2}{\sqrt{\frac{1}{n}\left(S_1^2 + S_2^2\right)}}$$

and replacing S_1^2 and S_2^2 with $\mathrm{MS}_{\mathrm{within}}$. After all, $\mathrm{MS}_{\mathrm{within}}$ is the best available estimate of the population variance common to all samples.

$$t = \frac{\overline{Y}_1 - \overline{Y}_2}{\sqrt{\frac{2}{n} MS_{within}}}$$

Instead of having 2(*n* - 1) degrees of freedom, this t test based on MS_{within} has *a*(*n* - 1) degrees of freedom, and in that sense is more powerful (since the critical value of *t* declines toward 1.96 as the number of degrees of freedom increases).

The next step is to replace the standard critical values of *t* with more appropriate values, to compensate for the compounding of errors that occurs when multiple related comparisons are performed. The Tukey-Kramer procedure assumes that the investigator intends to perform exhaustive pairwise comparisons between all means used in the ANOVA. Tables with appropriate adjustment of the critical values are available in the form of the Studentised Range, written $Q_{\alpha[a,a(n-1)]}$ (see Table 18 of Sokal and Rohlf, 1994). These tables give the critical values for the difference between two of a means. The tables are based on a rearrangement of the above formula, noting that *n* and MS_{within} are constant for all comparisons. The test will be significant if:

$$t = \frac{\left|\overline{Y}_1 - \overline{Y}_2\right|}{\sqrt{\frac{2}{n} MS_{within}}} > \left(tabulated\_critical\_value\right)$$

that is, if

$$\left|\overline{Y}_1 - \overline{Y}_2\right| > (critical\_value)\sqrt{\frac{2}{n}MS_{within}}$$

$$> Q_{\alpha[a,a(n-1)]}\sqrt{\frac{MS_{within}}{n}}$$

$$> MSR$$

MSR is the minimum significant range between two means. Thus, a table of differences between means is constructed and the differences are compared with the *MSR*. The *MSR* is calculated from MS_{within}, the sample size *n*, and the tabulated value of the studentised range $Q_{\alpha[a,a(n-1)]}$.

The adjustment of the critical value for significance in multiple comparison tests has important implications. In order to achieve an experimentwise error rate of 0.05, the level of significance of each individual comparison is necessarily much lower than 0.05. If there are many means in the ANOVA, the actual error for individual comparisons between means may be a tenth, a hundredth or even a thousandth of the experimentwise error. Multiple comparison procedures are typically conservative, compared with the conventional pairwise t-tests.

There is a plethora of multiple comparison procedures available, differing from the Tukey-Kramer Method, and from each other, in:

- the level of adjustment necessary to compensate for the number of comparisons possible under the experimental design, and
- the approach taken to make those adjustments.

Some options are described below. For a more detailed treatment of the subject of multiple comparison tests, refer to Keppel (1973) and Day and Quinn (1989).

Unplanned comparisons

Unplanned comparisons are a subset of possible comparisons chosen on the basis of information obtained from examination of the results of the ANOVA. In many cases, unplanned comparisons are exhaustive, comprising comparisons of all possible combinations of the available means. In this sense, unplanned comparisons are exploratory, looking for the most plausible explanation for the overall significant result obtained in the ANOVA. Of the techniques available for unplanned multiple comparisons, the Tukey-Kramer Method is recommended.

Tukey-Kramer Method (Option TUKEY)

The Tukey-Kramer Method is suitable for exhaustive comparisons among means where it is assumed that the pool of possible comparisons includes only those between the means taken a pair at a time. This technique has fared extremely well in Monte Carlo simulations (Dunnett 1980a,b; Day and Quinn, 1989), and has been shown to control the experiment-wide error rate to the desired level (Hayter, 1984). The rationale for the test has been described above.

Student-Newman-Keuls Method (Option SNK)

The Student-Newman-Keuls method is a stepwise test where the means are arranged in order and the level of significance assigned to a test depends on the level of separation between the two means being compared. The actual experiment-wide error rate for the SNK method is not easy to estimate, but it is not contained below or even in the vicinity of the desired level (usually 0.05). Indeed, as the number of means increases, the experimentwise error rate approaches unity (SAS Institute, 1989: 947), which is totally unacceptable.

The Student-Newman-Keuls method has not been recommended by statisticians for many years, and is effectively debunked in the SAS Manuals (SAS Institute, 1989: 947). Nevertheless, it is one of the most popular multiple comparison procedures among ecologists (Day and Quinn, 1989). One can only assume that ecologists are vaguely uncertain about accepting the inherent conservatism of multiple comparison procedures (see Keppel 1973), and are opting for the 'half-way-house' of the Student-Newman-Keuls method (Option SNK) which, in terms of conservatism, lies between the Tukey-Kramer method (Option TUKEY) and not making any adjustment at all to compensate for the number of related comparisons (Option LSD).

The Student-Newman-Kuels test is not recommended.

If you wish to use a stepwise procedure for multiple comparisons, the the REGWQ procedure is appropriate, at least for balanced designs. REGWQ stands for the Ryan-Einot-Gabriel-Welch range test. It is a more modern version of the SNK test that actually controls the experiment-wide error rate to less than 0.05.

Bonferroni and Sidak Methods (Options BON and SIDAK)

The Bonferroni method depends upon the observation that if we set the significance level for each of *k* comparisons at

$$\frac{\alpha}{k} = \frac{0.05}{k}$$

then the probability of obtaining one or more Type I errors across all *k* comparisons (the experimentwise error rate) will be less than 0.05. Hence, by setting the level of significance of tests between a means taken a pair at a time at

$$\frac{\alpha}{k}=\frac{0.05}{a(a-1)/2}$$

we can be sure that the experimentwise error rate is less than 0.05.

The Sidak method depends upon the observation that if we set the significance level for each of k comparisons at

$$1-(1-\alpha)^{1/k}=1-(1-0.05)^{1/k}$$

then the probability of obtaining one or more Type I errors across all *k* comparisons (the experimentwise error) will be less than 0.05. Hence, by setting the level of significance of tests between a means taken a pair at a time at

$$1-(1-\alpha)^{2/a(a-1)}=1-(1-0.05)^{2/a(a-1)}$$

we can be sure that the experimentwise error rate is less than 0.05.

Of the two procedures, Sidak's is less conservative than Bonferroni's and is therefore preferred.

The advantage of these procedures is that they can provide simultaneous tests of more than one related hypothesis in a wide range of contexts. All that is required is knowledge of the number of comparisons in the total pool of comparisons possible (ie *k*) and the level of significance required for the experimentwise error rate (i.e. $\alpha=0.05$). The pool of possible comparisons can vary, depending upon the experimental design, from exhaustive comparisons of all means against all other means taken singly or in combination, to exhaustive comparisons between pairs of means, to a restricted set of comparisons (see planned comparisons below). The disadvantage of the two techniques is that because they rely on inequalities, which merely set upper bounds to the experimentwise error rate, they are typically more conservative than other available procedures such as the Tukey-Kramer procedure and the Student-Newmann-Keuls procedure.

Planned comparisons

Planned comparisons are a subset of possible comparisons, chosen before the experiment is done so that they are not suggested by the results. Planned comparisons are typically decided as part of the overall experimental design. The pool of possible comparisons is typically severely restricted by the experimental design.

Comparing all treatments to a control

A special case of multiple comparisons is where the only comparisons contemplated at the time of designing the experiment are between a single control and a set of treatments. In this case, improved power can be legitimately achieved by adjusting the level of significance on individual tests only for this restricted class of comparisons. Dunnett (1956) proposed a test to cater for this situation, and it is now widely used. **Dunnett's test** (Option DUNNETT) holds the experimentwise error to a level not exceeding the stated level of significance, usually 0.05.

The alternative of using Bonferroni's or Sidak's method is considered unnecessarily conservative in comparison with Dunnett's test, but may be the best option when faced with more complex designs involving several control and treatment samples.

A priori unrelated comparisons

There are rare instances where relatively few, completely independent comparisons are contemplated using the results of an ANOVA based on a more extensive data set than required to address the hypotheses of interest. Providing the investigator is willing to accept an error rate of 0.05 on each comparison, then it may be legitimate to apply t-tests independently to each hypothesis. It is still sensible to use the best available estimate of the common population variance, MS_{within}, in place of the individual sample variances, and a suitable test in this case is the Least Significant Difference Test (Option LSD). The occasions for its use are rare indeed, and it should definitely not be used for unplanned comparisons or for comparisons with a control.

Deciding a personal policy

Appropriate choice of multiple comparison test remains a controversial subject and you may wish to address the issues yourself. You need to:

- Consider whether you accept at all, the need to adjust the level of significance on each test to compensate for the number of comparisons in an experiment.

Not all researchers accept this need, though nowadays, they would have difficulty publishing.

- Decide the total pool of comparisons contemplated as part of the study design, in advance of collecting the data.

For five means, there is a pool of 5(5-1)/2 = 10 exhaustive pairwise comparisons possible, but only four comparisons are of interest if the object is to compare four treatment means against a control. This is the most important criterion in choosing a multiple comparison procedure.

- Choose a multiple comparison procedure that adjusts the per-comparison error rate appropriately for the pool of comparisons identified above. It should be the most powerful of appropriate alternatives available.
- Decide the level of significance required for the experimentwise error rate.

There is general agreement on the acceptable per-comparison error rate, usually 0.05, but certainly in the range 0.10 to 0.01—not so large as to allow a large number of false conclusions regarding the presence of differences between means, but not so small as to greatly reduce our chances of detecting differences when they are present. There is no general agreement on an acceptable experimentwise error rate for decision making—is it 0.05, 0.10 or 0.20 (Keppel 1973)? For the purposes of this Module, I have chosen 0.05, but this is subject to debate.

There are a few misconceptions associated with multiple comparison procedures. A common error is to argue for a reduced pool of comparisons after the data have been examined. Following an analysis of variance, you may choose only to compare the largest and smallest of the means. You have chosen to do a single comparison only, but this does not justify ignoring the need to adjust the per-comparison error rate for the overall pool of such comparisons from which you selected one.

A second error is commonly made when the response to the confusing array of options available is to choose the option that gives the most satisfactory results. The folly of this approach should be obvious. You may be confronted with a confusing and uninterpretable array of overlapping non-significant subsets of sample means, using, say, the Tukey-Kramer procedure. You need to appreciate that failure to reject the null hypothesis that two population means are equal, should not lead you to conclude that they are in fact equal. It implies only that the difference between population means, if any, is not large enough to be detected with the given sample sizes. Your approach should be to gather more data, or to redesign and repeat the experiment giving more thought to planned comparisons. It even may be appropriate to increase the experimentwise error rate to 0.10 or 0.20, provided this is openly stated. It is not appropriate to cast your eye around for a more powerful but inapplicable multiple comparison test such as the LSD test.

Confusion may arise from the non-transitive nature of non-significance. The mean of sample A may be significantly different from the mean of sample B, but neither may be significantly different from a mean lying in between. When the sample sizes are unequal, the results may be even more counter-intuitive. If we have four cells with means ranked A>B>C>D, the difference between B and C, each based on 1,000 values, may be significant while the difference between A and D, each based on 5 values, might not.

Where have we come?

The full analysis is now available to you. As a researcher, when you are faced with the problem of comparing the means of several samples, classified according to a single factor, the approach is as follows:

- perform a single-factor ANOVA to determine whether the means are more variable than can be considered by chance alone;
- if the ANOVA yields a significant result, perform an appropriate multiple comparison test to determine where the differences lie.

Views on the application of multiple comparison tests are varied, and the subject remains controversial. You need to come to a reasoned position yourself. The recommendations I make are :

- If unplanned comparisons are required as part of the experimental design, apply the Tukey-Kramer procedure.
- If planned comparisons involving a single control are required, apply Dunnett's test.
- If the design is more complex, but where the pool of comparisons possible under the design is restricted, then the Sidak procedure is recommended.
- In rare cases, where a few unrelated comparisons are decided independent of the data at hand, and independent of each other, the LSD procedure may be applied.

What you do not yet know is that there are different models in ANOVA, and that the choice of model affects the direction your analysis takes. That is the subject of the next module.

Lesson 3: Models in ANOVA

Fixed and Random Models

One more class of analysis is yet to be introduced. Two models are recognised in single-factor ANOVA—fixed models and random models. The two differ little in the computation leading to the final F statistic. Where they differ is in the direction taken during follow-up analysis.

Fixed model ANOVA

In the fixed model ANOVA, the criterion upon which the factor levels are chosen are fixed and repeatable. For example, each sample corresponding to a factor level may be drawn from individuals of specified ages, or subject to different specified treatments, or belong to specified genetic strains. A case might involve a pastoralist who wishes to compare the ability of breeds of cattle to produce butterfat in the milk. Here, the factor is breed and the response variable is butterfat concentration. If the objective of the study is to select the best breed for butter production, then the approach would be to take five specific breeds (the factor levels) known to be candidates for the new herd, probably including the breed that makes up the existing herd.

This is a fixed design because:

- the breeds chosen for investigation are fixed in the sense that if the experiment were to be repeated, the same breeds would be chosen again,
- the investigation is designed to detect significant differences among specific breeds, in this case, to find the breed with the highest butterfat content in the milk,
- it would be sensible to follow up the analysis with multiple comparison tests to determine where the differences among the means, if any, lie.

Random model ANOVA

In the random model ANOVA, the criteria upon which the factor levels are chosen are random—that is, the factor levels are chosen at random from a substantial pool of possible choices. NSW Agriculture may wish to address a similar problem to that of the pastoralist, but without a specific interest in any one breed. Instead, they may be interested in the more general problem of whether the factor breed has any effect on butterfat production. From their perspective, there may be 50 breeds available for experimentation, but financial and logistic constraints preclude using all of them. They choose five breeds at random.

The questions that would be asked of such a design are quite different from those asked of a fixed design. NSW Agriculture are not at all interested in whether there are significant differences between specific breeds, because they were selected at random. All they are interested in is whether or not there are differences among breeds overall, that contribute to variation in butterfat production. In statistical jargon, they are interested in whether there is an added variance component due to variations among breeds in butterfat production. If there is, they might proceed to measure the relative strength of the added variance component.

In the random design:

- the factor levels (breeds) are randomly selected from a large pool of possible choices. If the experiment were to be repeated, there is no reason to expect that the same factor levels (breeds) would be chosen second time around;
- the investigation is not designed to detect significant differences among specific breeds, but rather to detect an overall effect of the factor—breed—on the response variable, butterfat concentration;
- it is not sensible to follow up the analysis with multiple comparison tests to determine where the differences among the means, if any, lie. Rather, the added variance component due to the overall effect of the factor — breed — is estimated.

Estimating the added variance component

Recall from the discussion of rationale of ANOVA that the mean squares are estimates of the common population variance, but that one, *MS_{among}*, is biased by the effect of the factor.

$$MS_{among} \approx \sigma^2 + n.\sigma_A^2$$

$$MS_{within} \approx \sigma^2$$

To estimate the added variance component, we need an estimate of σ_A^2. Such an estimate is provided by:

$$S_A^2 = \frac{MS_{among} - MS_{within}}{n} \approx \sigma_A^2$$

There are two intuitive interpretations of S_A^2. First, it provides an estimate of how much of the variability among means cannot be explained by observed variability within samples. It is a measure of the **strength of the real effect** of the factor, which can be expressed as a percentage:

$$\frac{S_A^2}{\frac{MS_{within}}{n}+S_A^2}x100\%$$

Second, it provides a measure of how reproducible measurements are across samples relative to the repeatability of measurements within samples. Later in this Module, an example is presented where the National Association of Testing Agencies (NATA) was interested to know to what degree chlorophyl-*a* determinations were repeatable within labs and to what degree results were reproducible across labs. A random sample of 25 laboratories Australia-wide were asked to extract and determine the concentration of chlorophyl-*a* in three samples labelled A, B and C. The laboratories involved were not told that the three samples were simply replicates of the same batch.

We need to ask, how much more variable would be a set of 25 determinations, one from each laboratory, than a set of 25 determinations from a single laboratory?

Variation in single determinations across laboratories is given by:

$$VAR\left[\overline{Y}\right] \approx \sigma_{\overline{Y}}^2 + \sigma_A{}^2 = \frac{\sigma^2}{n} + \sigma_A^2 = \sigma^2 + \sigma_A^2$$

since, for single determinations, n = 1. Variation in single determinations within a laboratory is given by:

$$VAR\left[Y\right] \approx \sigma^2$$

Hence, of the total variation among single determinations taken one from each laboratory, an amount of:

$$\frac{\sigma_A^2}{\sigma^2+\sigma_A^2}.100\%$$

is *additional* to what would be expected of values taken from a single laboratory, namely:

$$\frac{\sigma^2}{\sigma^2+\sigma_A^2}.100\%$$

Thus

$$\left(\frac{S_A^2}{MS_{within} + S_A^2}\right).100\%$$

is an estimate of the variation in single determinations from laboratories *over and above* what would be expected if the laboratories had common methods and equipment and could perfectly reproduce each others' results.

It is the percentage contribution to variation in single determinations among laboratories that can be attributed to differences in their equipment and procedures.

On this basis, we can define an index of **reproducibility**, the ability to reproduce results across laboratories, as:

$$\mathit{Reproducibility} = \left(1 - \frac{S_A^2}{MS_{within} + S_A^2}\right).100\%$$

Reproducibility ranges from zero to 100%, the latter being desirable in interlaboratory comparisons.

An estimate of the expected variation in single determinations across laboratories derived from variation in determinations within laboratories is:

$$\left(\frac{MS_{within}}{MS_{within} + S_A^2}\right).100\%$$

It is the percentage contribution to variation in single determinations <u>among laboratories</u> that can be attributed to the inability of laboratories to repeat their own determinations. It is the variation among laboratories that would exist even if all laboratories had identical equipment and procedures.

Thus, **repeatability** can be defined as the ability to repeat results in a single laboratory, relative to the ability to reproduce results across laboratories.

$$\mathit{Repeatability} = \left(1 - \frac{MS_{within}}{MS_{within} + S_A^2}\right).100\%$$

If there is no added variance component due to differences among labs ($S_A^2 \approx \sigma_A^2 = 0$), then the results will be perfectly reproducible. If you had 10 replicate bottles of water to evaluate, it would not matter whether you sent them to 10 different laboratories or all 10 bottles to the one laboratory. This is an unachievable ideal for NATA, though progress toward the ideal can be achieved by setting standards for equipment and procedures to be adhered to by member laboratories.

If the results from laboratories were perfectly repeatable $\left(MS_{within} \approx \sigma^2 = 0\right)$, then it would not make sense to send more than one replicate bottle to each laboratory. Rather, you would spread the bottles across laboratories to obtain the best average determination.

Of course, there are many possibilities in between.

Where have we come?

In this lesson, the two different models of ANOVA were introduced. These are important in single factor ANOVA because they determine the direction the analysis takes following a significant result in the ANOVA. They are even more important in more complex ANOVA designs because they influence the calculations in the ANOVA itself.

You should now appreciate

- The distinction between fixed and random models in ANOVA.
- The procedure used to follow up a significant result in a random model ANOVA.
- The distinction between repeatability and reproducibility, and how to construct meaningful measures of them from the ANOVA table.

We now move on to an important topic, that of power and how to interpret a non-significant result in ANOVA.

Lesson 4: Power analysis

Planning of experiments

The effectiveness of ANOVA is greatly influenced by sample size. Sample sizes need to be adequate to be reasonably certain of detecting an important effect when it exists. At the same time, sample sizes should not be so large that the cost of the study becomes excessive, nor is there much value in having weak, unimportant effects becoming highly significant. Planning the intensity of sampling using a **prospective power analysis** can be important in the design of experimental and observational studies that use ANOVA as the means of analysis.

As with the t-test, optimal sample size for an ANOVA will be affected by:

- **The size of the smallest effect or difference that it is important to detect**. The smaller the effect, the larger will be the sample size required to detect it, all other things being constant.
- **The variability of the data.** The more variable the data within samples, the more difficult it will be to demonstrate a given effect or difference among sample means against the backdrop of that variability.
- **The acceptable probability of detection**. The more certain you want to be of detecting a difference of a given size, the larger will be the samples required to give you that greater certainty.
- **The level of significance of the test.** It will take larger samples to be reasonably sure of detecting a given difference at the 1% level of significance than at the 5% level of significance.

There are obvious parallels with the procedure for determining appropriate sample sizes for the t-test. ANOVA has the added complication of needing a decision on exactly what effect it is that is of interest to us. A number of possibilities exist. The smallest effect of interest might be defined in terms of:

- the **smallest average effect** across samples that is regarded to be of importance;
- the **smallest single difference** between one sample mean from the overall average that is regarded to be of importance;
- a **minimal scenario** or set of minimal scenarios involving several sample means that is regarded to be of importance.

Estimating sample size under all of these scenarios is complex and not well covered by statistical packages. Only one approach is covered in this Module, for the fixed-factor ANOVA. The approach recognises that on achieving significance in the ANOVA, you will follow with multiple comparisons among the sample means. The objective of the power analysis is to set a sample size to be 80% sure, say, of detecting a true difference of given minimal magnitude δ at the level of significance after Bonferroni correction α'.

To be 80% sure ($P = 1 - \beta = 0.8$) of detecting a given difference between any two of ten means (δ) at the 5% level of significance ($\alpha = 0.05$), you will require a sample size of:

$$n \geq 2\left(\frac{\sigma}{\delta}\right)^2 \left(t_{\alpha'[\nu]} + t_{2(1-P)[\nu]}\right)^2$$

where *n* is the required size of each sample, σ is the true common parametric standard deviation, ν is the degrees of freedom for σ, $t_{\alpha'[\nu]}$ is the value from a two-tailed *t*-table with ν degrees of freedom and level of significance α' and $t_{2(1-\beta)[\nu]}$ is the value from a two-tailed *t*-table with ν degrees of freedom and level of significance $2(1-\beta)$. In this case, where we have 10 means, and therefore 45 potential pairwise comparisons,

$$\alpha' = \frac{\alpha}{45} = \frac{0.05}{45} = 0.001$$

The inequality above must be solved iteratively, as *n* is on both sides of the inequality, ν being a function of *n*.

To undertake such a prospective power analysis, you need also to make some hard decisions. First, you need to decide on what is the smallest difference between any two of your samples (δ) upon which you will place some importance. You must decide that differences smaller than that value are of little or no consequence.

Second, you need to estimate the common parametric standard deviation, σ, and this must often be estimated before you collect the data. You can use a ball-park figure based on experience, or you can undertake a pilot study to estimate σ using the root mean square error

$$\sqrt{MS_{within}} \approx \sigma$$

and then the desired sample size before expending resources on the major optimised study. You may find the ratio of δ to σ easier to estimate.

Third, you need to decide on the risk you are willing to take ($P = 1 - \beta$) in not finding an important difference when it actually exists. There is no general agreement on the value of P. The value of 0.80 seems to have currency in the same way as 0.05 has currency for α. Some would argue for higher values of 0.90 and 0.95, but ultimately it comes down to how important to you it is to detect a true difference of δ if it exists. What risk are you willing to take of missing it?

Finally, you need a clear idea of the experimental design and in particular, what comparisons will be made in following up a significant result. This is very important, as it will determine the level of Bonferonni correction that will be applied, and this in turn will affect the optimal sample sizes and expenditure on the project.

Regardless of the problems of its computation, the cost savings of this approach can be considerable, either through minimising the risk of undertaking the study only to find that no difference can be demonstrated (when it exists) or by avoiding the expense of collecting more data than is required for success.

Interpretation of non-significant results

Retrospective power analysis is used to decide if it is reasonable to accept the null hypothesis, that is, for dealing with the ambiguity of a non-significant result. There may well be no difference between samples, or the sample sizes may not be large enough to detect a difference that is there (you make a Type II error). The risk of such a false negative result cannot usually be quantified, unless the alternative hypothesis H_1 is known. A non-significant result, therefore, is difficult to interpret.

Strictly speaking, you would interpret a non-significant result equivocally, as having failed to demonstrate a difference. If however you do wish to draw a firm inference from a non-significant result, then a **retrospective power analysis** is mandatory.

In a retrospective power analysis, you ask, given your sample sizes, what might be the smallest difference (δ) you could be reasonably confident of detecting ($P = 1 - \beta = 0.80$). Using the MS_{within} as an estimate of σ^2, the formula for the smallest difference likely to be detected by an ANOVA, followed with Bonferoni comparisons, with sample sizes each of *n*, is:

$$\hat{\delta} \geq \left(t_{\alpha'[\nu]} + t_{2(1-P)[\nu]}\right)\sqrt{\frac{2MS_{within}}{n}}$$

where *n* is the size of each sample, MS_{within} is our best estimate of the true common parametric variance, ν is the degrees of freedom for MS_{within}, *P* is the intended power of the test, $t_{\alpha'[\nu]}$ is the value from a two-tailed *t*-table with ν degrees of freedom and level of significance α' and $t_{2(1-\beta)[\nu]}$ is the value from a two-tailed *t*-table with ν degrees of freedom and level of significance $2(1-\beta)$. In this case, where we have 10 means, and therefore 45 potential pairwise comparisons, again

$$\alpha' = \frac{\alpha}{45} = \frac{0.05}{45} = 0.001$$

If $\hat{\delta}$ is so small as to be of no consequence, then your interpretation of the negative result is acceptable. If, on the other hand, even a large difference would often go undetected with your sample sizes, you have nothing to report.

Retrospective power analysis is a controversial area, and the analyses for ANOVA have not adequately been incorporated into statistical packages. Many power analysis algorithms give you the optimal sample size to be reasonably sure of detecting the difference you observed in your analysis, which is not useful. Others differ on how to define the minimum effect size, with the decision having a strong effect on the outcome of the power analysis. The advice above is only one option available to you, and is over-engineered in the sense that the Bonferoni correction is one of the most conservative of multiple comparison procedures.

As with power analysis and the t-test, choice of the value for the probability of detecting the difference if it exists is controversial. It has been argued that just as a small α (Type I error) is required to declare a difference to be nonzero, so too a small β (Type II error) should be required to declare a difference to be zero. We have chosen $P = 1 - \beta = 0.80$ above, which is developing similar currency as $\alpha = 0.05$ as the defacto standard for the Type I error. Cogent arguments can be made for *P* = 0.84, 0.90 and 0.95.

Where have we come?

In this lesson, you learned about the concept of power, how to use it to plan experiments and how to use it to place interpretation on a non-significant result.

In particular, you should appreciate:

- The considerations necessary to determine the level of sampling intensity required to be reasonably sure of detecting a difference of a given magnitude when it exists.
- That non-significant results are ambiguous. It could be that there is no difference of any importance there to find, or it could be that there is an important difference to find, but that your sample sizes

are too small to demonstrate it. Power analysis allows you to make a judgement and so report a non-significant result with some level of confidence.

We have now covered the theory of single factor ANOVA. There are a number of matters to consider in applying this theory to real world problems, not least of which are the assumptions of the technique. The next lessons deal with nuances in the application of ANOVA.

Lesson 5: Application

Assumptions of ANOVA

Up to this point, ANOVA has been presented without much attention paid to the assumptions of the technique. This is the approach adopted by Sokal and Rohlf (1994), in the belief that non-mathematical audiences learn better if they come to understand the structure and purpose of the analysis, without being distracted from the central theme by whether or not the data are strictly amenable to such an analysis. However, it is essential for the practitioner to verify that the assumptions are reasonable. If they are not, steps should be taken to ensure that the assumptions are met.

In this section, I describe the assumptions of ANOVA, how to check if they are reasonable, and how to proceed in the face of perceived violations. Alternatives to parametric ANOVA are briefly described, but their practical application is beyond the scope of this Module.

Single-factor ANOVA has four assumptions, namely, randomness and independence in sampling, equality of variances across samples, and normality. Each of these will be dealt with in turn.

Randomness in sampling

Randomness in the selection of entities for measurement

In inferential statistics, we study samples intensively in order to infer attributes of the populations from which those samples are drawn. It is critical to any experiment to ensure that the measurements taken are representative of the system under study, if our inferences are to have any validity. The entities selected for measurement must be representative (unbiased) of the population from which the entities are drawn.

Take for example a study of stream invertebrates at each of several sites. We may choose to take 10 replicate collections of invertebrates from each site, in order to characterise invertebrate abundance at the sites and as a basis for comparisons among sites. The mean invertebrate abundance must be representative of the true parametric mean for the each site, and their variances must be representative of the true parametric variances for each site. If they are not, we may as well close up shop. Randomness in the selection of the entities for measurement is by far the safest way of achieving this unbiased representation.

Non-randomness may manifest itself as lack of independence of the entities, or in unequal variances or in non-normality. Violation of the assumption of randomness in sampling cannot be overcome easily, and typically the data must be discarded, the sampling protocols redesigned and the data recollected. Adequate attention must be paid at the time of designing an experiment, or when sampling from natural populations, to ensure adequate randomness in the selection of entities.

Randomness in the allocation of entities across factor classes

Where the allocation of entities to factor classes is within the control of the investigator, ANOVA expects that the items, individuals or entities are allocated to each of the factor classes at random. In a field trial, plots should be allocated at random to the treatments they are to receive. If we fail to do so, we run the risk of introducing a systematic bias that will confound our interpretation of a significant result. For example, if we consciously or unconsciously allocate the better looking plots first and so preferentially to a control factor class, and the poorer looking plots last and so preferentially to our manipulated factor classes, what are we to make of a significant result? We will not know if the significance is a result of our manipulations in comparison to the control, or a result of differences between better-looking plots and poorer-looking plots. Our experiment will have been **confounded**.

Often it is not possible to randomly allocate entities across factor classes. Having selected sites in a river for investigation, it is not possible to then randomly allocate invertebrate collections to them – the invertebrate collections are constrained to be those that are collected from the individual sites. But here the entities (invertebrate collections) are integrally connected with the characteristics of the site — confounding of the sort outlined above is not an issue. Provided we ensure that the entities within sites are selected at random, the ANOVA can proceed.

Independence

Independence requires that knowledge of the value of one measurement provides no information on the value of one of the other measurements, relative to its expected value. **Note that we demand that the residual values under the working model are independent, not the measurements themselves.** Human heights are not absolutely independent, for none of us are the size of gnats. However, randomly selected humans may have heights that are independent, in the sense that knowledge of the above average height of one individual provides no information on the height of the next, <u>relative to the average height</u>. Heights of twins are not independent, because if one twin is known to be of above average height, then the other is odds on to be of above average height.

Because independence is an attribute of the residuals (or if our focus is on the parent populations, the errors ε_i) independence is a relative concept. Dependence considered in one context (say ANOVA) may become independence in another (say regression).

Independence within factor classes

Measurements within factor classes must be independent. In other words, if you were to arrange the measurements in one factor class in some logical order independent of their magnitude (say in the order of collection), then their deviations from the average value, the residuals, should follow a random sequence. A run of large values followed by a run of small values would be cause for suspicion (see the runs test, Sokal and Rohlf, 1994). Adjacent plots on the ground in an experiment using plots spread out across a field, adjacent trees in a forest, identical twins among unrelated individuals, eggs from a single clutch in an experiment where eggs are taken from many clutches, successive hourly measurements of algal abundance over a four day period, are unlikely to be independent in their response to any experimental treatment applied to them.

The consequences of dependence within factor classes can be devastating. If replicates are more alike than randomly selected entities in the population (that is, pseudoreplicated), as a consequence of their interdependence, then MS_{within} will be deflated. Significant results will emerge from the analysis without basis. If replicates are less alike than randomly selected entities in the parent population, as a consequence of their interdependence, then MS_{within} will be inflated. Power of the test will be compromised.

Independence across factor classes

Measurements within factor classes must also be independent. If the value of a measurement in one factor class is more like the value in another factor class, by virtue of interdependence between the two measurements, then the effect will be to draw their respective means together, with consequential loss in power to detect a true difference between the factor classes. If the reverse is true, and the measurements have a negative dependence, then the effect will be to draw their respective means apart artificially, and a significant result may emerge without basis. Either way, the validity of the ANOVA is compromised.

A special case of repeated measures

A common scenario where measurements are dependent across factor classes is in repeated measures designs. The following data comprise the number of eggs in a clutch (clutch size) for successive clutches for a species of marine turtle. A numbered titanium tag attached to the front flipper established the female's identity. The measurements (clutch size) are repeated for each female as she returns to the nesting beach repeatedly in a single season.

While an inexperienced analyst might be tempted to undertake a single-factor ANOVA on these data, treating the females as replicates, this is not valid. Knowledge that the size of the first clutch of female X16017 is above average for first clutches provides information on the likelihood that her second, third and fourth clutches are likely to be above average. This invalidates the analysis, its power potentially dramatically reduced. The correct approach is to undertake a two-factor ANOVA (possible even in the absence of replication) with Clutch Number as one factor and Female as the second factor (see Module 5).

FEMALE	CLUTCH NUMBER			
Tag	1st	2nd	3rd	4th
X16005	136	.	133	129
X16013	111	105	107	113
X16017	192	191	164	188
X16024	111	121	111	122
X16029	138	.	123	135

Independence in random models

In random model ANOVA, the factor levels are selected at random from a large or infinite pool of possibilities. The object of such an analysis is to estimate the added variance component resulting from the influence of the factor. If there is dependence among the factor levels because of deficiencies in our experimental design (say pseudoreplication of factor classes), our estimate of the added variance component will be compromised.

Clearly the impact of dependence among factor classes in the random model, or among entities across or within factor classes in the fixed model, is profound.

Essentially, as with randomness in sampling, violations of the assumption of independence of errors cannot be overcome easily, and typically the data must be discarded, the sampling protocols redesigned and the data recollected. Adequate attention must be paid at the time of designing an experiment, or when sampling from natural populations, to ensure independence. Independence is achieved through appropriate experimental design.

Equality of variances

In developing the rationale of ANOVA, it was argued that the effect of the factor across samples should act differentially to increase or decrease the sample means, but not to differentially alter the sample variances. An assumption of the analysis is that the individual sample variances estimate a common population variance, that is, that the population variances are equal.

There are two approaches to deciding whether the assumption of equality of variances is tenable and taking remedial action if necessary. The first approach is quantitative, relying upon formal tests of equality of variances followed by transformation of the data, if necessary, and retesting. A test for the equality of two variances was introduced in Module 3, based on the two-tailed F-test. A variety of tests for equality of variances also are available where the number of means exceeds two, including Bartlett's, the F_{max} and the Scheffe-Box tests discussed by Sokal and Rohlf (1994). The difficulty with these tests, to varying degrees, is that they have assumptions of their own, and may well be more sensitive to violations of those assumptions than is the ANOVA itself. For example, Bartlett's test is particularly sensitive to departures from the assumption of normality, and a significant result may indicate non-normality rather than unequal variances.

The second approach, and the one adopted in this series, is more qualitative. It is based on visually examining the scatter of sample values about their predicted values, the factor class means. Ideally, this scatter should be random across the classes. There should be no systematic trend or difference in the scatter of values about their respective means.

The visual examination is achieved by constructing a plot of residuals, that is, by plotting the observed deviation of each sample value from its factor class mean against the predicted value for that class, namely the class mean. Often, the residuals are scaled by dividing by their standard errors, and referred to as studentised residuals. The method of plotting residuals is demonstrated in the worked examples that follow later in this Module.

If substantial heterogeneity of variances is revealed in the residual plot, then a transformation may be applied to bring the variance of the residuals closer to equality, or the original data may be scrutinised for a single suspect outlier in case an error has been made. Common transformations are described in a section below.

Normality

A single-factor ANOVA assumes that the individual measurements in each sample are drawn from a normally distributed population.

This assumption cannot be verified simply by pooling the data and applying a visual or statistical test for departures from normality, because the data for each sample may well be centered on quite different means. For example, two samples drawn from populations with perfectly normal distributions but with quite different means will, when the data are pooled, yield a bi-modal distribution with serious departures from normality. What we must do first is centre the data on the factor class means (that is, consider the residuals), examine the distribution of the residuals, and apply tests of normality.

This approach is conditional on the assumption of equality of variances. By a similar argument to the above, two samples drawn from populations with perfectly normal distributions but with quite different variances will, when the data are pooled, yield a distribution with serious departures from normality. It will be leptokurtic.

Pooling the residuals for a test of normality is only valid provided there is no evidence of a departure from the assumption of equality of variances.

Analysis of residuals

As outlined above, residual analysis is the recommended approach for assessing the validity of the assumptions of homogeneity of variances and normality. It is a graphical approach, and less sensitive than the range of statistical tests for homogeneity of variances and normality, but this is its strength. The ANOVA is a robust procedure, especially if the design is balanced, and violations of its assumptions of a magnitude likely to affect the outcome of the ANOVA will be evident in the graphical residual analysis. Less serious violations, likely to be detected by hypothesis testing (Bartlett's Test, Shapiro-Wilks Test), are unlikely to impact on the outcome of the ANOVA.

Analysis of residuals involves the following steps (Figure 4-4):

- Apply the working model (in our case, a single-factor ANOVA), and save the raw or studentised residuals;
- Plot the residuals against the predicted values of the working model (in our case, against the means for each sample);
- Transform if necessary to address any heterogeneity in the sample variances;

- Re-run the analysis, re-examine the residuals and apply another transformation if necessary;
- Once homogeneity of variances is achieved, construct a histogram of the residuals for an assessment of the assumption of normality.

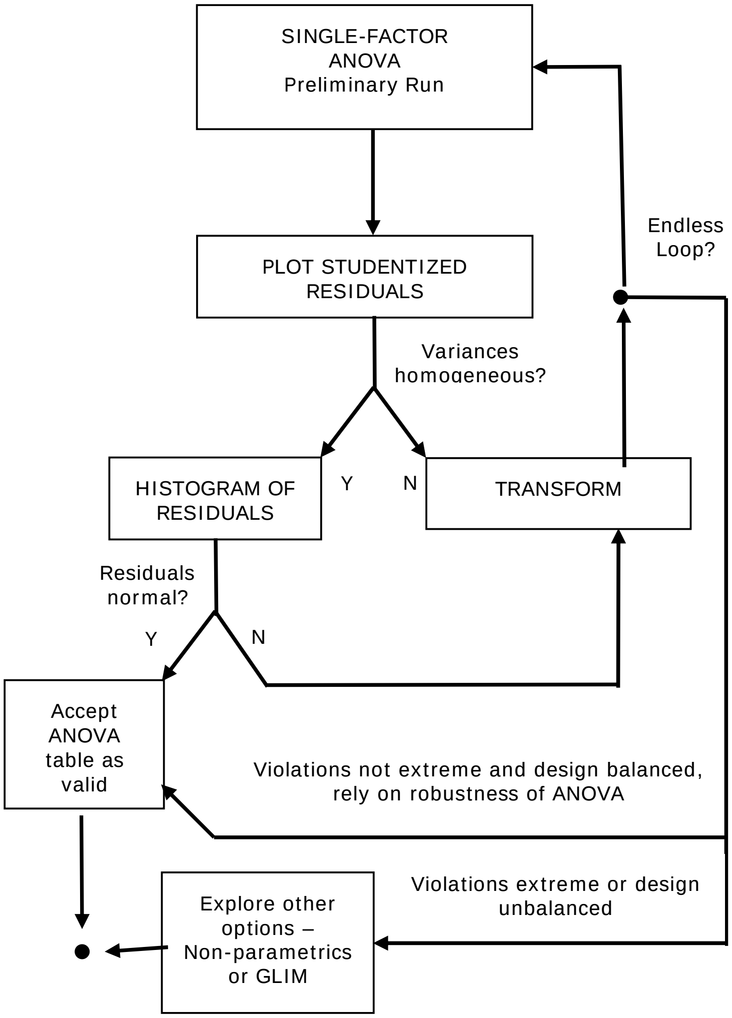
When limited data are at hand, such as is available for two-sample comparisons (Module 3), it is seldom possible to undertake a satisfactory examination of the validity of the assumptions. There are simply too few data to support a residual analysis. For this reason, you are advised to draw upon experience with the type of data at hand or on more extensive studies reported in the literature to decide an appropriate transformation. It is traditional to transform counts, for example, with either a square root (counts of independent entities) or log transformation (counts of aggregated entities).

With ANOVA, the sum total of data available is typically greater than for two-sample comparisons. It is reasonable therefore to conduct an ANOVA, examine the residuals, apply a transformation if deemed necessary, repeat the ANOVA and re-examine the residuals, and apply alternative transformations if the results of the first transformation are unsatisfactory.

Remember, though, the objective of transformation is to render normal, or of equal variance, the populations from which the samples were drawn, not the samples themselves. When it comes to the samples themselves, some deviation from normality and inequality of variances is acceptable. When small samples are involved, quite large apparent deviations from normality or homogeneity of variances can occur even when these assumptions are met for the parent populations.

Finally, it is important to appreciate that residual analysis allows an assessment of the validity of homogeneity of variances and normality. It does not necessarily address the assumptions of randomness or independence, or the adequacy of the ANOVA model to the data at hand. For example, pseudoreplication may result in deflation of the within-sample variance across the experiment. The variances may well remain homogeneous, and so the violation would not be detected. A residual analysis is not an unqualified ticket to proceed with the ANOVA.

Figure 4–4. A Decision Tree for Residual Analysis.


SINGLE-FACTOR ANOVA Preliminary Run
PLOT STUDENTIZED RESIDUALS
Variances homogeneous?
Endless Loop?
HISTOGRAM OF RESIDUALS
Y
N
TRANSFORM
Residuals normal?
Y
N
Accept ANOVA table as valid
Violations not extreme and design balanced, rely on robustness of ANOVA
Violations extreme or design unbalanced
Explore other options – Non-parametrics or GLIM

Summary of the assumptions of ANOVA

The assumptions of randomness and independence in sampling must be ensured by paying adequate attention to the random selection and allocation of items to the experimental classes or, if the design is constrained by the logistics of working with natural populations, by paying adequate attention to the random selection of items from within the experimental classes. If the assumptions of randomness or independence are violated, the results of the analysis can be profoundly affected, and the only recourse is to discard the data, redesign and repeat the experiment.

Departures from the assumption of equality of variances can be detected in a qualitative way by examining a plot of residuals, and a suitable transformation might be suggested by the pattern of scatter of those residuals. The effectiveness of the transformation may be evaluated by examination of the residuals following the transformation.

Having convinced yourself that the assumption of equality of variances is tenable, the assumption of normality may be tested by examining a histogram of the pooled residuals or a probability plot, depending upon your preference. Applying one or more of the tests introduced in Module 2 (Shapiro-Wilks test, probability plots etc) to the pooled residuals is likely to detect departures from normality that are of no practical consequence to ANOVA, especially where the design is balanced.

The residuals cannot be pooled for an assessment of the assumption of normality until homogeneity of variances across the factor classes has been achieved. A residual analysis will not necessarily detect violations of the assumptions of randomness and independence.

Transformations

Transformations are applied to either render the populations from which the samples are drawn normal, or to break a relationship between the mean and variance in order to comply with the assumption of homogeneity of variances. Very often both of these departures from the assumptions are simultaneously cured by the same transformation. The most commonly used transformations were first introduced in Module 3.

The following transformations are routinely used in the biological sciences.

The log transformation

$$Y' = \log_{10}\left(Y + 1\right)$$

is applied in cases where the standard deviation is proportional to the mean, or when the distribution of the parent population is skewed to the right.

The square root transformation

$$Y = \sqrt{Y + 0.5}$$

is applied when the data are counts for which a Poisson distribution is a satisfactory model, say for counts of organisms that are randomly distributed in the environment. It is usually unnecessary for such counts unless the mean count is less than 10.

The arcsine transformation

$$Y = SIN^{-1}\sqrt{p}$$

where *p* is a proportion. This transformation is appropriate for percentages and proportions. Data of this form are seldom satisfactorily modeled by a normal distribution if values occur outside the range 0.3 to 0.7 (30% to 70%). There is no need to apply the transformation if all values fall within this range.

There are other transformations, but they are beyond the scope of this Module. Refer to Sokal and Rohlf (1994, Chapter 13, section 6) or Zar (1984, Chapter 14) for further information. You can of course invent your own.

Robustness of ANOVA

The approach to checking assumptions recommended in this Module, that is, through qualitative examination of residuals, is not particularly rigorous. It relies in part on a general belief that analysis of variance is robust to moderate violations of the assumptions of normality and equality of variances. All but moderate violations would be evident on examination of the residuals in the manner described.

The foundation for this belief lies in Monte Carlo simulations undertaken in the middle of the twentieth century and reported by Lindquist (1953:78) and Keppel (1973). These studies show that moderate violations of normality do not constitute a serious problem and that, provided the samples sizes are equal or nearly so, nor do moderate departures from the assumption of equality of variances. If you are to rely heavily upon the robustness of ANOVA to violations of the assumption of equality of variances, in designing experiments, it is important to balance the design, that is, to ensure that the size of samples in each factor class are the same.

Non-parametric alternatives to ANOVA

If the assumptions of ANOVA are not met by the data, and no suitable transformation can be found to rectify the problem, or if the data are not measured at the interval or ratio level of measurement but rather the ordinal level, then we may decide to resort to a non-parametric alternative to ANOVA. One widely used non-parametric test is the Kruskal-Wallis one-way ANOVA by Ranks followed by multiple comparisons using a modified version of the Wilcoxon sum-rank test (Siegel and Castellan 1988; Sokal and Rohlf, 1994: Chapter 13). PROC NPAR1WAY in SAS is available for non-parametric ANOVA, but the subject will not be covered further in this Module. One word of caution though. The Kruskal-Wallis and Wilcoxon rank-sum tests may be non-parametric, but when used to address hypotheses specifically directed at means or medians, they are not assumption free. In addition to the universal assumptions of randomness in sampling and independence, these tests assume that the populations under consideration differ only in location, that is, that they have equal variances and that the shape of the distributions is the same.

ANOVA procedure in a nutshell

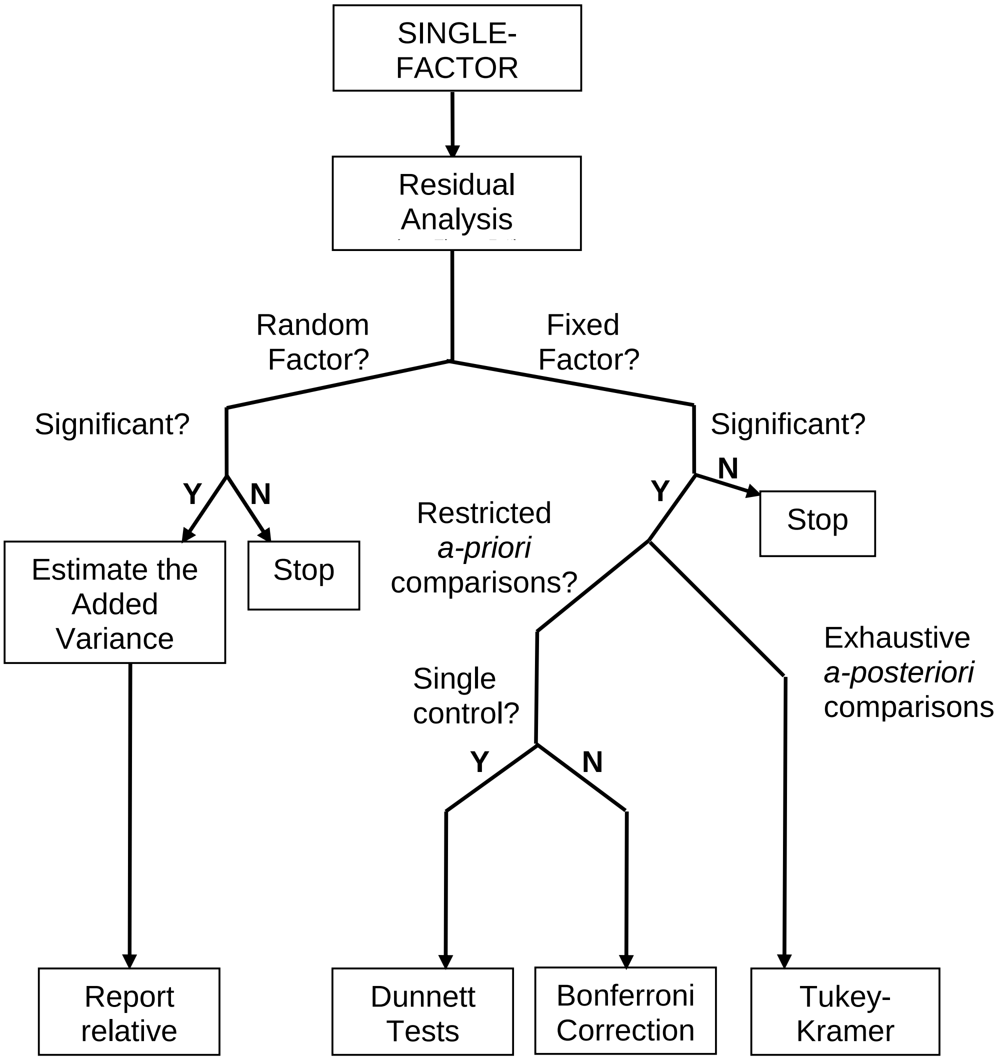
You should now have a grasp of one of the most important statistical concepts of use to biologists—the analysis of variance. Its fundamental objective is to determine whether the observed variation among a set of means is greater than would be expected by chance alone. It does this by comparing the observed variation among means with that expected on the basis of observed variation within samples.

The general procedure for undertaking a study involving single-factor ANOVA is summarised as follows:

- Decide the research question you wish to address.
- Carefully select your factor and factor classes so that differences among the factor classes will unambiguously address the research question. This would normally mean holding all other potentially influential factors constant.
- Design your experiment and sampling protocols to ensure that the entities to be measured are either randomly allocated to factor classes. If class membership is beyond your control, ensure that the entities are selected at random from the populations represented by each class. Ensure independence of the entities selected within and across factor classes.
- If the model is fixed, plan your comparisons in advance of beginning the experiment if at all possible. This will greatly increase power.
- Collect the data.
- Undertake an exploratory analysis, based on graphical techniques, preliminary runs of the ANOVA and examination of residuals to verify that the assumptions of ANOVA are tenable. Transform the data where necessary.
- Perform the final ANOVA, and follow by multiple comparison tests if the model is fixed or by estimating the added variance components if the model is random — see the Decision Tree of Figure 4-4.
- Interpret results statistically. If the ANOVA is not significant, and you wish to conclude that there is no effect, undertake a retrospective power analysis to support this conclusion. Otherwise conclude that you were unable to demonstrate a difference.

- Interpret results in the context of the initial question that you wished to address.

Figure 4–5. A Decision Tree for single-factor ANOVA.



Where have we come?

We have now covered all of the basics of analysis of variance where there is only a single driving factor. You should appreciate:

- an intuitive meaning for MS_{within}, MS_{among}, their sums of squares and degrees of freedom, and the F ratio itself;
- the difference between fixed and random models in ANOVA, and the practical consequences of these differences;

- the issues central to choosing an appropriate multiple comparison procedure, and a sensible position on a workable set of procedures to cover the common circumstances;
- the meaning and practical value of estimating the added variance component in the random model ANOVA;
- the assumptions of ANOVA, how to detect violations and how to overcome them, with emphasis on displaying and interpreting residuals.
- power analysis, and how to use it to interpret a non-significant result.

It is now appropriate to put this knowledge to use in worked examples and exercises. The practical application of the technique is much more straight-forward than you might think.

Lesson 6: Step-through Examples

Example 4-1: Macro-invertebrates of Crackenback River

This is an example of a one-way ANOVA where the factor is fixed, followed by unplanned (*a posteriori*) comparisons using the Tukey-Kramer Procedure.

The problem

David Tiller of the University of Canberra undertook a study of the effects of human disturbance on the benthic macro-invertebrate fauna of the Crackenback River in Kosciusko National Park. The river passes by the Thredbo Village, which discharges its sewage effluent, after treatment, 1.5 kilometres downstream. To assess the effects of this potential source of pollution on the fauna, Tiller chose the following sampling stations or sites:

- **Site 1:** 1 kilometre upstream of the village;
- **Site 2:** 1 kilometre below the village;
- **Site 3:** 1.5 kilometres below the village, ie immediately above the sewage effluent outflow;
- **Site 4:** 0.2 kilometres below the sewage effluent outflow;
- **Site 5:** 1 kilometre below the sewage effluent outflow;
- **Site 6:** 3 kilometres below the sewage effluent outflow;
- **Site 7:** 4.5 kilometres below the sewage effluent outflow;
- **Site 8:** 8 kilometres below the sewage effluent outflow.

Ten replicate collections of benthic invertebrates were made at each site using a Surber Sampler. A Surber Sampler is a square frame, 30 cm on a side, attached to a net. The sampler was placed on the bottom and all rocks to a depth of 10 cm lying within the square frame were washed and removed. Invertebrates dislodged by this process are carried by the flowing water into the collecting net. The collections were fixed in 4% formalin and returned to the laboratory for sorting, identification and counting. The data are raw counts pooled across species (Table 4–7).

David was interested to know if there were significant differences among the sites with respect to invertebrate abundance, and if so, where those differences lay.

Table 4–7. Raw counts of benthic invertebrates from the Crackenback River, upstream and downstream of Thredbo Village, Kosciusko National Park. The counts are pooled over species.

SITE							
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
286	325	496	1065	411	207	121	207
669	703	798	1539	1116	104	197	246
142	332	989	1174	681	153	292	468
65	265	640	880	1281	283	208	435
304	351	931	2113	1102	156	243	291
185	516	495	1172	578	386	260	246
210	350	469	1291	361	120	408	225
119	496	1160	1054	309	262	168	200
254	600	1139	1423	701	141	190	291
255	850	1072	1030	1242	294	110	174

The analysis

Data entry and exploratory examination

To analyse these data using SAS, they need to be set up in the form of two columns, one containing the measurements (in this case counts of benthic invertebrates) and the other containing a character string or number indicating from which site the measurement was taken. The data file THREDBO.DAT could be created to contain only two columns of data, but this is more than a little clumsy for editing purposes. Instead David chose to arrange the data as follows:

1	286	3	496	5	411	7	121
1	669	3	798	5	1116	7	197
1	142	3	989	5	681	7	292
1	065	3	640	5	1281	7	208
1	304	3	931	5	1102	7	243
1	185	3	495	5	578	7	260
1	210	3	469	5	361	7	408
1	119	3	1160	5	309	7	168
1	254	3	1139	5	701	7	190
1	255	3	1072	5	1242	7	110
2	325	4	1065	6	207	8	207
2	703	4	1539	6	104	8	246
2	332	4	1174	6	153	8	468
2	265	4	880	6	283	8	435
2	351	4	2113	6	156	8	291
2	516	4	1172	6	386	8	246
2	350	4	1291	6	120	8	225
2	496	4	1054	6	262	8	200
2	600	4	1423	6	141	8	291
2	850	4	1030	6	294	8	174

The data file THREDBO.DAT has been provided.

Double click on the SAS icon

Confirm that the data in THREDBO.DAT is as shown above by reading it into the EDITOR, then clear the window.

We must first read the data into SAS.

```
DATA TILLER;
    INFILE 'C:\MY DOCUMENTS\THREDBO.DAT';
    INPUT SITE $ COUNT @@;
RUN;
```

Site is read in as a character variable in this case. The @@ at the end of the INPUT statement indicates to SAS that more than one copy of SITE and COUNT are to be read from each line of raw data in the diskfile THREDBO.DAT.

Move to the first line of the EDITOR and type in the above data step. Submit the step for execution.

Use the EXPLORER window to locate the SAS workfile WORK.TILLER and examine its contents

To get a feel for the data, and the possible effects of the various potential impacts on the stream, the next step is to plot the counts against sites in the stream.

```
GOPTIONS RESET=ALL;
SYMBOL1 C=RED I=HILOT V=NONE;
SYMBOL2 C=RED I=STD2MB V=NONE;
AXIS1 LENGTH=10 CM VALUE=(H=1.5)
         LABEL=(H=2 "SITE")
         OFFSET=(2 PCT);
AXIS2 LENGTH=10 CM
         ORDER=0 TO 2500 BY 500
         VALUE=(H=1.5)
         LABEL=(H=2 A=90 "COUNT");
PROC GPLOT;
    PLOT COUNT*SITE=1
         COUNT*SITE=2 / OVERLAY
           HAXIS=AXIS1
           VAXIS=AXIS2;
RUN;
```

The first line of this program

```
GOPTIONS RESET=ALL;
```

cleans the slate so to speak, so that any parameters set in previous calls to PROC GPLOT and GCHART are reset to default values. The two axis statements set the characteristics of the axes. For AXIS2, for example, the length of the axis is set to 10 cm, the range of values shown on the axis are set to 0 to 2500 in increments of 500, the size of axis annotation is set and the axis title is given together with character size and orientation. These settings are then applied to the vertical axis (VAXIS) as an option appended to the PLOT statement.

The symbol statement

```
SYMBOL1 C=RED I=HILOT V=NONE;
```

defines the symbol that will be used on the plot. They can be quite complicated as in this case where I=HILOT indicates that a line should be drawn from the highest value to the lowest value (HILO) and that the line be terminated with horizontal bars (T=tick marks).

In the second symbol statement

```
SYMBOL2 C=RED I=STD2MB V=NONE;
```

the I=STD2MB requests a box (B) whose vertical extent is equivalent to two standard deviations (STD2) of the mean (M), that is, two standard errors on either side of the mean.

If we want both of these symbols on the same plot, to produce the traditional box plot, then we need to plot the data twice, once with each symbol definition, and use the OVERLAY option in the PLOT statement.

```
PLOT COUNT*SITE=1
    COUNT*SITE=2 / OVERLAY
         HAXIS=AXIS1
         VAXIS=AXIS2;
```

For further information on enhancing your plots with AXIS and SYMBOL statements, refer to Chapter 7 of the SAS/GRAPH User's Guide (SAS Institute, 1988).

The resulting graph is shown in Figure 4–6.

Type in the complete plotting program above and submit for execution.

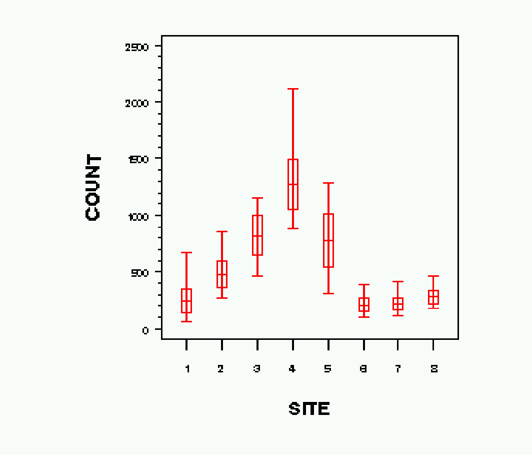


Figure 4–6. A graph showing counts of benthic invertebrates, for ten samples from each of eight sites in the Crackenback River. Vertical bars are ranges, boxes are two standard errors on either side of the mean.

At this point, you should mark on the plot, the locations of the various potential impacts. It would appear that the village itself has had an impact on the stream, perhaps aggravated by the sewage treatment plant, but at this point in the analysis, any interpretation would be pure supposition. The variation observed among the sites could well have happened by chance alone, and may not reflect a true difference in invertebrate abundance at the different sites. Tiller chose to perform an analysis of variance to determine if the observed differences among sites was significant, that is, if they reflected true differences at the sites from which they were drawn.

The within-sample variances differ considerably from site to site, and in fact appear to be correlated with the mean (higher abundances associated with higher variances). This is of some concern, as the analysis of variance assumes homogeneity of variances, but we will deal with this later.

Analysis of variance

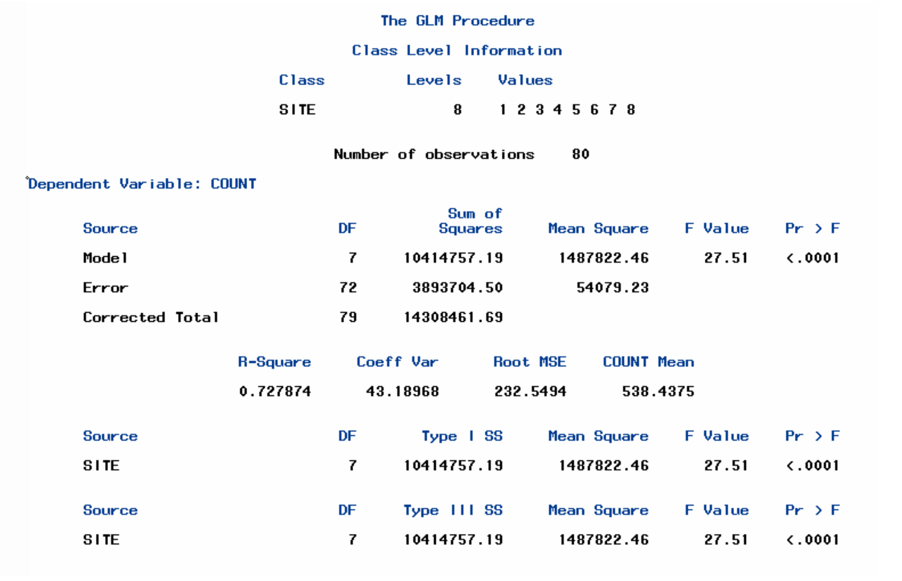
To perform the ANOVA, we use PROC GLM, principally because it is a more general procedure than PROC ANOVA. The latter procedure will only handle balanced designs (equal sample sizes).

```
PROC GLM;
    CLASS SITE;
    MODEL COUNT=SITE;
RUN;
```

Enter and submit the above step.

The output, with all extraneous information omitted, should be as in Box 4–1.

Box 4-1. *Abbreviated output of PROC GLM used to analyse macro-invertebrate abundances in Crackenback River.*



```
                             The GLM Procedure

                          Class Level Information

                   Class          Levels    Values

                   SITE                8    1 2 3 4 5 6 7 8

                        Number of observations    80
Dependent Variable: COUNT

                                        Sum of
       Source                  DF      Squares     Mean Square    F Value    Pr > F

       Model                    7   10414757.19     1487822.46      27.51    <.0001

       Error                   72    3893704.50       54079.23

       Corrected Total         79   14308461.69


                 R-Square     Coeff Var      Root MSE    COUNT Mean

                 0.727874      43.18968      232.5494      538.4375


       Source                  DF     Type I SS     Mean Square    F Value    Pr > F

       SITE                     7   10414757.19     1487822.46      27.51    <.0001

       Source                  DF   Type III SS     Mean Square    F Value    Pr > F

       SITE                     7   10414757.19     1487822.46      27.51    <.0001
```

Before launching into an interpretation of the ANOVA table, it would be wise to apply some diagnostic tests to determine if the ANOVA model is appropriate to the data at hand. We do this through an examination of residuals, that is, residual variation in the data after we have set the means for each sample to zero. It is customary to also standardise the residuals with the STUDENT option.

```
      OUTPUT OUT=RESPLT R=RESID P=PRED;
RUN;
```

Type in and submit the above statements for execution.

Note

Many SAS procedures remain ‘active’ until terminated by a QUIT statement or by calling another procedure. A RUN statement generally does not terminate a procedure, but merely requests SAS to act upon the statements submitted so far. Further GLM statements can be submitted, in this case an OUTPUT statement, and acted upon after one or more RUN statements.

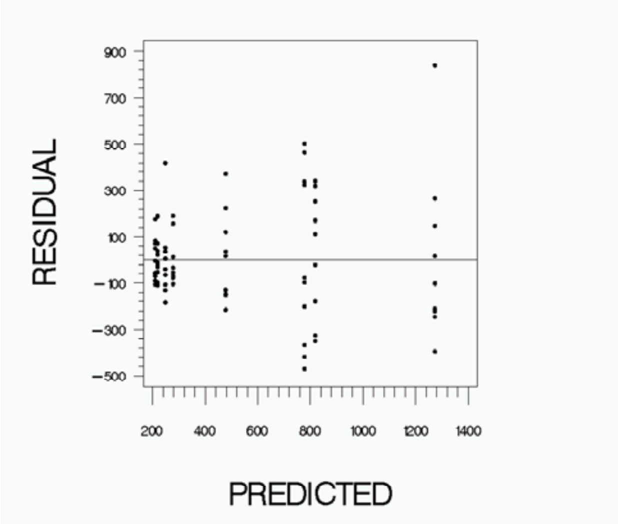
The output statement requests that the residuals for each data point relative to their sample mean and the predicted value for each data point (its sample mean) be written to a new SAS datafile called, in this case, WORK.RESPLT.

```
GOPTIONS RESET=ALL;
AXIS1 LENGTH=10 CM VALUE=(H=1.5)
    LABEL=(H=2 "PREDICTED");
AXIS2 LENGTH=10 CM VALUE=(H=1.5)
    LABEL=(H=2 A=90 "RESIDUAL");
PROC GPLOT DATA=RESPLT;
    PLOT RESID*PRED / HAXIS=AXIS1
    VAXIS=AXIS2 VREF=0;
RUN;
```

Type in and submit the above program for execution.

The resulting residual plot is shown in Figure 4–7.

Figure 4–7. A plot of residuals for counts of benthic invertebrates from eight sites in the Crackenback River. A correlation between the variance and mean is clearly indicated.



If the data conformed to the assumptions of homogeneity of variances and normality, we would expect the scatter of points to vary at random about the reference line. Instead, the scatter of points increases with increasing magnitude of the predicted value (mean). This correlation between the variance and mean is quite usual for counts of benthic invertebrates, and was not totally unexpected by Tiller. Tiller thought it prudent, on examination of the residual plot, to transform the counts before analysis. Two transformations would be appropriate to rectify the tendency for the variance to increase with the mean:

$$Y' = \sqrt{Y + 0.5}$$

or the stronger

$$Y' = \log_{10}(Y + 1)$$

Let us try the square root transformation first, and re-examine the residuals.

```
DATA TILLER;
    SET TILLER;
    SQCOUNT=SQRT(COUNT + 0.5);
RUN;
PROC GLM DATA=TILLER;
    CLASS SITE;
    MODEL SQCOUNT=SITE;
    OUTPUT OUT=RESPLT R=RESID P=PRED;
RUN;
GOPTIONS RESET=ALL;
AXIS1 LENGTH=10 CM VALUE=(H=1.5)
    LABEL=(H=2 "PREDICTED");
AXIS2 LENGTH=10 CM VALUE=(H=1.5)
    LABEL=(H=2 A=90 "RESIDUAL");
PROC GPLOT DATA=RESPLT;
    PLOT RESID*PRED / HAXIS=AXIS1
         VAXIS=AXIS2 VREF=0;
RUN;
```

Type in and submit the above program for execution.

The resulting residual plot is shown in Figure 4–8, and the distribution of the residuals about the reference line is vastly improved. There is no longer an obvious trend in the residual variances, and we can proceed to investigate whether the distribution of residuals is skewed.

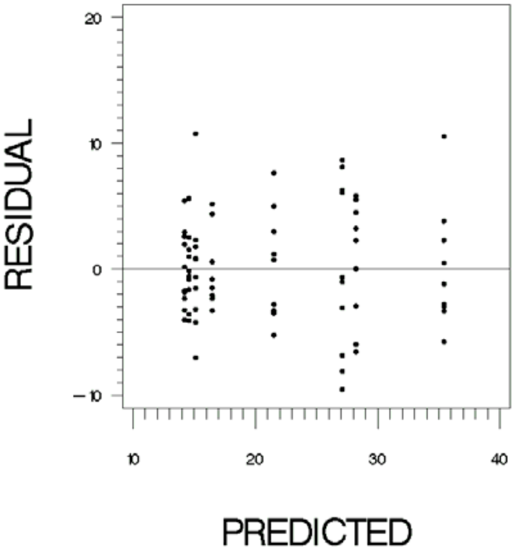


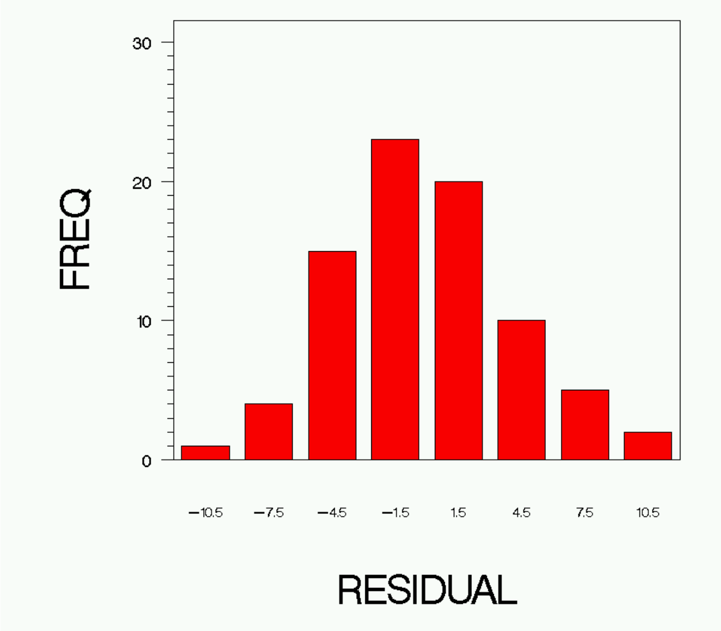
Figure 4–8. *Residuals for counts of benthic invertebrates from eight sites in the Crackenback River. The counts were transformed by square root before analysis.*

```
GOPTIONS RESET=ALL;
AXIS1 LENGTH=15 CM VALUE=(H=1)
    LABEL=(H=2 "RESIDUAL");
AXIS2 LENGTH=10 CM VALUE=(H=1.5)
    LABEL=(H=2 A=90 "FREQ");
PROC GCHART DATA=RESPLT;
    VBAR RESID / MAXIS=AXIS1
    RAXIS=AXIS2 SPACE=0;
RUN;
```

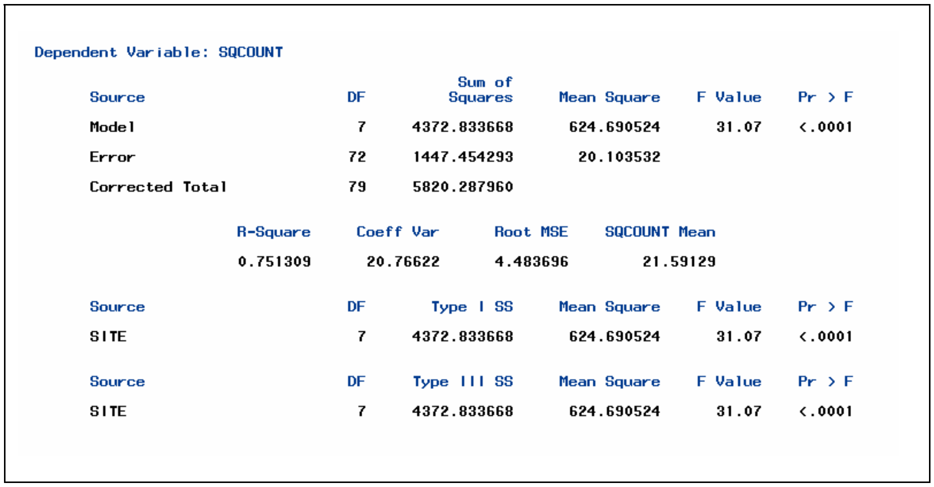
Type in and submit the above program for execution.

By inspection, it can be concluded that the residuals are not particularly skewed
(Figure 4–9).

__Figure 4–9.__ The distribution of residuals for counts of benthic invertebrates following transformation by square root.



__Box 4–2.__ Output of PROC GLM used to analyse macro-invertebrate abundances in Crackenback River, following a square root transformation.



```
Dependent Variable: SQCOUNT

                                            Sum of
      Source                      DF       Squares     Mean Square    F Value    Pr > F

      Model                        7   4372.833668      624.690524      31.07    <.0001

      Error                       72   1447.454293       20.103532

      Corrected Total             79   5820.287960

                      R-Square     Coeff Var      Root MSE    SQCOUNT Mean

                      0.751309      20.76622      4.483696        21.59129

      Source                      DF     Type I SS     Mean Square    F Value    Pr > F

      SITE                         7   4372.833668      624.690524      31.07    <.0001

      Source                      DF   Type III SS     Mean Square    F Value    Pr > F

      SITE                         7   4372.833668      624.690524      31.07    <.0001
```

We can now interpret the analysis of variance table based on the square rooted counts with confidence (Box 4–2).

The implications of the ANOVA of Box 4–2 are clear. The F value of 31.07 had a probability of occurring through chance alone of only <0.0001. As this is much less than the conventional cut-off value of 0.05, we conclude that the observed variation among sites is significant.

But where do the differences lie? To answer this question, Tiller chose to perform Tukey-Kramer multiple comparisons. Because we have performed plotting analyses since PROC GLM, the GLM procedure is no longer active. We must re-run it to perform the multiple comparisons.

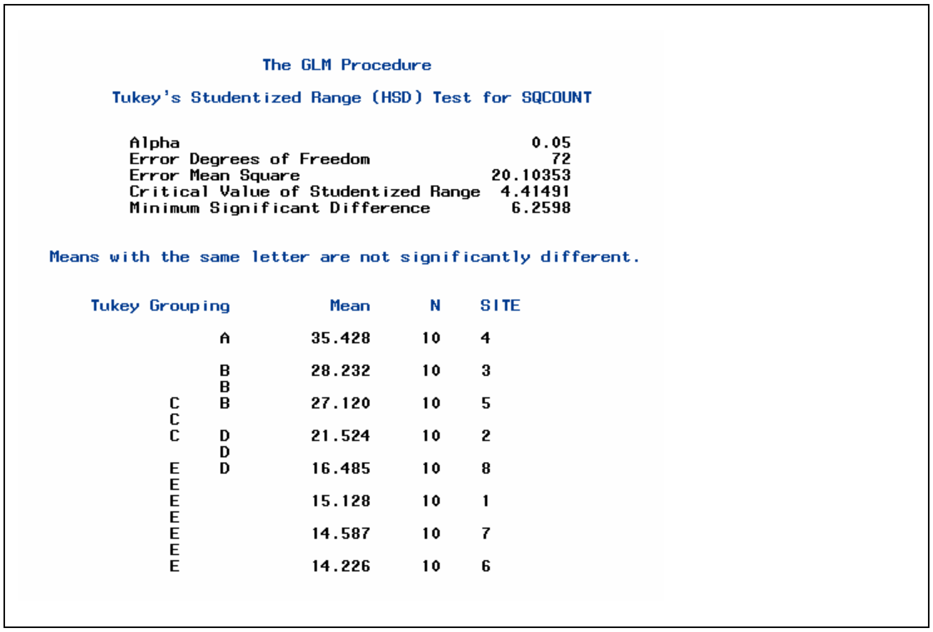
```
PROC GLM DATA=TILLER;
    CLASS SITE;
    MODEL SQCOUNT=SITE;
    MEANS SITE / TUKEY;
RUN;
```

Type in and submit the above statements.

There are two points to note at this stage of the analysis. First, Tukey-Kramer multiple comparisons are only appropriate following a significant result in the ANOVA (it is a so-called protected test). Had the result not been significant, then the analysis would have been complete with the production of the analysis of variance table. Hence, it is important not to routinely run the Tukey comparisons with the ANOVA—the Tukey analysis is conducted only after a significant result is demonstrated by the ANOVA.

The output, edited to remove unnecessary complexity, is shown in Box 4-3.

***Box 4–3**. Output from a Tukey Multiple Comparisons Test to determine which sites differed significantly from which others, following a significant result in the analysis of variance. The data are square rooted abundances of macro-invertebrates from Crackenback River.*



```
                      The GLM Procedure

        Tukey's Studentized Range (HSD) Test for SQCOUNT

          Alpha                                        0.05
          Error Degrees of Freedom                       72
          Error Mean Square                        20.10353
          Critical Value of Studentized Range       4.41491
          Minimum Significant Difference             6.2598

  Means with the same letter are not significantly different.

      Tukey Grouping          Mean       N     SITE

                   A        35.428      10     4

                   B        28.232      10     3
                   B
              C    B        27.120      10     5
              C
              C    D        21.524      10     2
                   D
              E    D        16.485      10     8
              E
              E             15.128      10     1
              E
              E             14.587      10     7
              E
              E             14.226      10     6
```

The Tukey test lists the sample means in order of decreasing magnitude. Sites grouped together beside the same letter (left hand columns) are not significantly different from each other. For example, Sites 1, 6, 7 and 8 are not significantly different from each other but are significantly different from Sites 3, 4 and 5. Site 4 stands out alone as significantly different from all other sites. It is below suspected sources of pollution, and shows a significant increase in invertebrate abundance compared with that of neighbouring sites upstream of the sources. It appears that both the village and the sewage outlet have an impact on the stream, but the good news is that the effect has dissipated 3 kilometres downstream. Overlapping non-significant subsets are common in analyses of this sort, and are often difficult to interpret. They can only be resolved by increasing the sample sizes so that true differences, if any, become more apparent.

Results summary

The results of the analysis could be reported in the Results section of a report or paper along the following lines.

The differences among the eight sites in the abundance of benthic invertebrates (Figure 4–10) was significant, as demonstrated by a single-factor ANOVA applied to the square-root transformed counts (F = 31.07; df = 7,72; p < 0.0005) (Table 4–8).

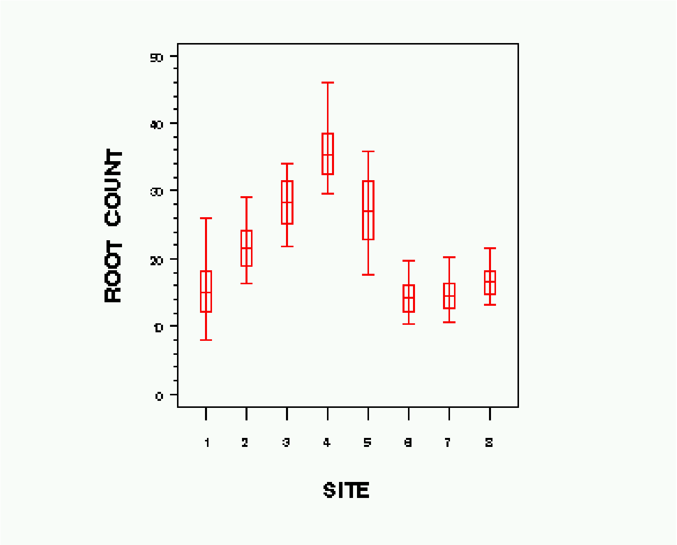


Figure 4–10*. A graph showing counts of benthic invertebrates, for ten samples from each of eight sites in the Crackenback River. Vertical bars are ranges, boxes are two standard errors on either side of the mean.*

Multiple comparisons based on the Tukey-Kramer procedure ($\alpha = 0.05$) revealed that macro-invertebrate abundance was greatest at site 4, immediately downstream of the sewage outflow. Site 3, immediately above the outflow, and Site 5, 1 kilometre below the outflow were similar in terms of macro-invertebrate abundance, second only to Site 4. The upstream Site 1 and the downstream sites 6, 7 and 8 were not significantly different, suggesting that any impact the village and its sewage discharges may have had on the stream had dissipated by the time the stream water had flowed 3 kilometres beyond the impacts.

Table 4–8. *Summary of the analysis of variance used to compare macro-invertebrate abundance at eight sites on the Crackenback River near Thredbo Village.*

Source	Degrees of freedom	Sums of squares	Mean square	F value	Prob under H_0
Among sites	7	4372.83	624.69	31.07	P<0.0001
Within sites	72	1447.45	20.10		
Total	79	5820.29			

Discussion

The analysis can now be discussed in the context of the reasons for conducting the study. What advice can you give to the managers of the village or to the authorities responsible for the health of the Crackenback River and Lake Jindabyne downstream? How do the results of this study compare with those elsewhere, and what can be concluded about the effectiveness of the treatment system at Thredbo? These are the sorts of points that would be covered in a discussion of the results.

Adequacy of the design

At this point, it is constructive to consider the adequacy of the experimental design. First, the conclusions drawn from this experiment strictly relate only to the time of year that the experiment was undertaken, for there is no evidence that the pattern of differences observed in this study is repeated in all months of the year. Nor with a single experiment such as this are we able to gain insight into variation among years, and the pattern of differences among sites may vary from year to year. With an experiment such as the one described here, we are very limited in the degree to which we can draw inferences on the impact of human disturbance in the river beyond the period in which we conducted the study. David Tiller addressed these concerns in his broader study, with data collected every month for each of three years. Having replicated his treatments across years, Tiller was able to assess the level of variability between years and the interaction between the effect of location in the stream and the year or month chosen for study. However his broader analysis is beyond the scope of single-factor ANOVA.

The second point relates to the adequacy of the upstream site as a control. It may well have been that Site 1 was different from Sites 2, 3, 4 and 5 quite irrespective of the human impact, and this may have been so even before Thredbo Village had been built. Our interpretation that the development has had an impact on the stream, is predicated on the assumption that Site 1 resembles what Sites 2, 3, 4 and 5 would have been like had the development of the village not gone ahead. Quite an assumption, and the only support for it in this case is indirect, provided by the observation that Site 1 is not significantly different from sites below Site 5.

A third point on design is that the upstream site was not replicated. Tiller had only one site upstream of the suspected impacts. Without some measure of the variation among replicated sites upstream, we cannot tell if Site 1 was typical of sites upstream of the village. The whole analysis is vulnerable to the chance influences that might make Site 1 untypical of upstream sites.

A final point relates to Tiller's choice of follow-up analysis. The power of the analysis could have been substantially increased if Tiller had planned follow-up comparisons as part of the overall design before undertaking the study. By restricting the pool of possible comparisons under the study design, and using Sidak's tests in place of the exhaustive comparisons of Tukey-Kramer procedure, less adjustment of the experimentwise error would have been necessary. The resulting comparisons would have been less conservative, that is, more sensitive, with a corresponding increase in resolution of differences among sites. Planned comparisons are preferable to ad hoc unplanned comparisons in this regard.

All of these considerations must come into play, preferably when designing the study, and certainly when interpreting the results in preparation for publication.

Example 4-2: Rock lobster in aquaculture

This is a one-way ANOVA with a fixed factor and a single control treatment.

The Problem

The western rock lobster is recognized as a valued fishery estimated at 300 million dollars a year. To protect this valuable commodity and increase the production, the idea of culturing the lobster has become increasingly attractive. The aquaculture of these lobsters is still in its infancy. To achieve progress in this area, the development of an artificial diet and culturing environment is required as well as methods to allow the assessment of condition of cultured animals.

Tsvetnenko et al. (1999) developed a set of body condition factors to evaluate condition of cultured rock lobsters in conjunction with a nutritional study of the western rock lobster juvenile phase (post-pueruli or after settlement). A large sample of juvenile lobsters were collected at various locations and held in a tank at the Fisheries Marine Research Lab for two months as an acclimation period. During the acclimatisation period the lobsters were fed a diet of mussels and prawn pellets.

Table 4-1. *Proximate composition of the experimental diets fed to rock lobster.*

Parameter	D2*	D3*	D4**	D5**
Dry matter (%)	60.0	60.0	90.0	90.0
Dig. energy (MJ/kg)	9.0	10.0	13.6	15.1
Crude protein (%)	30.1	33.8	45.3	50.6
Crude fat (%)	4.8	7.4	7.2	11.1

* Semi moist; ** Dry

Subsequently, juvenile lobsters were fed for nine weeks on either fresh mussel diet (D1) or one of four artificial diets, two in semi-moist form (D2 and D3) and two in dry pelleted form (D4 and D5) (Table 4-1). The central hypothesis to be addressed was whether the lobsters performed as well on the artificial diets (D2-D5) as they did on the natural diet (D1). In this context, the natural diet of mussel was regarded as the control treatment, and the artificial diets were regarded as the experimental treatments.

A number of indices of condition were measured for comparison across treatments. One was the weight of wet tail muscle expressed as a percentage of the weight of the whole animal, wet. At the conclusion of the experiment, 10 animals were sampled at random from each diet treatment and their MSI condition measured.

Table 4-2. *Muscle somatic index (MSI) of lobster maintained on five dietary treatments.*

D1 (Control)	D2	D3	D4	D5
18.825	15.289	15.949	16.079	18.787
17.086	15.348	14.569	16.411	14.583
18.321	15.709	19.855	14.873	15.319
13.783	14.568	17.304	14.090	18.821
22.698	15.067	15.179	17.377	21.979
19.974	13.719	14.936	12.592	20.188
23.706	14.706	16.749	14.133	17.080
15.839	14.692	13.772	15.958	15.539
18.584	13.233	16.021	14.479	16.002
15.850	18.983	14.920	15.932	12.210

Data entry and exploratory examination

SAS has a myriad of ways of reading in raw data. These data could be arranged as two columns only, one containing the dietary treatment and the other containing the measurement of muscle-somatic index (MSI). The resulting file is very unwieldy, and it is more satisfactory to type the data in as shown in Table 4-2 and modify its format on input during the DATA step. This can be done in the following way:

```
DATA LOBSTER;
  INPUT A B C D E;
  DIET="D1"; MSI=A; OUTPUT;
  DIET="D2"; MSI=B; OUTPUT;
  DIET="D3"; MSI=C; OUTPUT;
  DIET="D4"; MSI=D; OUTPUT;
  DIET="D5"; MSI=E; OUTPUT;
  DROP A B C D E;
DATALINES;
18.825  15.289    15.949    16.079    18.787
17.086  15.348    14.569    16.411    14.583
18.321  15.709    19.855    14.873    15.319
13.783  14.568    17.304    14.090    18.821
22.698  15.067    15.179    17.377    21.979
19.974  13.719    14.936    12.592    20.188
23.706  14.706    16.749    14.133    17.080
15.839  14.692    13.772    15.958    15.539
18.584  13.233    16.021    14.479    16.002
15.850  18.983    14.920    15.932    12.210
;
```

A little consideration will reveal what is going on here. The five columns of data represent the MSI values for each diet D1 to D5. We first read them into five temporary variables A-E. We then set the DIET variable to take on the value "D1". We then set the MSI variable to take on the value of temporary variable A, which contains 18.825 or the first value for diet D1. Finally, we output the data processed so far to the workfile WORK.LOBSTER. Note that we have requested that SAS drop the five temporary variables A-E on output, so that the resulting workfile should have only two variables, DIET and MSI.

This process is repeated four more times (D2-D5) for each line of data so that we ultimately have two columns of data comprising the response variable MSI and the breakdown or class variable DIET. You should verify that this is so using the explorer window to look at WORK.LOBSTER. File this little programming trick away in your recipe book.

Enter the above data step. You will need to cut and paste the data from the datafile LOBDIET.DAT if you wish to use the DATALINES option for data entry.

Submit the program for execution, and peruse the results of your data manipulation in the Explorer Window.

At this point, we might like to define some value labels to the diets, as D1-D5 is not very informative and may lead to confusion later. We do this with PROC FORMAT as outlined in Module 1.

```
PROC FORMAT;
  VALUE $ DIETFMT
    "D1" = "CONTROL"
    "D2" = "MOIST #1"
    "D3" = "MOIST #2"
    "D4" = "DRY #1"
    "D5" = "DRY #2";
RUN;
```

Enter and submit the above data step. Check the LOG Window to see that it has executed correctly.

A plot of the data reveals a clear trend (Figure 4-19), at least in the sample data. The boxes are only one standard error on either side of the mean, so the likely significance of the trend is in doubt. We will have to wait and see what the ANOVA tells us.

```
GOPTIONS RESET=ALL;
SYMBOL1 C=RED I=HILOT V=NONE;
SYMBOL2 C=RED I=STD2MB V=NONE;
AXIS1 LENGTH=5 CM VALUE=(H=1)
    LABEL=(H=1 FONT=SWISSB "DIET")
    OFFSET=(2 PCT);
AXIS2 LENGTH=5 CM VALUE=(H=1)
    LABEL=(H=1 FONT=SWISSB A=90 "MSI (g)");
RUN;
PROC GPLOT DATA=LOBSTER;
    PLOT MSI*DIET=1 MSI*DIET=2 /OVERLAY
         HAXIS=AXIS1 VAXIS=AXIS2;
RUN;
```

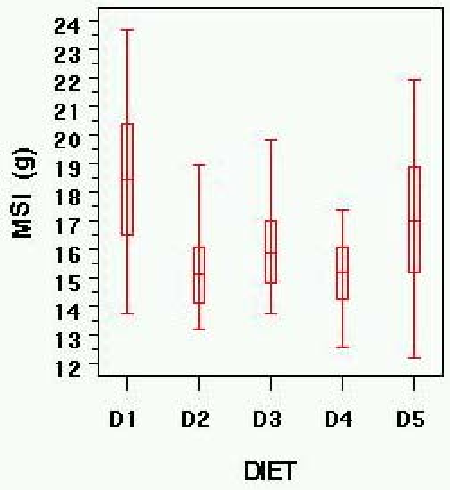


Figure 4–19*.* *A box plot showing variation in lobster MSI for different diets. Note, the boxes show two standard errors on either side of the mean. The vertical bars are ranges.*

Generate the plot for yourself by entering and submitting the above program.

Preliminary examination of the plot indicates some promising variation among the treatments. The ANOVA will determine which differences we can regard as significant.

First, we should attend to the assumptions, with a residual analysis.

```
PROC GLM DATA=LOBSTER;
   CLASS DIET;
   MODEL MSI=DIET ;
   OUTPUT OUT=RESPLT STUDENT=RESID P=PRED;
RUN;
GOPTIONS RESET=ALL;
AXIS1 LENGTH=10 CM VALUE=(H=1)
      LABEL=(H=1 "PREDICTED");
AXIS2 LENGTH=15 VALUE=(H=1)
      LABEL=(H=1 A=90 "RESIDUAL");
PROC GPLOT DATA=RESPLT;
        PLOT RESID*PRED /HAXIS=AXIS1 VAXIS=AXIS2
VREF=0;
RUN;
```

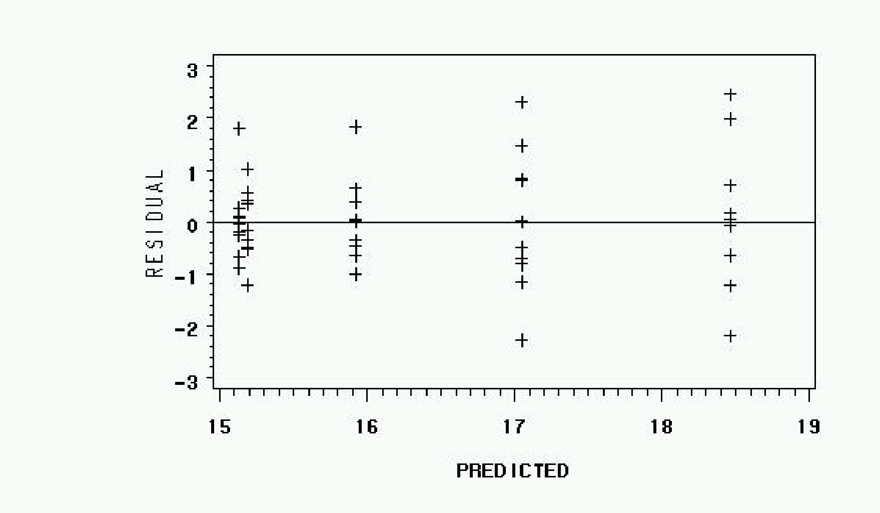


Figure 4–2. *A residual plot for lobster MSI across different diets.*

Generate the plot for **yourself** by entering and submitting the above program.

The residuals look quite respectable, with only a slight trend in variance with increasing magnitude of the response variable. We could try to rectify this with a transformation, but in this case we will follow the authors lead and run with the raw data.

The residuals also pass a test of normality.

```
GOPTIONS RESET=ALL;
PROC GCHART DATA=RESPLT;
  VBAR RESID;
RUN;
PROC UNIVARIATE DATA=RESPLT NORMAL PLOT;
  VAR RESID;
RUN;
```

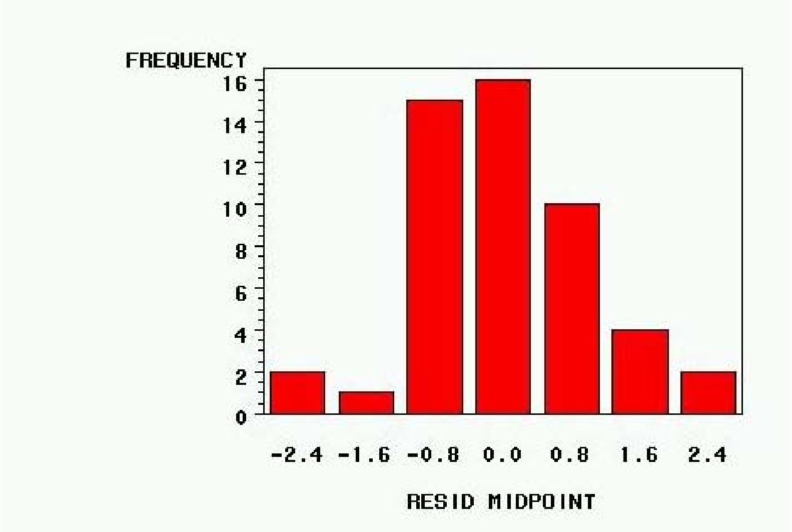
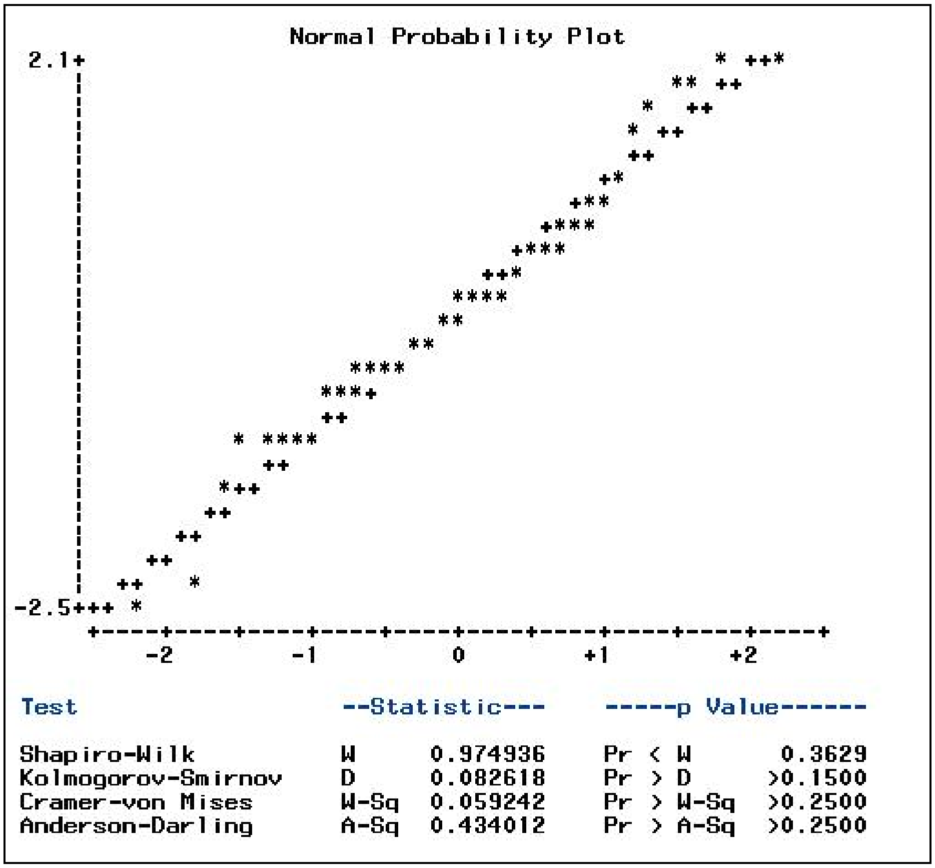


Figure 4–3. *A histogram of the residuals from and ANOVA on lobster MSI across different diets.*

There is no indication of substantial deviation from normality by the residuals in the histogram (Figure 4-3), the probability plot or in terms of significance of the Shapiro-Wilkes test (Box 4-1). We can now proceed to interpret the ANOVA printout.

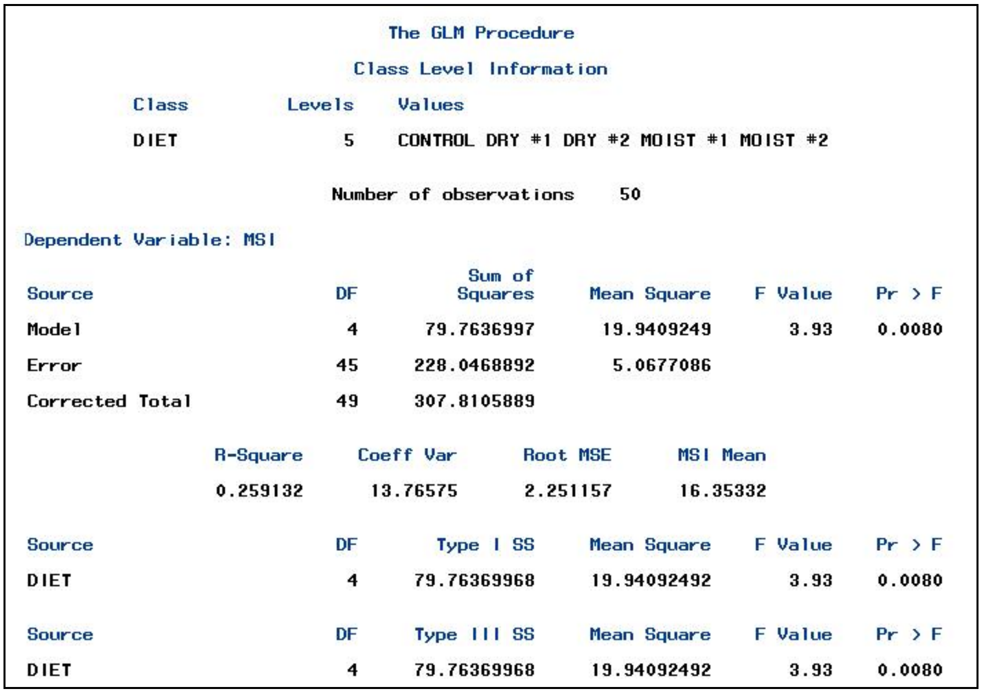
Box 4–1. *Tests of Normality of the residuals from and ANOVA on lobster MSI across different diets.*



```
Test                    --Statistic---     -----p Value------

Shapiro-Wilk            W     0.974936     Pr < W      0.3629
Kolmogorov-Smirnov      D     0.082618     Pr > D     >0.1500
Cramer-von Mises        W-Sq  0.059242     Pr > W-Sq  >0.2500
Anderson-Darling        A-Sq  0.434012     Pr > A-Sq  >0.2500
```

Box 4–2. *Results of an ANOVA comparing lobster MSI values for different diets.*



```
                              The GLM Procedure

                          Class Level Information

         Class          Levels    Values

         DIET                5    CONTROL DRY #1 DRY #2 MOIST #1 MOIST #2

                         Number of observations    50

Dependent Variable: MSI

                                         Sum of
Source                      DF          Squares    Mean Square   F Value   Pr > F

Model                        4       79.7636997     19.9409249      3.93   0.0080

Error                       45      228.0468892      5.0677086

Corrected Total             49      307.8105889

                R-Square     Coeff Var      Root MSE      MSI Mean

                0.259132      13.76575      2.251157      16.35332

Source                      DF        Type I SS    Mean Square   F Value   Pr > F

DIET                         4      79.76369968    19.94092492      3.93   0.0080

Source                      DF      Type III SS    Mean Square   F Value   Pr > F

DIET                         4      79.76369968    19.94092492      3.93   0.0080
```

Lobsters raised on different diets differed significantly in condition as indicated by the muscle-somatic index (MSI). The question that now must be addressed is whether the artificial diets promote or retard body condition compared with the more natural diet (control).

This question can be addressed using Dunnett's Test, which is designed to adjust the level of significance to compensate for multiple comparisons where experimental treatments are each compared against a single control. Tukey's multiple comparison procedure would be far too conservative, provided we are not interested in comparing among the artificial diets.

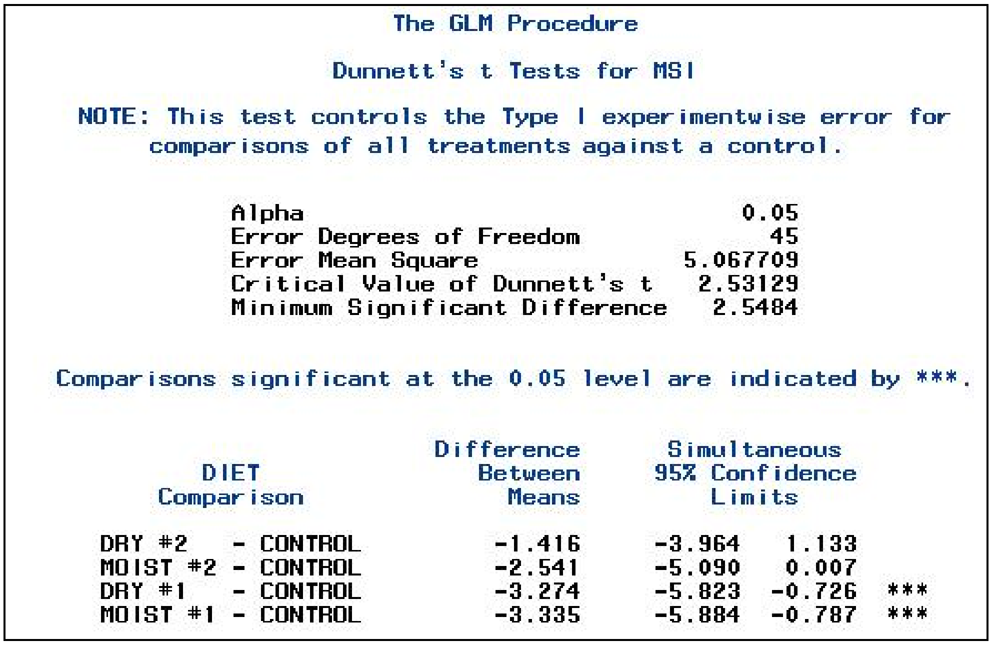
The GLM procedure remains active, so we can continue to feed it instructions:

```
    MEANS DIET / DUNNETT(CONTROL);
  RUN;
```

This statement asks SAS to compare the mean MSI for the experimental diets against the CONTROL diet D1 (mussel). Note that in specifying the control treatment, SAS expects the label specified in the FORMAT statement, that is "CONTROL" not "DI". The output is as shown in Box 4-3.

All artificial diets yielded lower wet muscle-somatic index than lobsters fed control diet (D1), however this result was significant only for lobsters fed diets that included formula 1 (D2 and D4) (Box 4-3).

Box 4–3. *Results of a Dunnett's multiple comparison procedure to follow a significant result in an ANOVA comparing lobster MSI values for different diets.*



```
                         The GLM Procedure

                     Dunnett's t Tests for MSI

  NOTE: This test controls the Type I experimentwise error for
        comparisons of all treatments against a control.


                 Alpha                                  0.05
                 Error Degrees of Freedom                 45
                 Error Mean Square                  5.067709
                 Critical Value of Dunnett's t       2.53129
                 Minimum Significant Difference       2.5484


 Comparisons significant at the 0.05 level are indicated by ***.


                                Difference      Simultaneous
          DIET                   Between       95% Confidence
       Comparison                 Means            Limits

    DRY #2   - CONTROL           -1.416      -3.964    1.133
    MOIST #2 - CONTROL           -2.541      -5.090    0.007
    DRY #1   - CONTROL           -3.274      -5.823   -0.726   ***
    MOIST #1 - CONTROL           -3.335      -5.884   -0.787   ***
```

Results Summary

A fixed-effects, single factor ANOVA found a significant difference in wet muscle somatic index among diets fed to Rock Lobsters (F=3.93; df=4,45; p <0.01). Lobsters fed diets based on formula 1, whether semi-moist or in dry pellet form had significantly lower wet muscle somatic index, 3.3 g lower in each case, than lobsters fed a more natural Mussel control diet (Dunnett's multiple comparison test, α = 0.05, MSD = 2.55 g). No difference could be demonstrated between the muscle somatic index of lobsters fed on formula 2 and those fed on the more natural mussel diet.

Discussion

Lobsters fed the natural mussel diet grew significantly faster than those fed the artificial diets, but this difference was statistically significant only in the case of formula 1 diets, whether moist or dry. If muscle somatic index were the only consideration, then the artificial diet based on Formula 2 would be the preferred choice among the two artificial diets.

Western rock lobsters naturally eat molluscs in the wild and supplement this feed with coralline algae. It should be noted that when all analyses were considered, the results agreed with previous research in that natural diets produce significantly better growth rates in lobsters than artificial feeds. Tsvetneko et al. (1999) later determined that the artificial feeds may have had too much lipid for good dietary performance, which seems to be born out in the partial results given here for MSI.

Source

The data that form the basis of this example were kindly provided by Dr. Elena Tsvetnenko, Muresk Institute of Agriculture, Curtin Univeristy of Technology, Suite 3, Enterprise Unit 1, 11 Brodie Hall Drive, Technology Park, Bentley WA 6102.

Tsvetneko, E., J. Brown, B. D. Glencross and L.H. Evans. 1999. Measures of condition in dietary studies on western rock lobster post-pueruli. Pp. 100-109 in Proceedings, International Symposium on Lobster Health Management, Adelaide, September 1999.

Example 4-3: Atmospheric SO_2 and Soybean Growth

This is a one-way ANOVA with a fixed factor followed by a retrospective power analysis.

The problem

The economy of China has been developing rapidly, supported largely by coal-fired power stations. Many regions regularly experience high atmospheric concentrations of sulphur dioxide emitted by the power stations. In the highly industrial area of Shenyang in the Lioaning Province, the average atmospheric SO_2 concentration during the summer is 38 ppb.

China is the world's third largest producer of soybeans. Soybean is an important cash crop, and vegetable oil produced from soybean is of high quality. Soybean (*Glycine max*) is sensitive to atmospheric SO_2, decreasing in yield by up to 4% at SO_2 concentrations as low as 0.05 ppm.

Open-top chambers (cylinders of 2.4 m high and 3 m in diameter) were used to fumigate soybean plants with high (488.6 ppb) and low (97.3 ppb) concentrations of SO_2. A control chamber was also established with background levels of SO_2 (1.2 ppb). Seeds of soybean were planted in 2 L plastic pots filled with a 2L an artificial potting material and randomly allocated to fumigation chambers.

The response variable was growth of soybean measured as total plant mass at the end of the experiment (g/pot).

***Table 4-9.** Total mass (g) of soybean following treatment with atmospheric SO2 at background levels (control), low concentrations (97.3 ppb) and high concentrations (488.6 ppb).*

Replicate	Control	Low SO_2	High SO_2
1	79.1	61.8	61.2
2	64.7	58.9	65.2
3	67.0	76.3	51.0

The Analysis

Data entry and exploratory examination

SAS has a myriad of ways of reading in raw data. These data could be arranged as two columns only, one containing the treatment and the other containing the measurement of soybean biomass. The resulting file would be a little repetitive, so we might choose to arrange the data in the same form as in the table above and use some simple programming for input.

```
DATA SO2;
  INPUT REP A B C;
  TRT="CONTROL"; BIOMASS=A; OUTPUT;
  TRT="LOW"; BIOMASS=B; OUTPUT;
  TRT="HIGH"; BIOMASS=C; OUTPUT;
  KEEP TRT BIOMASS;
DATALINES;
1 79.1  61.8  61.2
2 64.7  58.9  65.2
3 67.0  76.3  51.0
;
```

Understanding this data step requires a little thought and knowledge of how SAS manipulates and stores data. The step reads the four columns of data into the workfile — REP and the temporary variables A B and C. The statement

```
TRT="CONTROL"; BIOMASS=A; OUTPUT;
```

puts the string "CONTROL" into the variable TRT, the contents of A into BIOMASS and then outputs TRT and BIOMASS to the workfile WORK.SO2. The contents of variables B and C are handled similarly so that the workfile ends up with two columns of data—one called TRT, containing the labels given to each treatment, and the other called BIOMASS, containing the measurements of soybean growth in grams. The unneeded variables REP, A, B and C are not kept.

Enter and submit the above data step.

Verify that the data has been read correctly first by perusing the LOG window:

```
NOTE: The data set WORK.SO2 has 9

observations and 2 variables.

NOTE: DATA statement used:

real time           0.01 seconds
cpu time             0.01 seconds
```

If the data has been read correctly, there should be two variables in WORK.SO2, the class variable TRT and the measurement variable BIOMASS. As there are 9 measurements of BIOMASS, the work file should contain 9 observations. All would appear well from the above log, but just to make sure, list the contents of WORK.SO2:

Enter and submit the following Proc Step.

```
PROC PRINT;
RUN;
```

The data should appear in the OUTPUT window in the usual form, a factor or class variable and a measurement variable, suitable for analysis by the GLM procedure.

A plot of the data reveals a clear trend (Figure 4-11), at least in the sample data. The boxes are only one standard error on either side of the mean, so the likely significance of the trend is in doubt. We will have to wait and see what the ANOVA tells us.

```
GOPTIONS RESET=ALL;
SYMBOL1 C=RED I=HILOT V=NONE;
SYMBOL2 C=RED I=STD1MB V=NONE;
AXIS1 LENGTH=5 CM VALUE=(H=1)
    LABEL=(H=1 FONT=SWISSB "TREATMENT")
    ORDER="CONTROL" "LOW" "HIGH"
    OFFSET=(2 PCT);
AXIS2 LENGTH=5 CM VALUE=(H=1)
    LABEL=(H=1 FONT=SWISSB A=90 "BIOMASS
(G/POT)");

PROC GPLOT;
    PLOT BIOMASS*TRT=1 BIOMASS*TRT=2 /OVERLAY
         HAXIS=AXIS1 VAXIS=AXIS2;
RUN;
```

Generate the plot for yourself by entering and submitting the above program.

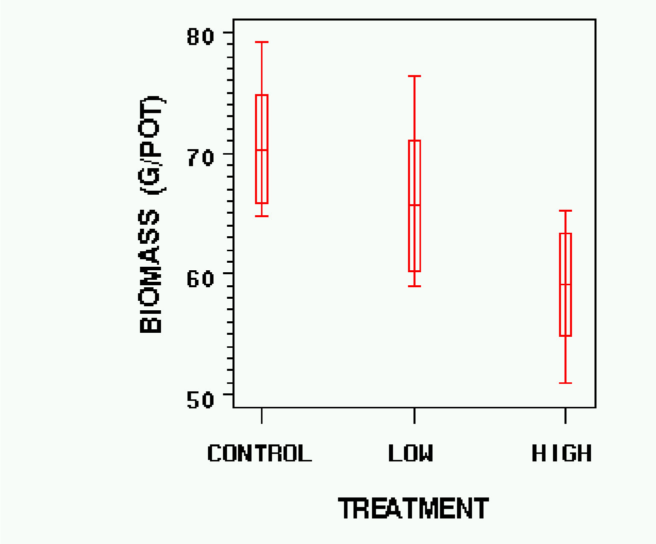


Figure 4–11. A box plot showing the results of treatment of soybean with three concentrations of atmospheric SO_2. Note, the boxes show only one standard error on either side of the mean, and so are not 95% confidence limits.

Although the sample sizes are exceptionally small (n=3), we should undertake a residual analysis before proceeding with the full ANOVA.

```
PROC GLM;
    CLASS TRT;
    MODEL BIOMASS=TRT ;
    OUTPUT OUT=RESPLT STUDENT=RESID P=PRED;
RUN;
GOPTIONS RESET=ALL;
AXIS1 LENGTH=10 CM VALUE=(H=1)
      LABEL=(H=1 "PREDICTED");
AXIS2 LENGTH=15 VALUE=(H=1)
      LABEL=(H=1 A=90 "RESIDUAL");
PROC GPLOT DATA=RESPLT;
         PLOT RESID*PRED /HAXIS=AXIS1 VAXIS=AXIS2
VREF=0;
RUN;
```

Generate the residual plot for yourself by entering and submitting the above program.

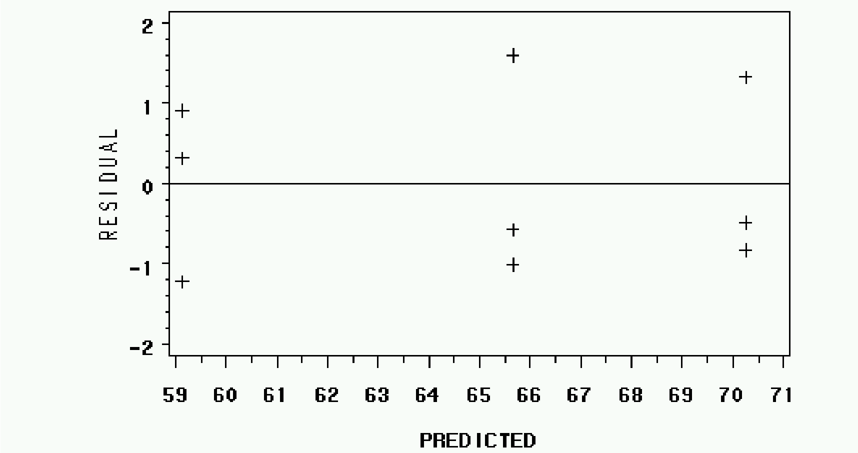


Figure 4–12. *A residual plot for an ANOVA of soybean growth against a treatment of three concentrations of atmospheric SO_2.*

The spread of the residuals looks good, so we should now consider normality.

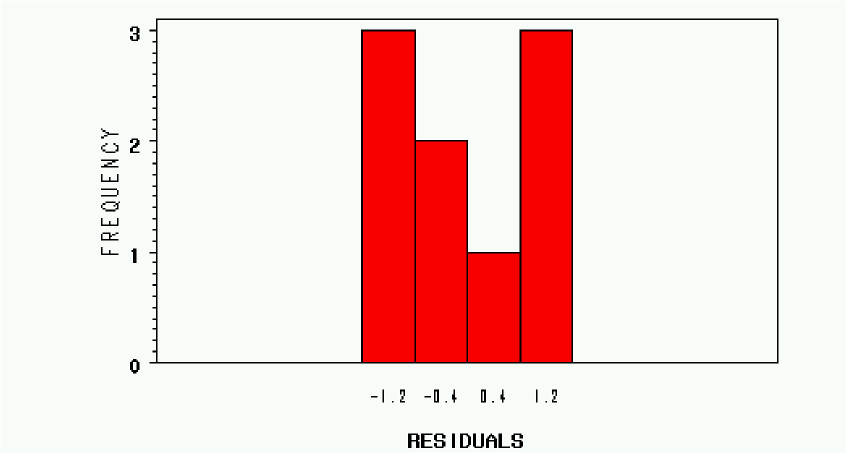


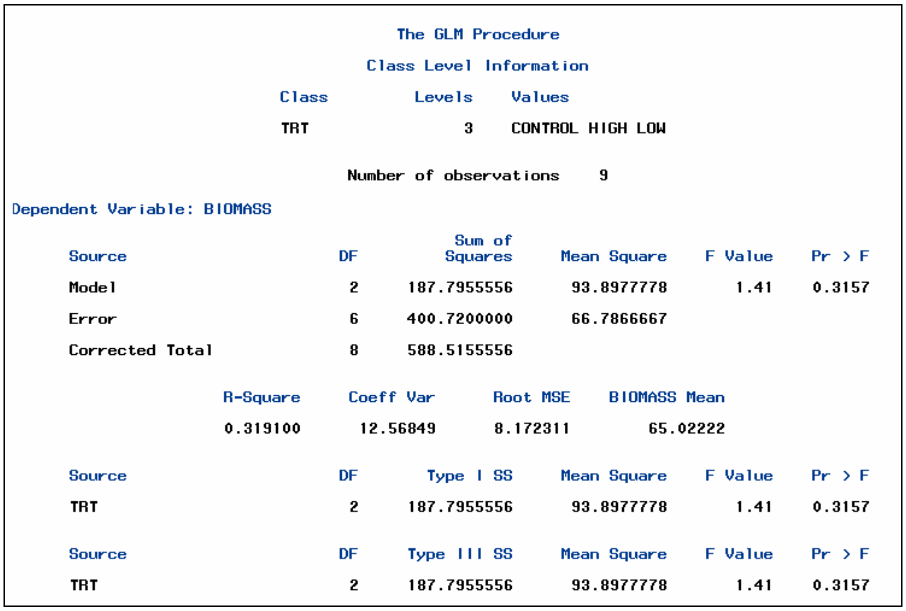
Figure 4–13. A *histogram* of residuals *from* an ANOVA of soybean growth against a treatment of three concentrations of atmospheric *SO_2*.

There is no strong indication of a problem with the distribution of the residuals, though with only nine values, the assessment is a bit moot.

Generate the histogram for yourself by entering and submitting the above program.

We can now proceed to interpret the ANOVA printout.

Box 4–4. *Output from PROC GLM used to undertake an ANOVA of soybean growth against a treatment of three concentrations of atmospheric SO_2.*



```
                                      The GLM Procedure

                                   Class Level Information

                            Class          Levels    Values

                            TRT                 3    CONTROL HIGH LOW


                                 Number of observations    9

Dependent Variable: BIOMASS

                                                Sum of
     Source                      DF          Squares     Mean Square    F Value    Pr > F

     Model                        2      187.7955556      93.8977778       1.41    0.3157

     Error                        6      400.7200000      66.7866667

     Corrected Total              8      588.5155556


                      R-Square     Coeff Var      Root MSE    BIOMASS Mean

                      0.319100      12.56849      8.172311        65.02222


     Source                      DF        Type I SS     Mean Square    F Value    Pr > F

     TRT                          2      187.7955556      93.8977778       1.41    0.3157


     Source                      DF      Type III SS     Mean Square    F Value    Pr > F

     TRT                          2      187.7955556      93.8977778       1.41    0.3157
```

The analysis of variance is not significant (F=1.41; df=2,6; p =0.3157) so the trend, which appeared so clear in the original box plot diagram, is not supported. The differences we observed in the sample data may well have occurred by chance.

Had we got a significant difference, we would have proceeded to perform a Dunnett Test to compare each manipulated SO_2 treatment against the control.

We can report that we were unable to demonstrate an effect of SO_2 on the growth of soybean, which may have resulted either because SO_2 does not influence soybean growth, or because our sample sizes were so small that any effect could not be detected by the ANOVA. Because of our small samples, we cannot say is that SO_2 does not affect soybean growth.

To proceed further, we need to do a **retrospective power analysis**. Such an analysis will tell us one of two things. First, it might tell us that we have sufficient power to detect all but insubstantial differences between treatment and control. If so, then our non-significant result can be regarded as evidence that SO_2 has no substantial effect on soybean growth. Alternatively it might tell us that our samples are far too small to be reasonably sure of detecting even a substantial effect of SO_2 on soybean growth, in which case we will have to carry our studies further with larger sample sizes.

Given our sample sizes (n=3), what is the smallest difference that we would be 80% sure of detecting at the 0.05 level of significance? We have:

$$P = 1 - \beta = 0.80\text{; } \alpha = 0.05\text{; } MS_{within} = 66.79\text{; } n = 3\text{; } \nu = 6$$

We have decided a priori that we are only interested in comparing the two manipulated SO_2 treatments with the control so applying the Bonferroni correction, we have:

$$\alpha' = \frac{\alpha}{2} = \frac{0.05}{2} = 0.025$$

From tables, we have $t_{0.025[6]}$=2.9687 and $t_{2(1-P)[\nu]}$=0.9057. If you do not have tables, you can obtain these figures from SAS using the TINV function (equivalent to one-tailed tables):

```
DATA TEST;
  A=TINV(0.025/2,6);
  B=TINV(0.40/2,6);
RUN;
PROC PRINT; RUN;
```

The figure we require is given by

$$\hat{\delta} \geq \left(t_{\alpha'[\nu]} + t_{2(1-P)[\nu]}\right)\sqrt{\frac{2MS_{within}}{n}} = (2.9687 + 0.9057)\sqrt{\frac{2\,x\,66.79}{3}} = 25.8532$$

So the smallest difference we could be reasonably sure of detecting with samples of size 3 is 26 g, or about a 37% reduction from the average for the growth of soybean in the experimental control (70 g). Clearly, our experiment is not nearly sensitive enough.

So if we are to run the experiment again, what sample sizes should we use to be reasonably sure of detecting an effect? To answer this question, we need to do a **prospective power analysis**.

Taking a 10% drop in yield as our smallest important difference, and striving for 80% chance of detecting such a difference, we have for our first iteration

$$\delta = 0.10 * 70 = 7.0\ g\text{; } P = 1 - \beta = 0.80\text{; } \alpha = 0.05\text{; } \alpha' = 0.025\text{;}$$
$$MS_{within} = 66.79\text{; } n = 3\text{; } \nu = 6$$

$$n \geq 2\left(\frac{\sigma}{\delta}\right)^2 \left(t_{\alpha'[\nu]} + t_{2(1-P)[\nu]}\right)^2 = 2\frac{66.79}{7.0^2}(2.9687 + 0.9057)^2 = 40.92$$

and for our second iteration

$n = 40\,;\nu = 117$

$$n \geq 2\left(\frac{\sigma}{\delta}\right)^2 \left(t_{\alpha'[\nu]} + t_{2(1-P)[\nu]}\right)^2 = 2\frac{66.79}{7.0^2}(2.2706 + 0.8447)^2 = 26.45$$

and for our third iteration

$n = 26\,;\ \nu = 75$

$$n \geq 2\left(\frac{\sigma}{\delta}\right)^2 \left(t_{\alpha'[\nu]} + t_{2(1-P)[\nu]}\right)^2 = 2\frac{66.79}{7.0^2}(2.2873 + 0.8464)^2 = 26.77$$

Hence, the minimum sample size for us to be 80% sure of detecting a difference in yield as small as 10% at the 0.05 level of significance in an ANOVA with one control and two treatments is n=26. Our current sample size of n=3 is definitely inadequate, but served the purpose of allowing us to estimate how many measurements per factor level we need to take.

If a sample size of 26 is impracticable, we have the choice of revising our decision on the minimum effect that is important to us, or to in some way reduce the variability in growth between plants within treatments. We might do this by taking germination as our starting point in the experiment rather than time of planting of the seed, or by reducing the genetic variability of the seeds we use, but with loss of generality.

Results summary

A fixed-effects, single-factor ANOVA failed to detect any significant differences in plant growth among fumigation treatments with SO_2 either at high (488.6 ppb) or low (97.3 ppb) concentrations (F=1.41; df=2,6; p>0.05) compared to the experimental controls (1.2 ppb).

A retrospective power analysis indicated that a difference in soybean growth between the experimental control and one of the SO_2 treatments would have to be 15 g (a 20% reduction) or more to have a reasonable chance (80%) of being detected at the 5% level of significance. Clearly sample sizes of n=3 are not sufficient to detect all differences of importance, so we cannot conclude that SO_2 has no impact on soybean growth.

Sample sizes of 62 or more would be needed to have an 80% probability of detecting a difference between control and treatment of 4%.

Discussion

The exposure of soybean to high and low concentrations of SO_2 (488.6 and 97.3 ppb respectively) was expected to reduce the production of soybean as measured by the total biomass at the end of the experiment (g/pot). That we failed to demonstrate such an effect leaves us with an inconclusive result. Either SO_2 has no effect on soybean growth under the conditions we applied, or the effect is less than likely to be detected with three replicates per treatment.

The recommendation is that the work be expanded, and that the researchers consider alternative designs for increasing the power of the analysis. Such options include:

- re-evaluating the size of the smallest difference to be considered important, perhaps defining the smallest important difference as the difference that will trigger management intervention;
- considering whether it can be argued that SO_2 will either have no effect or will impede growth, allowing for a series of one-tailed tests following a significant result in the ANOVA;
- increasing the number of SO_2 treatments to enable analysis by regression rather than ANOVA;
- increasing the sample size substantially.

Source

The data that form the basis of this example were kindly provided by Frank Stagnitti and Xianzhe Xiong of Deakin University. They are preliminary data from a pilot study of the effects of metals and sulphur dioxide on the production of primary crops.

Example 4-4: Chlorophyll-a proficiency testing program

This is a one-way ANOVA with a random factor followed by estimation of variance components.

The problem

In November 1986, the National Association of Testing Authorities (NATA) asked the Water Research Centre at the University of Canberra to participate in a Chlorophyll-a Proficiency Testing Program (NATA, 1988). The Centre and 24 other laboratories Australia-wide were asked to extract and determine the concentration of chlorophyll-a in three samples labelled A, B and C. The laboratories involved were not told that the three samples were simply replicates of the same batch. NATA was interested to know to what degree chlorophyll-a determinations were repeatable within labs and to what degree results were reproducible across labs. The data arising from the exercise are stored in the file CHLORO.DAT on the data disk, in the form shown below. The first variable is the LAB and the remaining three are the chlorophyll determinations from each lab.

1	8.18	8.04	9.00
2	27.60	28.00	28.80
3	.	.	.
4	31.22	27.92	27.05
5	8.49	17.64	17.73
6	34.83	33.58	32.24
7	43.15	46.74	37.30
8	21.89	14.42	15.49
9	34.60	33.30	32.00
10	13.24	13.13	15.26
11	20.47	33.25	30.49
12	38.98	38.26	43.19
13	34.85	36.82	34.20
14	33.44	37.78	34.84
15	38.96	38.39	37.88
16	16.02	17.95	16.02
17	35.28	34.21	44.90
18	32.08	33.55	33.00
19	56.30	56.92	58.80
20	40.67	38.90	40.26
21	23.00	32.00	35.00
22	60.48	42.53	45.89
23	28.09	34.21	41.27
24	13.88	14.10	15.86
25	7.58	11.05	11.23

The analysis

Data entry and exploratory examination

SAS has a myriad of ways of reading in raw data. These data could be arranged as two columns only, one containing the laboratory and the other containing the measurement of chlorophyll-a. The resulting file would be a little unwieldy for editing, printing and checking, so we might choose to arrange the data columns across the page and use the @@ option for input as in the above two examples. There is, however, a third way more convenient for this example. The data are entered and saved in the raw data file as they are seen above, and the following data step is used to read them in:

```
DATA CHLORO;
    INFILE "C:\MY DOCUMENTS\CHLORO.DAT";
    INPUT LAB A B C;
    CHL=A; OUTPUT;
    CHL=B; OUTPUT;
    CHL=C; OUTPUT;
    DROP A B C;
RUN;
```

Understanding this data step requires a little thought and knowledge of how SAS manipulates and stores data. The step reads the four columns of CHLORO.DAT into the factor LAB and the temporary variables A B and C. The statement

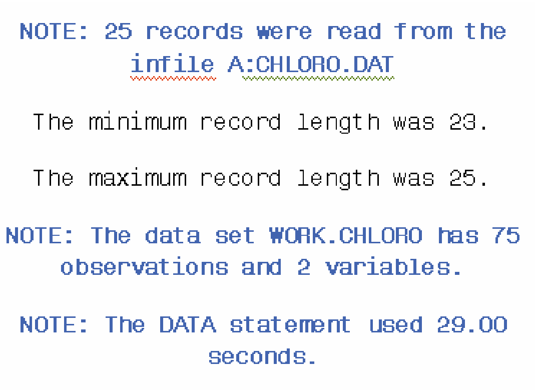
```
CHL=A; OUTPUT;
```

puts the contents of A into CHL and then outputs LAB and CHL to the workfile. The contents of variables B and C are handled similarly so that the workfile ends up with two columns of data—one called LAB, containing the labels given to each laboratory, and the other called CHL, containing the measurements of chlorophyll-a.

The DROP statement indicates to SAS that the variables A, B and C are not to be retained in the SAS workfile WORK.CHLORO.

Enter and submit the above data step.

Verify that the data has been read correctly first by perusing the LOG window:



```
NOTE: 25 records were read from the
        infile A:CHLORO.DAT

 The minimum record length was 23.

 The maximum record length was 25.

NOTE: The data set WORK.CHLORO has 75
    observations and 2 variables.

NOTE: The DATA statement used 29.00
              seconds.
```

If the data has been read correctly, there should be two variables in WORK.CHLORO, the class variable LAB and the measurement variable CHL. As there are 75 measurements of CHL, the work file should contain 75 observations. All would appear well from the above log, but just to make sure, list the contents of WORK.CHLORO:

Enter and submit the following Proc Step.

```
PROC PRINT;
RUN;
```

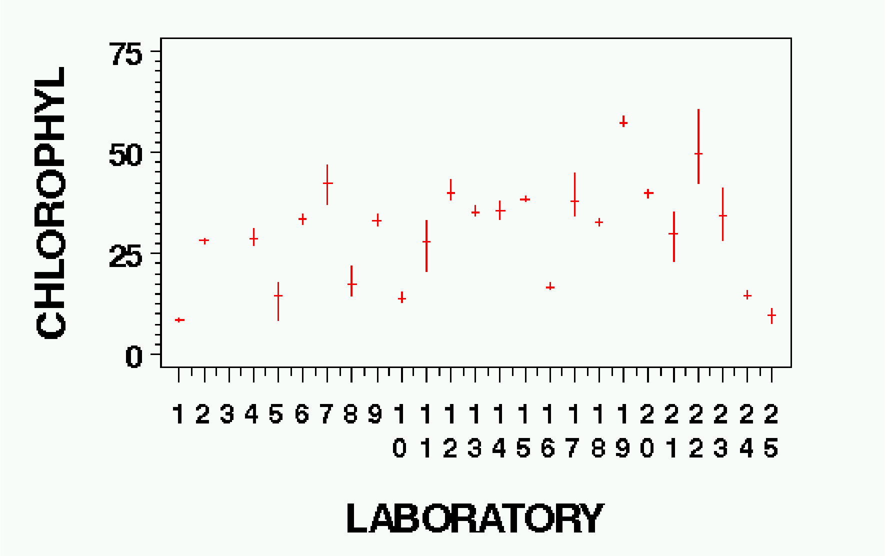
The data should appear in the OUTPUT window in the usual form, a factor or class variable and a measurement variable, suitable for analysis by the GLM procedure.

Perusal of these data reveal values ranging from 7.58 to 60.48 mg/L, a disturbing result given that all labs were Provided with the same material. A plot of the data reveals a veritable dog's breakfast (Figure 4–14).

```
GOPTIONS RESET=ALL;
SYMBOL1 C=RED I=HILO V=NONE;
AXIS1 LENGTH=10 CM ORDER=0 TO 75 BY 25
         VALUE=(H=1.5)
         LABEL=(H=2 A=90 "CHLOROPHYL");
AXIS2 LENGTH=15 CM VALUE=(H=1)
         LABEL=(H=2 "LABORATORY")
         OFFSET=(2 PCT)
         ORDER=1 TO 25 BY 1;
PROC GPLOT;
PLOT CHL*LAB=1 / HAXIS=AXIS2 VAXIS=AXIS1;
RUN;
```

Generate the plot for yourself by entering and submitting the above program.

Figure 4–14. *A plot showing the results of three chlorophyll-a determinations from each of 25 laboratories included in a NATA Proficiency Testing Program. All samples tested were identical. Symbols show the means and ranges.*



The data of Figure 4–14 are quite revealing. Many labs have reported quite repeatable results for their three determinations, as the range of determinations within those labs is quite low (indicated by a +). For other labs, the range of determinations for the three samples is quite high, indicating a poor ability to analyse chlorophyll-a with good repeatability. Also revealed is a disturbing range of determinations across labs, indicating that laboratories in Australia are unable to reproduce each other's results well.

A histogram of the means for each laboratory might give a clearer indication of the problem. The following data steps will produce such a histogram. We must first create a workfile that contains the means for each laboratory.

```
PROC SORT;
    BY LAB;
RUN;
PROC MEANS NOPRINT;
    VAR CHL;
    BY LAB;
    OUTPUT OUT=TEMPFILE MEAN=MEANS;
RUN;
```

The first two steps calculate the mean chlorophyll-a determination for each lab. The statement:

```
OUTPUT OUT=TEMPFILE MEAN=MEANS;
```

writes the variable LAB and the means for each laboratory to an internal SAS file called WORK.TEMPFILE. WORK.TEMPFILE becomes the default data set for subsequent analyses, and failure to recognise this may cause problems later.

The contents of WORK.TEMPFILE are as follows:

OBS	LAB	MEANS
1	1	8.4067
2	2	28.1333
3	3	.
4	4	28.7300
5	5	14.6200
6	6	33.5500
7	7	42.3967
8	8	17.2667
9	9	33.3000
10	10	13.8767
11	11	28.0700
12	12	40.1433
13	13	35.2900
14	14	35.3533
15	15	38.4100
16	16	16.6633
17	17	38.1300
18	18	32.8767
19	19	57.3400
20	20	39.9433
21	21	30.0000
22	22	49.6333
23	23	34.5233
24	24	14.6133
25	25	9.9533

Variables from this temporary SAS file can then be called up for use by subsequent steps.

The statements

```
GOPTIONS RESET=ALL;
AXIS1 LENGTH=10 CM ORDER=0 TO 10 BY 5
    LABEL=(H=2 A=90 "FREQUENCY");
AXIS2 LABEL=(H=1.5 "MEAN CHLOROPHYL");
PROC GCHART DATA=TEMPFILE;
    VBAR MEANS / RAXIS=AXIS1 MAXIS=AXIS2;
RUN;
```

produce a histogram of the mean determinations for the 25 labs (Figure 4–15).

> Generate the histogram for yourself by entering and submitting the above program.

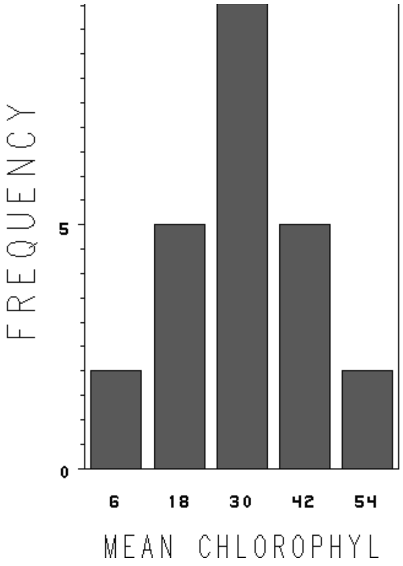


Figure 4–15. *A histogram showing the distribution of mean chlorophyll-a determinations (n = 3) from each of 25 randomly chosen laboratories around Australia.*

Analysis of variance

Determinations from the various labs range considerably, such that the extreme values of 7.58 and 60.48 cannot be considered solely as the result of a minority of aberrant labs. It should not come as any surprise to find that there are significant differences among labs (F = 32.50; d.f. = 23,48; p < 0.0005, Box 4-5), as indicated by an analysis of variance:

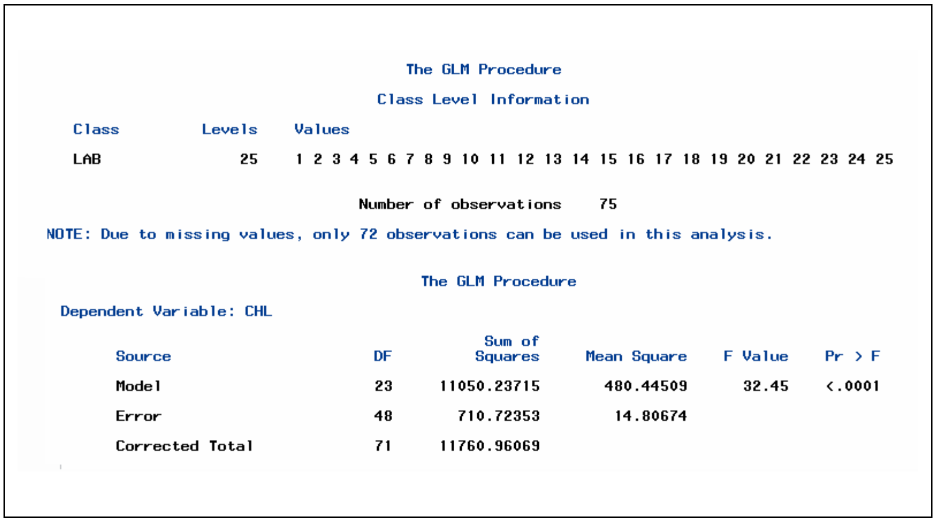
```
PROC GLM DATA=CHLORO;
    CLASS LAB;
    MODEL CHL=LAB;
RUN;
```

Note

The PROC GLM statement has to specify that the analysis is to proceed on data contained in the SAS workfile WORK.CHLORO created when we read in the raw data, and not on data contained in WORK.TEMPFILE created by the steps designed to produce the above histogram. SAS always defaults to using the last workfile to be accessed unless otherwise specified.

Enter and submit the above program.

Box 4-5. *Analysis of variance for chlorophyl-a determinations across 25 laboratories.*



```
                                 The GLM Procedure
                              Class Level Information
   Class         Levels    Values
   LAB               25    1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

                             Number of observations    75
NOTE: Due to missing values, only 72 observations can be used in this analysis.

                                   The GLM Procedure
 Dependent Variable: CHL
                                               Sum of
       Source                      DF         Squares     Mean Square    F Value    Pr > F
       Model                       23     11050.23715       480.44509      32.45    <.0001
       Error                       48       710.72353        14.80674
       Corrected Total             71     11760.96069
```

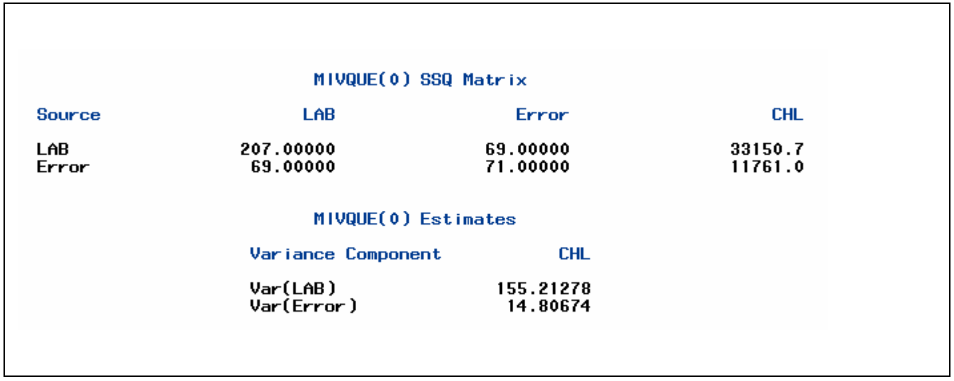
The labs involved in the NATA exercise can be considered a random selection of labs available in Australia, and NATA are interested in inferring from this study, some generalities about all labs in Australia. Besides, the NATA researchers were not interested in identifying which labs produced the best results, only in the degree to which results of chlorophyll-a determinations can be reproduced across labs. For these reasons, the design in this case is a random design single-factor ANOVA. The appropriate follow-up procedure in such cases is to estimate the variance components due to variation in determinations within and across labs. This can be achieved using the VARCOMP procedure.

Perform the following analysis using the VARCOMP procedure.

```
PROC VARCOMP DATA=CHLORO;
    CLASS LAB;
    MODEL CHL=LAB;
RUN;
```

The output is given in Box 4-6, and here we have a statement of the problem in a nutshell. Variation in the mean determinations among labs, over and above that expected on consideration of variation in determinations within single labs, was 155.26. When compared with the variation among determinations within labs of only 14.79, this is a sad testimony of the system's inability to conform to standard procedures leading to reproducible results for the determination of chlorophyll-a in Australian laboratories.

Box 4–6. *Estimation of the added variance component following a random model analysis of variance for chlorophyll-a determinations across 24 laboratories.*



```
                        MIVQUE(0) SSQ Matrix

Source               LAB              Error              CHL

LAB            207.00000           69.00000          33150.7
Error           69.00000           71.00000          11761.0

                        MIVQUE(0) Estimates

            Variance Component              CHL

            Var(LAB)                  155.21278
            Var(Error)                 14.80674
```

Expressing these results in percentage terms, we have:

$$\frac{S_A^2}{\frac{MS_{within}}{n}+S_A^2}.100\% = 96.9\%$$

Thus, only 3.1% of observed variation among mean determinations for the laboratories is what we would expect to occur if the laboratories could perfectly reproduce each others’ results. 96.9% of the variation among laboratories is attributable to differences in their procedures or equipment.

Consider total variation among single determinations taken one from each laboratory.

$$\left(\frac{S_A^2}{MS_{within}+S_A^2}\right).100\% = 91.3\%$$

Thus, 91.3% of variation is additional to what would have been expected if we sent all our samples to a single laboratory. This is the percentage contribution to variation that can be attributed to differences in equipment and procedures across laboratories. Hence, reproducibility is exceptionally poor (perfect = 100%).

$$\text{Reproducibility } = \left(1 - \frac{S_A^2}{MS_{within} + S_A^2}\right).100\% = 8.7\%$$

Repeatability is the ability to repeat results in a single laboratory, relative to the ability to reproduce results across laboratories.

$$\text{Repeatability } = \left(1 - \frac{MS_{within}}{MS_{within} + S_A^2}\right).100\% = 91.3\%$$

Results summary

The results of the analysis could be reported in the Results section of a report or paper along the following lines.

There was a significant difference among the 25 laboratories in their abilities to measure chlorophyll-a, as demonstrated by a single-factor ANOVA (F = 32.5; df = 23,48; p < 0.0005) (Table 4-10).

The added variance component due to differences among labs in their determinations of chlorophyll-a was 155.26 representing some 96.9% of observed variation among the means (n = 3). Reproducibility across labs was extremely low at 8.7%. Under ideal circumstances, it should be close to 100%.

Table 4–10. *Summary of the analysis of variance used to compare the ability of various laboratories (n = 24) to measure chlorophyll-a in identical samples prepared and distributed by NATA.*

Source	DF	Sum of Squares	Mean Square	F value	Pr F
Model	23	11052.70	480.55	32.50	0.0001
Error	48	709.70	14.79		
Corrected Total	71	11762.40			

Discussion

Some variation among labs was not totally unexpected by NATA, but its magnitude proved alarming. The exact cause of the high variation remained unidentified. Extraction of chlorophyl-a from the algal type used in the program (*Scenedesmus obliquus*) is exceptionally difficult, but this alone would not be expected to cause the observed variation in the results. Furthermore, many preliminary trials and statistical analyses were performed to ensure reasonable homogeneity between samples, so sample variability was ruled out as a possible cause.

Clearly, if there is to be attention given to improving the ability of Australian laboratories to measure chlorophyll-a in the laboratory, particular attention should be given to improving comparability of methods and equipment used across laboratories.

Adequacy of the design

This study was conducted by a professional body, NATA, well versed in statistical design. They went to great lengths to ensure that samples sent out to the laboratories were as close to identical as possible. This had two desirable effects. First, by keeping actual variability between samples to a minimum, the within laboratory variance would be kept to a minimum so increasing the chances of detecting a difference between laboratories. Second, ensuring homogeneity among samples would ensure that any added variance component was due entirely to differences among laboratories and not in part because different laboratories received samples that differed in concentration of chlorophyll.

Lesson 7: Some Challenging Exercises

Exercise 4-1: Turbidity in Lake Burley Griffin

Turbidity in lakes and storages is of interest to water scientists because it has a profound affect on aquatic biota, and it is especially implicated with the switch from systems where rooted aquatic plants dominate *(e.g. Vallisneria)* to systems where planktonic algae dominate (e.g. *Microcystis).*

Turbidity is relatively easy to measure, and may be used as a surrogate for phosphorus in programmes monitoring water quality.

In a pilot study, Kurt Hammerschmidt collected ten replicate samples of water from each of ten sites in Lake Burley Griffin. The sites were specifically chosen at set intervals along the main channel leading from the inflow to the Scrivener Dam wall so that they could be revisited if necessary. Turbidity (in ntu) was measured for each replicate sample taken at each site, and the data are shown in the table below.

__Figure 4-15.__ A map of Lake Burley Griffin showing the location of sampling sites.

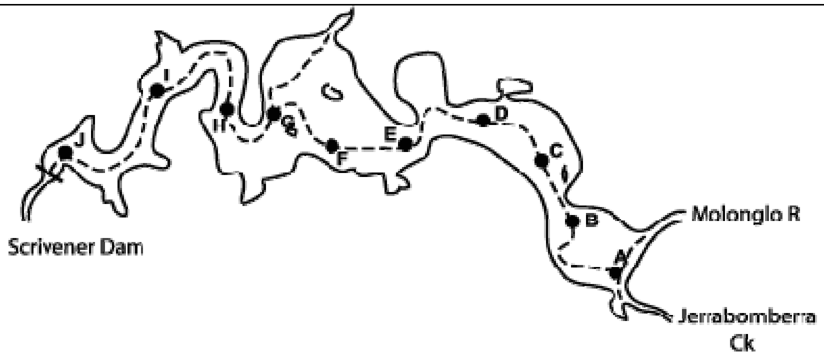


__Table 4-11.__ Turbidity measurements taken from each of ten sites along the channel in Lake Burley Griffin.

SITE									
A	B	C	D	E	F	G	H	1	J
43	25	23	32	17	23	14	13	15	13
28	28	24	32	21	21	18	26	15	15
43	28	30	32	18	17	14	18	14	14
28	25	32	33	17	18	16	15	12	13
42	25	25	32	25	19	14	15	17	16
43	25	28	29	17	24	9	14	19	19
40	26	23	26	18	14	14	17	15	16
35	25	25	38	14	17	26	15	14	15
42	23	26	27	15	17	10	11	16	11
43	25	27	29	15	18	15	14	14	15

Kurt was interested to know whether there were significant differences among the sites in mean turbidity, and if so, where those differences lay. The study was conducted to determine whether it is sensible to take measurements of turbidity in urban lakes and storages at only a few sites, as is current practice.

(a) Describe as completely as possible an appropriate analysis and give reasons for your choice. Be sure to specify the nature of the Factor (s) involved, and to state clearly the null hypotheses to be addressed.

(b) Enter the data in a form suitable for the nominated analysis, and conduct an exploratory analysis based on graphical presentations with box diagrams. Include the box diagrams below. What would you anticipate the results of an appropriate ANOVA to be?

(c) Perform the Analysis of Variance and summarise the results in the form of an ANOVA table.

(d) Before interpreting the ANOVA table, examine a plot of the residuals to determine whether the assumptions of the analysis are tenable. If not, try some potential remedies, and repeat the ANOVA. Please include any graphs from your residual analysis below.

(e) If the above analyses demonstrate a significant difference among the mean turbidity values, perform an appropriate follow-up analysis to determine where the differences lie. Present your results below.

(f) Write a summary of the results of the entire analysis, as might be included in the results section of a report or manuscript. Refer in your summary to an ANOVA table and a figure showing the variation among sites (box diagrams). Include in your results, a statement of any clear and statistically significant trends in turbidity, but do not at this stage attempt to explain them.

(g) Discuss the analysis in the context of the reasons for conducting the study. What might be the causes of the observed variation in mean turbidity among sites? What advice can you give to the water scientists charged with the responsibility of monitoring turbidity in Lake Burley Griffin?

Exercise 4-2: Point Impact Assessment

Phosphorus is an important nutrient in aquatic ecosystems, which can be changed dramatically through artificial discharges into streams and lakes. Phosphorous loads can be increased by agricultural activities of adjacent catchment areas, from discharge of household effluents, especially detergents, and from discharge of industrial wastes.

The following data are for concentrations of phosphorus (μg/l) in samples of water taken at various distances up and downstream of an industrial wastewater outlet. The figures in the body of the table are replicates taken from their respective locations at the one time.

Table 4-12. *Phosphorus concentration with distance downstream.*

Distance Downstream (km)				
- 0.5	**0.0**	**1.0**	**2.0**	**3.0**
4.86	6.16	6.82	5.86	5.31
4.86	5.83	6.67	5.73	4.98
5.19	6.93	6.34	5.62	4.98
4.31	6.16	6.08	4.83	5.46
4.99	6.93	5.73	5.49	4.66

The water scientist wishes to know whether the mean phosphorous levels differed among the sites and if so, were they significantly lower or higher downstream from the effluent discharge compared to the upstream "control" site. He or she also needed to assess if the impact, if any, could be considered local or if it persisted well downstream.

(a) Describe as completely as possible an appropriate analysis and give reasons for your choice. Be sure to specify the nature of the Factor (s) involved, and to state clearly the null hypotheses to be addressed.

(b) Enter the data in a form suitable for the nominated analysis, and conduct an exploratory analysis based on graphical presentations with box diagrams. Include the box diagrams below. What would you anticipate the results of an appropriate ANOVA to be?

(c) Perform the Analysis of Variance. Before interpreting the ANOVA table, examine a plot of the residuals to determine whether the assumptions of the analysis are tenable. If not, try some potential remedies, and repeat the ANOVA. Please include any graphs from your residual analysis below.

(d) Once you have the residuals in an acceptable form, repeat if necessary the Analysis of Variance and summarise the results in the form of an ANOVA table.

(e) If the above analyses demonstrate a significant difference among the mean phosphorus values, perform an appropriate follow-up analysis to determine where the differences lie. Present your results below.

(f) Write a summary of the results of the entire analysis, as might be included in the results section of a report or manuscript. Refer in your summary to an ANOVA table and a figure showing the variation among sites (box diagrams). Include in your results, a statement of any clear and statistically significant trends in turbidity, but do not at this stage attempt to explain them.

(g) Discuss the analysis in the context of the reasons for conducting the study. What impact did the industrial effluent have on stream phosphorus levels, and how persistent was that impact. If the impact is moderated by distance downstream, what biological processes might you suggest to explain this? What advice would you give to the Environmental Protection Authorities charged with responsibility for monitoring and maintaining water quality in our streams?

(h) Discuss the adequacy of the experimental design, especially with regard to the use of an upstream site as a control.

Exercise 4-3: Duration of chase in Australian Chats

Three species of Australian Chat (*Epthianura*) can be found in micro-sympatry in mesic coastal, semi-arid and xeric arid regions of Western Australia. *Epthianura aurifrons* is the most physiologically competent to handle aridity, *Epthianura albifrons* is the least physiologically competent and *Epthianura tricolor* is intermediate in competence.

Territorial behaviour is expensive in terms of maintaining water balance, so data was collected for each species in the arid zone to see if physiological competence has a bearing on the duration of the territorial chase. A bird is said to engage in a territorial chase when it sees another bird off its territory. The data comprise a variable giving the species and a variable giving the duration of chase (in seconds).

Table 4-13. *Duration of chase in three species of Australian chat.*

SPECIES	DURATION OF CHASE			
Albifrons	48	24	32	39
Tricolor	66	66	54	51
Aurifrons	72	74	76	70

Analyse the data using an appropriate ANOVA model to address hypotheses on differences in duration of chase.

(a) Describe as completely as possible an appropriate analysis and give reasons for your choice. Be sure to specify the nature of the Factor (s) involved, and to state clearly the null hypotheses to be addressed.

(b) Enter the data in a form suitable for the nominated analysis, and conduct an exploratory analysis based on graphical presentations with box diagrams. Include the box diagrams below. What would you anticipate the results of an appropriate ANOVA to be?

(c) Before preparing an ANOVA table, examine a plot of the residuals to determine whether the assumptions of the analysis are tenable. If not, try some potential remedies, and repeat the ANOVA. Please include any graphs from your residual analysis below.

(d) Perform the Analysis of Variance and summarise the results in the form of an ANOVA table.

(e) If the above analyses demonstrate a significant difference among the mean chase durations, perform an appropriate follow-up analysis to determine where the differences lie. Present your results below.

(f) Write a summary of the results of the entire analysis, as might be included in the results section of a report or manuscript. Refer in your summary to an ANOVA table and a figure showing the variation among species (box diagrams). Include in your results, a statement of any clear and statistically significant trends in chase duration, but do not at this stage attempt to explain them.

(g) Discuss the analysis in the context of the reasons for conducting the study. What might be the causes of the observed variation in chase duration among species taking into account differences in their physiological tolerance to aridity?

Exercise 4-4: Home range Estimation in Badgers

Badgers are widespread in Britain. In 1988, there were estimated to be around 42,000 social groups of badgers, and just under 200,000 adult badgers. By 1997 this had risen to just over 50,000 social groups and 310,000 adult badgers. The population is now probably stable. However, badgers are a high profile species, and their biology and conservation is of great interest.

Badgers live in social groups of four to 12 adults. They are a focus system for development of behavioral models in social grouping behavior that are uniquely relevant to carnivores. One of the prevailing models is known as the Resource Dispersion Hypothesis (RDH). The RDH hypothesis suggests that the dispersion and richness of resources in the environment provide a passive mechanism for the formation of groups, even without the direct benefits of group living. However, few studies have empirically tested the RDH in the field.

Dominic Johnson and his colleagues tested several hypotheses about RDH with their data on badgers in Wytham Woods, near Oxford, UK. In order for them to make this a meaningful test though, they had to standardise on an accepted method of describing home range. A preferred method of calculating home range is a contentious issue debated among wildlife biologists, so Dominic wished to compare the estimates of home range area by three calculation methods: the interpolated mapping method (INT), the minimum convex polygon method (MCP), and the Dirichlet tessellation method (TES).

The data reside in the file badger.dat and comprise a class variable containing the method of home range area estimation and the home range areas (km^2) as data pairs.

(a) Describe as completely as possible an appropriate analysis and give reasons for your choice. Be sure to specify the nature of the Factor (s) involved, and to state clearly the null hypotheses to be addressed.

(b) Conduct an exploratory analysis based on graphical presentations with box diagrams. Include the box diagrams below. What would you anticipate the results of an appropriate ANOVA to be? Do you think it likely that the assumptions of the analysis will be upheld. If not, why not?

(c) Perform the Analysis of Variance. Before interpreting the ANOVA, examine a plot of the residuals to determine whether the assumptions of the analysis are tenable. If not, try some potential remedies, and repeat the ANOVA. You may need to hit the data with a sledge hammer to meet the assumption of normality – Y' = LOG10(LOG10(Y+1). If there is a conflict in meeting the two assumptions of normality and homogeneity of variances, which takes precedence in this case? Why? Please include any graphs from your residual analysis below.

(d) After selecting your final transformation, repeat the ANOVA. If the above analyses demonstrate a significant difference among the home range areas, perform an appropriate follow-up analysis to determine where the differences lie. Present your results below.

(e) Write a summary of the results of the entire analysis, as might be included in the results section of a report or manuscript. Refer in your summary to an ANOVA table and a figure showing the variation among sites (box diagrams). Include in your results, a statement of any clear and statistically significant differences or trends in home range area, but do not at this stage attempt to explain them.

(f) Discuss the analysis in the context of the reasons for conducting the study. What are the management implications of these results?

Exercise 4-5: T-tests versus ANOVA

You would appreciate by now that the single factor fixed design analysis of variance can be applied to compare several means, but you may not have realised that it can be used for the two sample case in place of the Student's t-test. Under such circumstances, when two statistical procedures are equally appropriate, it is satisfying to learn that they are mathematically equivalent and so always yield the same result. In fact, there is a simple relationship between F and t.

$$F_{0.05(1)[1,\nu]} = t^2_{\ 0.05(2)[\nu]}$$

so the *F* value for the one-tailed test of analysis of variance, with 1 and ν degrees of freedom, is equal to the square of the *t* value for the equivalent t-test, with ν degrees of freedom.

This exercise is designed to demonstrate the point empirically. Consider again the study of *Antechinus* conducted by Geoff Smith at Cooloola in 1977 and 1978. Recall that he was interested to see if the weights of male marsupial mice *Antechinus flavipes* differed for the two years.

Table 4-14. *Weights of Antichinus flavipes in two consecutive years.*

YEAR			
1977		1978	
66	66	52	52
72	57	52	49
53	53	54	54
62	61	54	50
59	59	45	61
		58	

(a) Analyse the data using a student's t-test to determine if the weights of *Antechinus flavipes* differ significantly between years. Record the sample value of t, the degrees of freedom, and the level of significance (*p* value). Refer to statistical tables to obtain the tabulated value of t at the 95% level of significance.

(b) Now analyse these data as a single factor fixed model analysis of variance to address the same hypothesis. Complete the following table of results.

(c) Does the above relationship hold for the sample values of t and F?

(d) What do you conclude about the likely outcomes of the two approaches to the same problem?

Where have we come?

Lesson 7 is where the real learning occurs. In earlier lessons, you have read and understood written material and been led through worked examples. In Lesson 7 you were required to recall and integrate the information to complete some challenging real-world exercises. Recall in the context of problem solving is one of the best ways of achieving lasting learning.

In completing this module successfully, you will have achieved a number of core competencies, namely,

- Knowledge of the options available to you for analysing a single sample in terms of reporting its mean and the level of precision associated with it – the confidence limits.
- Knowledge of the options available to you for comparing two samples to see if the differences you observe are sufficient to conclude that the difference is real.
- Practical skills in the operation of SAS to undertake the necessary analyses.
- The ability and confidence to to interpret the results of the analyses in a biological context based on demonstrated understanding of the analyses.
- The ability to present findings in a style appropriate to the scientific literature.
- Appropriate attitudes and efficient strategies for extending your abilities to conduct analyses and solve problems beyond the scope of this module, by using resource materials such as statistical texts, software manuals, and your colleagues.

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