Model Based Statistics in Biology.
Part IV. The General Linear Model. Multiple Explanatory Variables.
Chapter 13.4 Fixed x Random Effects (Randomized Complete Block)

ReCap.	Part I (Chapters 1,2,3,4), Part II (Ch 5, 6, 7)
ReCap	Part III (Ch 9, 10, 11)
ReCap	Multiple Regression (Ch 12)
13.1	Fixed Effects ANOVA (no interactive effects)
13.2	Fixed Effects ANOVA (interactive effects)
13.3	Fixed*Random Effects (Paired t-test)
13.4	Fixed*Random Effects (Randomized Block)
13.5	Fixed*Random Effects (Repeated Measures)
13.6	Nested Random Effects (Hierarchical ANOVA)
13.7	Random within Fixed (Hierarchical ANOVA)
13.8	More Than Two Factors (to be written)

on chalk board

Tribolium growth data
Sokal and Rohlf Box 11.4
Ch13.xls

ReCap Part I (Chapters 1,2,3,4) Quantitative reasoning is based on models, including statistical analysis based on models.
ReCap Part II (Chapters 5,6,7)
Hypothesis testing uses the logic of the null hypothesis to declare a decision.
Estimation is concerned with the specific value of an unknown population parameter.
ReCap (Ch 9, 10,11) The General Linear Model with a single explanatory variable.
ReCap (Ch 12) GLM with more than one regression variable (multiple regression)
ReCap (Ch 13) GLM with more than one categorical variable (ANOVA).
Two fixed factors (Ch 13.1, Ch13.2)
One fixed and one random factor (Paired t-test)

Today: Randomized Blocks (Special case of Two way ANOVA)
One factor fixed by design, the other factor is random.

Wrap-up.
The randomized block design is analyzed with a general linear model consisting of two explanatory variables on a nominal scale.
One of these is fixed (two or more classes),
the other is random (two or more classes).
We are interested in the fixed effects controlled for the random effects.

Introduction.

Research context.

The flour beetle *Tribolium casteneum* was a model lab organism for establishing the basic facts and principles of quantitative genetics, including inbreeding and response to selection. Once the whole genome was sequenced, *Tribolium* was used in immunohistochemistry, in situ hybridization, gene sequencing for characterization of microRNAs, and gene editing.

Economic context.

Tribolium is a major pest of stored grain. Economic losses consist of reduced weight and product quality, difficulties in baking, reduced marketability of infested products, and an accompanying unpleasant smell. Pest numbers are reduced by sieving or by adding inert dusts that cause death by dessication.

Statistical context.

This study illustrates a randomized block design, which has a fixed and a random factor. The randomized block is an example of statistical control, in which the effects of one variable (the random factor) are removed in order to arrive at a better analysis of the fixed factor. This analysis is a more sensitive test because it removes some of the noise in the data before testing. The paired t-test is a special case of the randomized block, in which the fixed factor has just two categories.

Statistical control is used when manipulative control is not possible.

Epidemiology: manipulative control is unethical.

Many field situations: manipulative control is not feasible.

Manipulative control is impossible at large scales.

Manipulative control can be expensive, even at small scales.

Manipulative control can generate artefacts, a study with well designed statistical controls can be more informative.

1. Construct model

Data are from Box 11.4 in Sokal and Rohlf 1995, p 350. Dry weights (mg) of 3 genotypes of *Tribolium castaneum* in 4 experiments. Within each experiment the order of weighing was random, resulting in a randomized-complete-block design with 4 blocks.

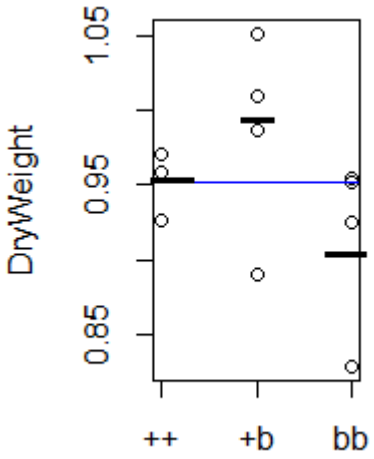
	Genotypes		
Blocks	++	+b	bb
1	0.958	0.986	0.925
2	0.971	1.051	0.952
3	0.927	0.891	0.829
4	0.971	1.010	0.955

Verbal model.

Does weight of flour beetle *Tribolium* vary among genotype, after controlling for differences among experiments ? Genotype bb is a mutant strain, for which the expected weights are least for the homozygote bb and greatest for the heterozygote +b.

The analysis removes the effects of blocks. This results in a more sensitive test of whether weight varies among genotypes.

1. Construct model



Graphical model.

Response variable is beetle mass
The first explanatory variables is genotype, a fixed factor under the control of the experimenter.
The second explanatory variable is block, a random factor beyond the control of the investigator.
The graphical model consists of 3 means, relative to the grand mean.

Table of variables

Symbol	Name	Fixed or Random?	Units	Type of measurement scale
M	beetle mass		mg	ratio
G	genotype	Fixed	++ +b, bb	categorical (nominal)
B	block	Random	Exp 1,2,3,4	categorical (nominal)

Formal model.

Write GLM:	M	=	β_o	+	$\beta_B \cdot B$	+	$\beta_G \cdot G$	+	$\beta_{GxB} \cdot G \cdot B$	+ residual		
S&R81	Y_{ijk}	=	μ	+	B_j	+	α_i	+	$(\alpha \cdot B)_{ij}$	+	ε_{ij}	

Common practice is to use roman letters to distinguish random from fixed effects.
Genotype is a treatment effect, so Greek letters are used.
Block is a random effect so Roman letters are used.

In this example we have insufficient degrees of freedom to estimated both the interaction term and the residual. The interaction term becomes part of the residual in the revised model

full GLM:	M	=	β_o	+	$\beta_G \cdot G$	+	$\beta_B \cdot B$	+	$\beta_{GxB} \cdot G \cdot B$	+ ε
revised GLM	M	=	β_o	+	$\beta_G \cdot G$	+	$\beta_B \cdot B$	+	ε	

This is called a ***mixed model***. It has a random and a fixed factor.

The test over the residual term with the reduced model is equivalent to a test over the interaction term, which is computationally correct. A test over the residual term with the full model is not computationally correct because it assumes that a random component, the interaction term, is negligible. This has been called "pseudoreplication" even though the problem is misallocation of the mixed (interaction) term in forming the F-ratio.

2. Execute analysis.

Place data in model format:

Column labelled M, with response variable mass

Column labelled X_B with explanatory variable, X_B = B1,B2,B3, or B4

These are labels (categories), not numbers on ratio scale.

Note the labelling B1,B2,B3,B4 instead of 1,2,3,4

This forces the factor to be categorical, not ratio scale.

Column labelled X_G with explanatory variable, X_G = bb, +b, ++

Code model statement in statistical package according to the GLM

$$M = \beta_o + \beta_B \cdot X_B + \beta_G \cdot X_G + \varepsilon$$

Write the code for the model, using XB and XG for factors.

The grand mean.

$\hat{\beta}_0 = 12^{-1} \Sigma M = 12^{-1} \cdot 11.426 = 0.95217$ mg

Wt	grand mean	Gtype Effect	Block Effect	Fits	Res
0.958	0.952	0.00458	0.004	0.9609	-0.0029
0.971	0.952	0.00458	0.039	0.9959	-0.0249
0.927	0.952	0.00458	-0.070	0.8869	0.0401
0.971	0.952	0.00458	0.027	0.9833	-0.0123
0.986	0.952	0.03233	0.004	0.9887	-0.0027
1.051	0.952	0.03233	0.039	1.0237	0.0273
0.891	0.952	0.03233	-0.070	0.9147	-0.0237
1.010	0.952	0.03233	0.027	1.0110	-0.0010
0.925	0.952	-0.03692	0.004	0.9194	0.0056
0.952	0.952	-0.03692	0.039	0.9544	-0.0024
0.829	0.952	-0.03692	-0.070	0.8454	-0.0164
0.955	0.952	-0.03692	0.027	0.9418	0.0133

The fitted values are computed from the genotype and block means.

mean(M_{G++}) = 0.95675 mg

$\hat{\beta}_{G++}$ = (0.95675 – 0.95217)

= 0.00458 mg

etc.

The table shows calculations in spreadsheet format.

3. Evaluate the model.

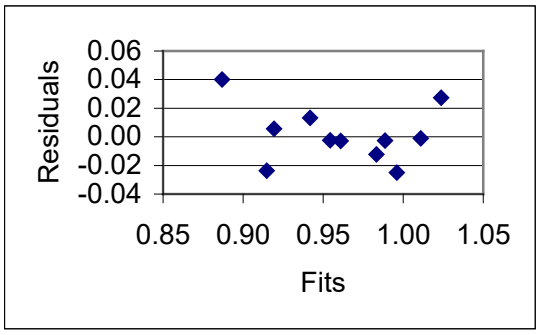
Plot residuals versus fits.

<u>Structural model.</u>

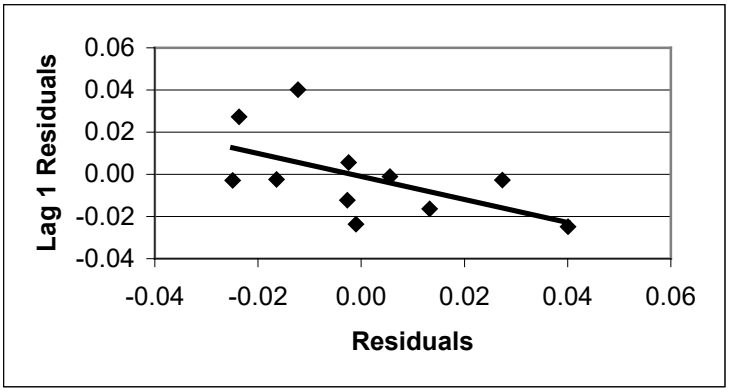
No line fitted in model, so skip this evaluation.

<u>Error model</u>

Homogeneity. No systematic change in residuals with increase in fitted values (*i.e.* no cones) so residual homogeneous, no need to revise error model.



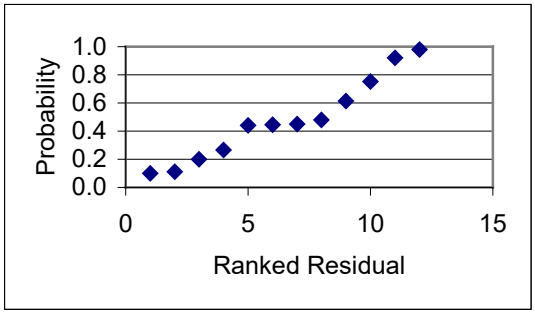
3. Evaluate the model.



Homogeneous? Yes

Independent? The graph suggests a downward trend for residuals listed in order of blocks within genotype. There is a suspicious negative association going from block 1 to 2, 2 to 3, 3 to 4. There appears to be a negative carryover effect, assuming the experiments are presented in the order in which they were conducted.

Normal ?



The residuals deviate somewhat from normal, as judged relative to the trend line in the normal probability plot. There is some indication of clustering of values around the median value of the residuals,-- the plot tends horizontally from the 5th ranked to the 8th ranked residual.

4. Partition df and SS. Calculate LR

GLM	$M-\beta_o$	=	$\beta_B \cdot X_B$	+	$\beta_G \cdot X_G$	+	ε
Source	Total	=	Block	+	Genotype	+	Resid
df	12–1	=	4 –1	+	3–1	+	12–3–2
SS	0.0353	=	0.021391	+	0.009717	+	0.004184

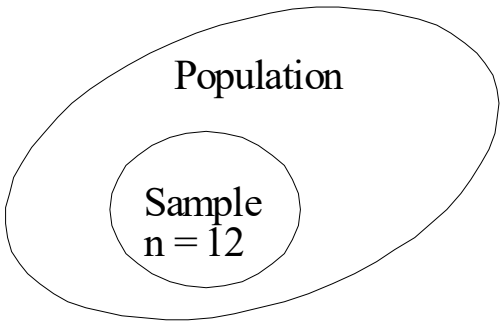
$LR = (0.353/0.004184)1^{-12/2} = 3.6 \times 10^5$

5. Choose mode of inference.

In a randomized complete block design all treatments are found in each block. Inference is to many repetitions of the experiment, where each block is an experimental run. Control of Type I error is implicit in the analysis of such experiments. So we will use a fixed tolerance for Type I error.

5. State population and whether sample is representative.

The sample was taken from a population of beetles maintained by the investigator (R.R. Sokal). Inference is to the population of beetles in this lab and presumably to all beetles of this species, which share the same genetics. Inference is to the same chance set-up, measurement of weight of beetles.



6. State H_A H_o pairs, test statistic, distribution, tolerance for Type I error.

Interaction term. There is no H_A for the interaction term. The term is the product of a random and fixed term. This results in a mixed term, which is treated as random. To eliminate bias from this term, genotypes were weighed in random order (S&R95 p351). Note that the interaction and error term cannot both be estimated.

> Note: no df left if Var(GxB) estimated
> $df_{total} = 11$ $df_G = 2$ $df_B = 3$ $df_{GxB} = 2*3 = 6$
> $df_{res} = 11 - 2 - 3 - 6 = 0$

Dropping the interaction term from the model flushes this component of variance to the residual, which now consists of a mixed term (which can be estimated) in addition to a residual term that cannot be estimated in this example.

Block term (experiment)

There is no H_A about this term. We are not interested in this effect. Instead, we want to estimate the variance component due to blocks and remove this variance from the error term, to produce a more sensitive test with a better chance of detecting main effects.

Experimental term

This is a fixed effect, the means are of interest.

$E(M_{bb})$ is the expected value (true mean) of the weight of genotype bb
$E(M_{+b})$ is the expected value (true mean) of the weight of genotype +b
$E(M_{++})$ is the expected value (true mean) of the weight of genotype ++

H_o: $E(M_{bb}) = E(M_{+b}) = E(M_{++})$
H_A: the means differ

The hypothesis pair above is equivalent to the following pair concerning variance.

H_A: $Var(\beta_{Genotype}) > 0$ There is variance due to experimental factor.
H_o: $Var(\beta_{Genotype}) = 0$

Are there more specific hypotheses about parameters? No

State test statistic	F-ratio
Distribution of test statistic	F-distribution
Tolerance for Type I error	5% (conventional level)

7. ANOVA - Calculate then partition df and SS according to model.

```
Model at top of board on left.
ANOVA table at top of board on right.
```

GLM	$M-\beta_o$	=	$\beta_B \cdot X_B$	+	$\beta_G \cdot X_G$	+	ε
Source	Total	=	Block	+	Genotype	+	Resid
df	12–1	=	4 –1	+	3–1	+	12–3–2
SS	0.0353	=	0.021391	+	0.009717	+	0.004184

$SS_{tot} = Var(M) \cdot df_{total} = 11 * Var(M) = 11 * 0.0032084 = 0.0353$

$SS_{tot} = \Sigma Y^2 - n^{-1}(\Sigma Y)^2 = 10.914748 - 12^{-1} \cdot 11.426^2 = 0.0353$

SS_{total} computed by Minitab.

```
MTB> let k1 = ssq('weight')
MTB> print k1
```

GLM commands in other packages perform in similar ways, to partition the variance.

GLM commands compute MS and variance ratio F. MS block was not computed, there is no interest in testing whether this term is significant. The interest is in estimating it.

Source	df	SS	MS	F---->	p
blocks	3	0.021391			
gtype	2	0.009717	0.004858	6.97	0.027
residual	6	0.004184	0.000697		
total	11	0.0353			

Calculate Type I error.

$p = 0.027$ calculated from F-distribution with df = 2, 6

Statistical control

Compare this partitioning to that when the Block term was not included in model

```
MTB > anova 'weights' = 'gtype'
Factor     Type Levels Values
gtype     fixed      3     1     2     3
Analysis of Variance for weights

Source      DF         SS         MS       F      P
gtype        2   0.009717   0.004859    1.71  0.235
Error        9   0.025575   0.002842
Total       11   0.035292   0.003208
```

Compare SS	SS_{error} shrinks from 0.0256 to 0.0042
Compare MS	MS_{error} shrinks from 0.02842 to 0.000697
Compare R^2	R^2 increases from 28% to 88%
Compare LR	LR increase from 6.9 to 3.6×10^5
Compare F-ratio	F-ratio increases from 1.71 to 6.97
Compare Type I errors	p-value shrinks from 0.235 to 0.027

7. ANOVA - Statistical control

Because the block effects are estimated and removed, the residual SS is much smaller. This allows smaller genotypic differences to be detected.

Sokal and Rohlf 1995 (p 350), provide a calculation of the increased efficiency of the randomized block design. Reducing the error variance via statistical control is one of the key concepts of experimental design.

8. Decide whether to recompute p-value.

Residuals were homogeneous, perhaps not independent, and slightly deviant from a normal distribution.

Sample size *n* is small, but p = 0.027 and hence would need to change be a factor 2-fold to change our assessment of Type I error.

Given this information, we would not usually undertake randomization, even though the residuals were not independent.

How good was this judgement?

The p-value via randomization in this case is 128/5000 = 0.0256

The p-value changed by a factor of 0.027 / 0.0256 = 1.05.

Our judgement (no need for randomization) was correct.

Having computed the p-value based on randomization, we report it because it is free of assumptions.

9. Report statistical conclusion.

Only one term, the fixed factor, is tested.

We reject the null hypothesis, H_o of not difference.

There is significant variation in mean dry weight among genotypes.

$F_{2,6} = 6.97$ $p = 0.027$

No parameters are reported for the block term because it is a random factor and so the means are of no interest.

mean(M_{bb}) = 0.957mg	st.err = 0.0104 mg
mean(M_{+b}) = 0.9845 mg	st.err = 0.0339 mg
mean(M_{++}) = 0.915 mg	st. err = 0.0295 mg

The differences in weight among genotypes are small and not detectable with these standard errors. The differences become detectable (as in the ANOVA table) when variation among experiments (blocks) is removed from error term.

10. Report science conclusion.

The differences among means were small. At the same time they were greater than those from chance, once we control for among block variance. Because of the substantial variation among experiments, statistical control was necessary to detect differences in dry weight among genotypes.