Solution to Exercise 6.5 (Version 1, 26/10/14)

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Exercise 6.5 (Data: courtesy M. Torrance, Rothamsted Research)

An experiment investigated detection of IgG antibodies ingested by parasitoids with enzyme-linked immunosorbent assay (ELISA). At the start of the experiment, parasitoids were fed either honey spiked with antibodies or normal honey (negative control, labelled Control). Those fed spiked honey were either tested immediately afterwards (positive control to check that the antibodies had been ingested, labelled Day0) or after one, two or three days (labelled Day1, Day2, Day3, respectively), having been fed on normal honey in the interim. The five treatments were each allocated at random to 10 parasitoids as a CRD and the insect samples were placed into 50 wells of a standard 96-well microplate for testing. The resulting optical density readings (variate *OpticalDensity*) are in file PARASITOIDS.DAT with the unit number of each parasitoid (*DParasitoid*) and the treatment to which it was allocated (factor Treatment).

The main aim of the experiment was to assess for how long after ingestion the antibodies could be detected, that is, comparisons between the negative control treatment and samples after one, two or three days. Analyse these data appropriately using one-way ANOVA and discuss whether this aim can be fully realized. What conclusions can you draw?

Data 6.5 (PARASITOIDS.DAT)

Optical density readings for parasitoids fed with honey spiked with antibodies (and tested immediately or after 1, 2 or 3 days) or normal honey (Control) according to a CRD:

	Days after ingestion of antibodies				
Parasitoid	0	1	2	3	Control
1	3.212	3.286	3.212	0.837	0.094
2	3.365	1.586	3.054	1.571	0.086
3	3.462	3.337	0.941	0.469	0.090
4	3.279	1.198	0.588	0.422	0.097
5	3.365	0.749	0.488	0.321	0.092
6	3.559	2.066	3.358	0.355	0.082
7	3.580	3.358	2.531	0.115	0.088
8	3.112	3.103	0.761	0.523	0.097
9	3.258	1.180	1.641	0.128	0.087
10	3.279	0.392	1.878	0.978	0.142

Solution 6.5

A priori, we expect heterogeneity in this experiment. The control group (negative control) is given no antibodies, and so we expect their readings to be zero, or very close to zero (assuming no contamination). A preliminary scan of the data suggests that this is the case. Similarly, the Day0 group is a positive control group to check that the antibodies can be detected. In this group, there has been no

time for the parasitoids to process the antibodies and so we expect the readings to reflect the full dose and hence to be consistent across parasitoids. Again, this seems to be the case. For the remaining groups, we expect more variability as the rate at which the antibodies are processed might be expected to vary between parasitoids. We cannot predict whether this variability might change over the course of the experiment but Table S6.5.1 shows the treatment sample means and variances, and suggests that there is variance heterogeneity among the Day1, Day2 and Day3 groups.

Table S6.5.1 Treatment sample means and variances.

	Days after ingestion of antibodies				
	0	1	2	3	Control
Sample mean	3.347	2.026	1.845	0.572	0.095
Sample variance	0.0226	1.3486	1.2830	0.1983	0.0003

If we didn't stop to think about the structure of the treatments, we might just rush ahead and fit a single factor model with symbolic form

Response variable: *OpticalDensity*
Explanatory component: [1] + Treatment

The residual plots (based on standardized residuals) from the resulting one-way ANOVA are shown in Figure S6.5.1. It is clear from the fitted values plot (top right) and absolute residuals plot (bottom left) that the variances are heterogeneous, being much smaller for treatments with the smallest and largest fitted values (Control and Day0 groups, respectively). Unsurprisingly, Bartlett's test confirms that the observed between-group heterogeneity is larger than would be expected if all groups had the same underlying variance ($\chi^2 = 87.87$ on 4 df, $P < 0.001$).

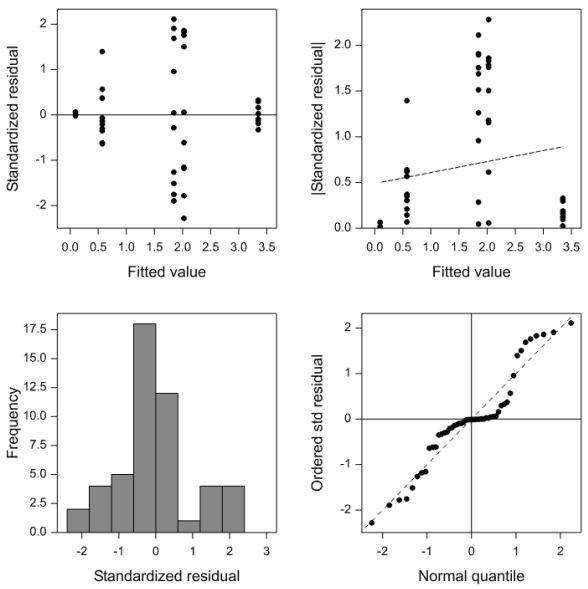

Figure S6.5.1. Composite set of residual plots based on standardized (std) residuals obtained from analysis of optical density readings.

Of the two control treatments, the positive control (Day0 group) is of no scientific interest. We are interested in how long the antibodies can be detected for, i.e. comparisons against the Control group, and the positive control was included only to check that antibodies could be detected immediately after ingestion. In Section 8.5, we discuss the use of controls within experiments and state that the data from control groups should usually be included in the analysis. The only exception occurs when the controls are uninformative and might bias estimates of background variation. The Day0 group fits this definition and so we will exclude it from the analysis. The Control group does not fit the definition as comparisons with it are of scientific interest; however, if we include it in the analysis then we must find a way to get a realistic estimate of background variation if the results are to be valid. As there appears to be a pattern of increasing variance once the Day0 group has been removed, we try taking a logarithm transformation (base 10), as $\mathit{LogDensity} = \log_{10}(\mathit{OpticalDensity})$, and then refit the single factor model to the transformed response excluding the Day0 group. The resulting residual plots are shown in Figure S6.5.2, and it appears that although the variation within the treatment groups (Day1, Day2 and Day3) is similar, the variation within the Control group is still much smaller. We have not been able to find a transformation to achieve homogeneity of variances and so we conclude that we cannot fully realize the aims of this experiment using a single factor model and one-way ANOVA. However, we can get a valid analysis of the three treatment groups (Day1, Day2 and Day3) on the $\log_{10}$ scale and so we will do this.

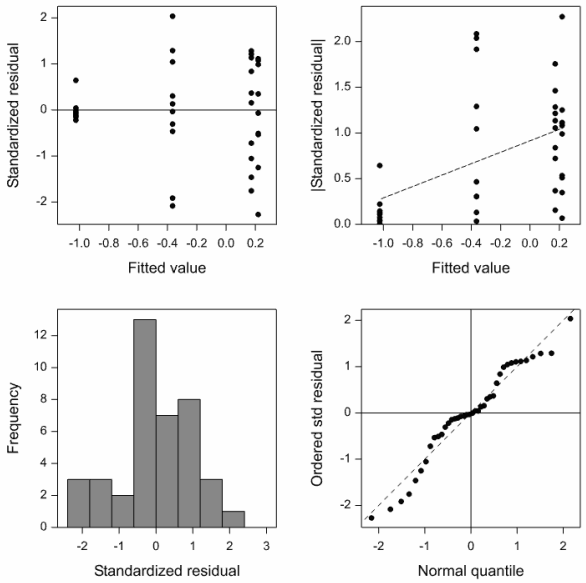

Figure S6.5.2. Composite set of residual plots based on standardized (std) residuals obtained from analysis of the $\log_{10}$-transformed data excluding the Day0 group.

The one-way ANOVA table for analysis of the $\log_{10}$-transformed readings of the three treatment groups is Table S6.5.2, and a composite set of residual plots is Figure S6.5.3. The residual plots are broadly acceptable, although there is non-linear trend in the normal plot and a suggestion of skewness in the distribution of the residuals. There is strong evidence of a difference in population means between the three treatments ($F_{2,27} = 9.496$, $P < 0.001$). Predicted treatment means are shown in Table S6.5.3 and Figure S6.5.4. Basing comparisons on the LSD, there is no evidence of a change in population mean log optical density between the first and second days after feeding (groups Day1 and Day2), but there is evidence that the population mean 3 days after feeding (group Day3) is smaller than that on the previous two days.

Table S6.5.2 ANOVA table for $\log_{10}$-transformed optical density readings with three antibody treatments.

Source of variation	df	Sum of squares	Mean square	Variance ratio	*P*-value
Treatment	2	2.1077	1.0539	9.496	< 0.001
Residual	27	2.9964	0.1110		
Total	29	5.1041			

Table S6.5.3 Mean $\log_{10}$-transformed optical density readings with back-transformed values. Log scale: SED = 0.1490; LSD = 0.3057 (α_s = 0.05, df = 27).

	Days after ingestion of antibodies		
	1	2	3
Mean ($\log_{10}$ scale)	0.219	0.172	−0.365
Back-transformed mean	1.656	1.487	0.431

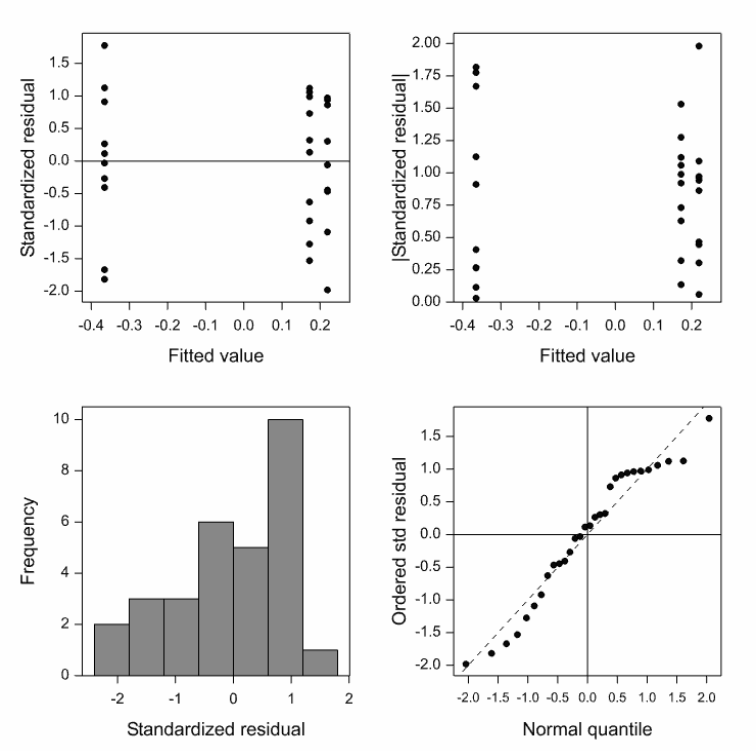

Figure S6.5.3. Composite set of residual plots based on standardized (std) residuals obtained from analysis of the $\log_{10}$-transformed data (excluding both positive and negative control treatments).

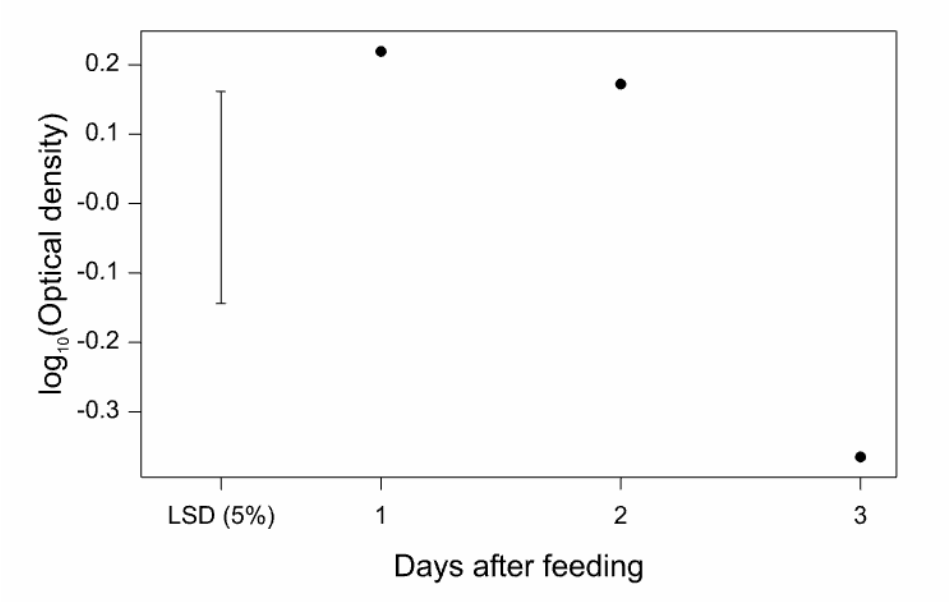

Figure S6.5.4. Mean $\log_{10}$-transformed optical density readings obtained 1, 2 and 3 days after feeding with antibody-spiked honey, with LSD ($\alpha_s = 0.05$, df = 27).

We can conclude that, on average, fewer antibodies are present 3 days after feeding than on the first or second day after feeding, but this analysis cannot determine whether the level present after 3 days is equivalent to that for insects that were not fed the antibodies, as it does not include the Control group. To pursue this aspect, we might think of using a t-test without the assumption of equal variances. We also need to think carefully about the form of our test: we are interested in whether the antibodies can still be detected after 3 days, i.e. whether the reading from Day3 is equivalent to the Control group reading. As discussed in Section 10.5, where we are more interested in the similarity between groups rather than the difference, then our null hypothesis and statistical tests must be amended. We might therefore take the TOST (two one-sided t-tests) approach described in Section 10.5, modified to allow for unequal variances between groups. We leave the details as an exercise for the reader.