

## Model Based Statistics in Biology.

### Part IV. The General Linear Model. Multiple Explanatory Variables.

#### Chapter 14.2 ANCOVA - Statistical Control

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)
ReCap	Multiple Categorical Variables (Ch 13)
14.1	Comparing Regression Lines
14.2	Statistical Control
14.3	Model Revision
14.4	More than two explanatory variables (to be written)

CrwTb9_1.xls Ch14.xls
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on chalk board

**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).

**ReCap** (Ch 14) ANCOVA with GLM - Comparing regression lines.

Today: Statistical control, with ANCOVA.
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Statistical control allows the effects of one variable to be removed, in order to arrive at a better analysis of the effects of another variable.
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#### Wrap-up.

Statistical control improves analysis by removing the effects of a secondary variable, to achieve lower residual mean square and better analysis of the variable of interest.

In ANCOVA either the ratio scale or the nominal scale explanatory variable can be the control variable. A ratio scale response variable (*e.g.* fish production from lakes) can be analyzed relative to a ratio scale explanatory variable (*e.g.* size of lake) controlled for a nominal scale variable (*e.g.* temperate versus tropical lakes). Or a nominal scale explanatory variable (*e.g.* experimental treatment versus control) can be tested controlling for the effects of a ratio scale explanatory variable (*e.g.* metabolic rate of the animal).

Of these two possibilities, the more commonly encountered is that of a classification (nominal scale) explanatory variable, controlled for a ratio scale variable. An example of this was worked through today.

## Introduction.

ANCOVA is applied to data situations that have a mixture of both ratio and nominal scale explanatory variables. We have already looked at ANCOVA where we use the interaction term to compare slope across a categorical variable. Today we will look at another application of ANCOVA, where we use the regression variable to control for its effects in comparing means across a categorical variable. This analysis assumes homogeneous slopes, which we can evaluate with the interaction term.

Data are from Table 9.1 in M.J. Crawley (1993) *GLIM for Ecologists*. The data are reported on p 288 in Crawley (2003) *Statistical Computing* as fruit biomass of the plant *Ipomopsis*. The Scarlet Gilia *Ipomopsis aggregata* subsp *weberi* is a rare and endangered plant endemic to the Park Mountain Range in Colorado and the Sierra Madre Range in Wyoming. The biomass of the fruit is a measure of seed production by each plant.

As reported in Crawley (2003) 40 plants were allocated to two treatments, grazed or not grazed by rabbits.

The grazed plants were exposed to rabbits during the first two weeks of stem elongation, then protected from subsequent grazing.

Seed production depends on plant size, which is measured as the diameter at the top of the root stock (in mm). Root diameter was measured before exposure to grazing.

At end of growing season, fruit biomass ( $M_{fruit}$  = mg dry wt) was recorded for each of the 40 plants.

### 1. Construct model

#### Verbal model.

Rabbit grazing reduces fruit biomass and hence seed production, once we control for the relation of production to root size.

Fruit (mg)	Root (mm)	Grazed
59.77	6.225	n
60.98	6.487	n
14.73	4.919	n
19.28	5.13	n
34.25	5.417	n
35.53	5.359	n
87.73	7.614	n
63.21	6.352	n
24.25	4.975	n
64.34	6.93	n
52.92	6.248	n
32.35	5.451	n
53.61	6.013	n
54.86	5.928	n
64.81	6.264	n
73.24	7.181	n
80.64	7.001	n
18.89	4.426	n
75.49	7.302	n
46.73	5.836	n
80.31	8.988	y
82.35	8.975	y
105.1	9.844	y
73.79	8.508	y
50.08	7.354	y
78.28	8.643	y
41.48	7.916	y
98.47	9.351	y
40.15	7.066	y
116.1	10.25	y
38.94	6.958	y
60.77	8.001	y
84.37	9.039	y
70.11	8.91	y
14.95	6.106	y
70.7	7.691	y
71.01	8.515	y
83.03	8.53	y
52.26	8.158	y
46.64	7.382	y

# 1. Construct model

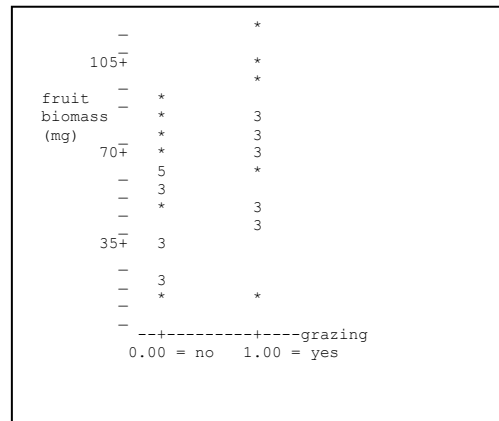
Table of variables

Symbol	Name	Units	Scale type	Functional placement	Random/Fixed
$M_{fruit}$	Fruit	mg dry weight	ratio	Response	
$Root$	Root	mm (diameter)	ratio	Explanatory	Fixed
$Gr$	Grazed	Y/N	categorical	Explanatory	Fixed

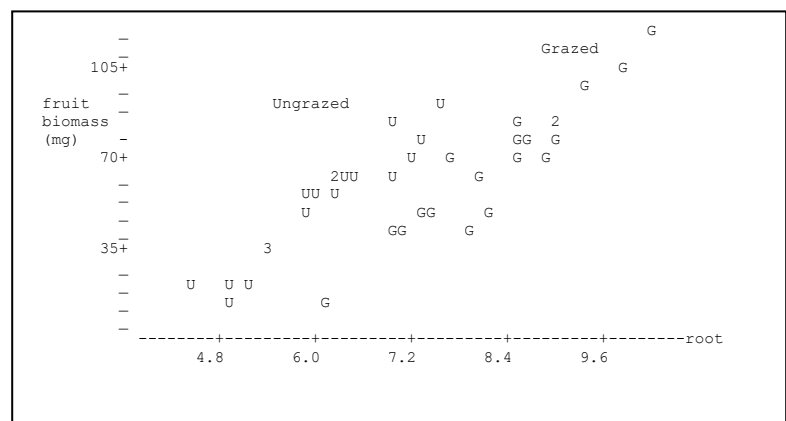
The Grazed term is fixed by the experimental protocol to two categories, yes or no. Root diameter is listed here as fixed because we are inferring only to the experimental material. We are not inferring beyond the material, to a population of random slopes.

## Graphical model.

Fruit biomass in relation to grazing pressure.



Fruit biomass in relation to root diameter



In the graph above left, add a short horizontal line showing your visual estimate of the mean in the grazed and ungrazed plants. Comparing the means, we see that grazed plants (68 mg) have a higher mean than ungrazed (51 mg).

This difference is due to the larger size of the grazed plants. It becomes clear that plants were not randomly assigned to one of the two treatment groups, a standard practice in experimental design.

In the graph showing root diameter, sketch a regression line, ignoring the groups. Then sketch a regression line for each of the two groups. We see that the lines look parallel for the two groups, but are not parallel to the line drawn through all the data. Comparing the slopes we find that the overall slope is  $\hat{\beta}_{root} = 14.0$  mg/mm.

The ANCOVA estimate of the slope (14 mg/mm) from both groups differs markedly from the slope estimate for each group: 24 mg/mm for ungrazed, 23.3 mg/mm for grazed. This arises from the lateral offset: ungrazed plants are smaller, hence to the left of the grazed plants in the graph. This lateral offset reduces the overall slope from around 23 mg/mm in each group to 14.0 mg/mm averaged across both groups.

## Graphical model.

In the context of statistical control the covariate (Root) and the  $Root \times Gr$  term are not of interest. The text example makes no mention of hypotheses concerning the interaction term. However, if grazing alters the relation of fruit biomass to plant size, we might expect the regression slopes to differ. We will include the interactive effects, to check the assumption of parallel slopes.

## Formal model

Write the formal model (GLM).

$$M_{Fruit} = \beta_o + \beta_{Root} * Root + \beta_{Gr} * Gr + \beta_{Root*Gr} * Root * Gr + \varepsilon$$

Above each term sketch a graph showing data dots with means or lines

## **2. Execute analysis.**

Place data in model format:

Column labelled  $M_{fruit}$  the response variable fruit biomass (mg dry wt)

Column labelled Grazed with explanatory variable Gr: grazed=Y, ungrazed=N

Column labelled Root with explanatory variable Root = diameter

Code the model statement in a statistical package according to the GLM.

$$M_{Fruit} = \beta_o + \beta_{Root} Root + \beta_{Gr} Gr + \beta_{Root \cdot Gr} Root \cdot Gr + \varepsilon$$

```
MTB > glm 'Mfruit' = 'root' 'Gr' 'root'*'Gr';
SUBC> covariate 'root'.
```

Fits and residuals are calculated in any of several ways:

- model statement output of fitted values and residuals (as above), or
- parameters reported by GLM routine, or
- direct calculation of parameters (means and slopes) from the data.

The mean for grazed and ungrazed is expressed as a deviation from  $\hat{\beta}_o = 59.4$  mg

$$\hat{\beta}_o + \hat{\beta}_{Gr} = \begin{cases} \text{mean}(M_{Gr=no}) = 59.41 - 8.53 = 50.88 \text{ mg} \\ \text{mean}(M_{Gr=yes}) = 59.41 + 8.53 = 67.94 \text{ mg} \end{cases}$$

The slope parameter for grazed and ungrazed together is  $\hat{\beta}_{root} = 14.0$  mg/mm

```
MTB > regress 'fruit' 1 'root'.
```

The regression equation is  
fruit = - 41.3 + 14.0 root

Predictor	Coef	Stdev	t-ratio	p
Constant	-41.31	10.73	-3.85	0.000
root	14.026	1.464	9.58	0.000

The regression slopes differ substantially from each other:  $125/94 = 1.33$

$$M_{Gr=No} = -94.367 + 23.996 Root$$

$$M_{Gr=Yes} = -125.28 + 23.254 Root \quad \text{The slope is 33\% greater than ungrazed.}$$

### 3. Evaluate the model

Plot residuals versus fits.

Straight line assumption

questionable. Residual plot shows pattern of rise from left to right in each group, with drop in the transition from ungrazed to grazed.

Error model.

If n small, evaluate assumptions for p-values from chisquare (t, F) distributions.

n = 40, so even substantial deviations will have little distorting effect on calculation of parameter estimates and p-values.

a. Homogeneous? No

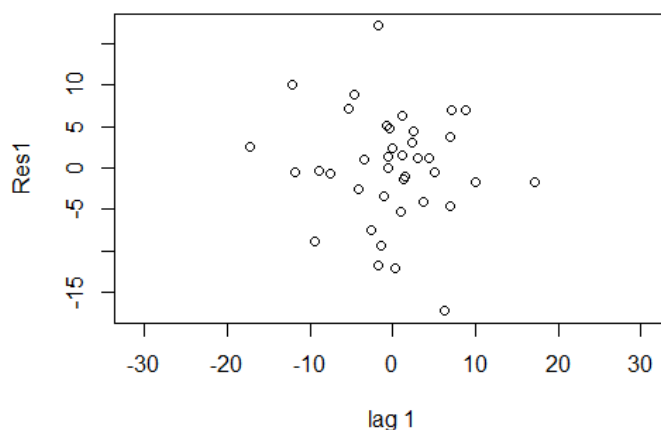
Residuals show a spindle shape: high dispersion at the center, tapering to less dispersion at the extremes. The dispersion is greater where the two groups overlap, in the middle of the plot.

b. Normal?

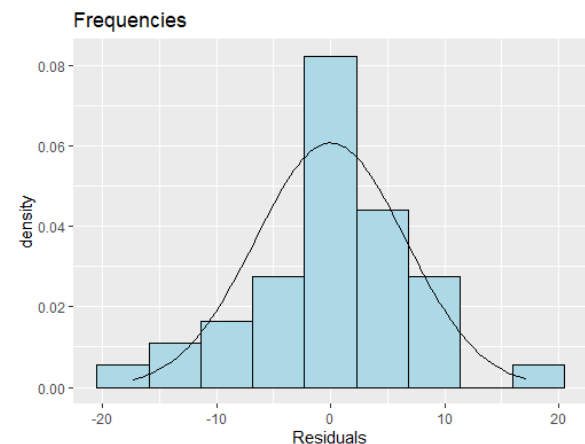
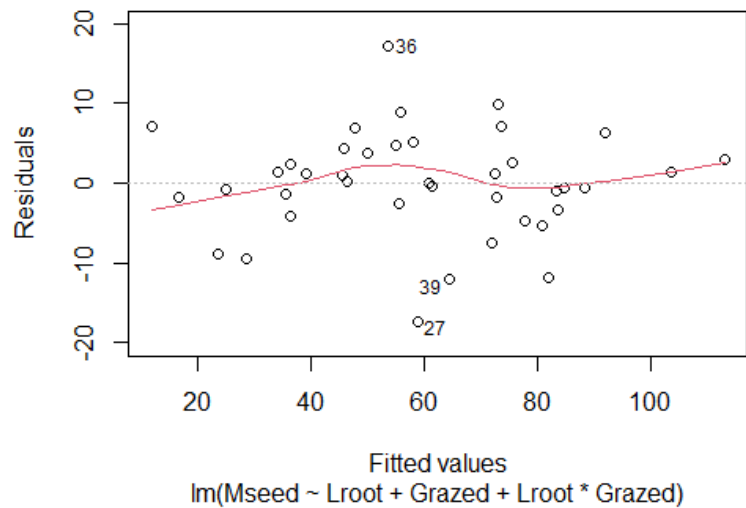
The residuals look normal, except for excess number around the overall mean. The QQ plot echoes the pattern in the heterogeneity plot – the residuals trend away from normal in the lower left and upper right of the plot.

c. Independent?

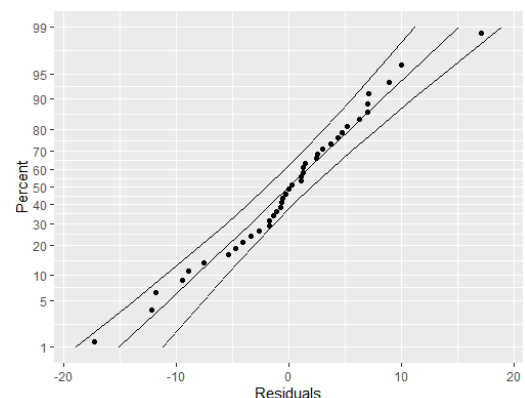
Each residual plotted against its neighbor.



The residuals are clearly not homogeneous, but this does not appear to produce outliers that can have undue influence on accurate estimates of the regression slopes.



No evidence of non-independence.



#### 4. Partition df and SS according to the model.

$$\begin{array}{rclclclclcl}
 M & = & \beta_o & + & \beta_{root}Root & + & \beta_{Gr}Gr & + & \beta_{Root \times Gr}Root \cdot Gr & + & res \\
 40-1 & = & & & 1 & + & 1 & + & 1 & + & 39 \\
 23752.2 & = & & & 16800.4 & & 5266.7 & & 4.6 & + & 1680.5
 \end{array}$$

#### Calculate likelihood ratio for overall (omnibus) model.

$$1 - R^2 = (1680/23752.2) = 0.07075 \quad LR = (1 - R^2)^{-40/2} = 2.7 \times 10^{22}$$

#### 5. State population and whether the data are a representative sample.

The principles of sound experimental design are randomization, replication, and local control. In this case we have acceptable replication—20 grazed and 20 ungrazed plants. Randomization and local control were not achieved. Grazed plants were larger at the outset. This introduces an uncontrolled source of variance that is confounded with the presence/absence of grazing. The reason for larger plants in the group was not reported in the text example.

The sample of 40 plants will be taken as representative of a population based on multiple repeats of the same experimental protocol, keeping in mind the limitations of that protocol.

#### 5. Justify mode of inference.

For ANCOVA with statistical control by the covariate, we assume that the slopes are homogeneous and that the interaction term is negligible. We will use likelihood ratios to evaluate this assumption. We will not use hypothesis testing to judge whether the interactive effect is important (cf. Chapter 11). Instead, we will focus on measures of evidence and on an accurate estimate of the difference in fruit biomass, accompanied by a measure of uncertainty.

#### 6. State hypothesis pairs and likelihood ratios.

First, a check on whether slopes are parallel.

$$\beta_{root*Gr=0} \neq \beta_{root*Gr=1} \quad (\text{slope not parallel})$$

$$\beta_{root*Gr=0} = \beta_{root*Gr=1} \quad (\text{slopes parallel})$$

Next the factor of interest, Grazing or not.

$$\beta_{Gr=0} > \beta_{Gr=1} \quad (\text{Grazing reduces fruit biomass})$$

$$\beta_{Gr=0} \leq \beta_{Gr=1} \quad (\text{Grazing does not reduce fruit biomass})$$

#### 7. ANOVA

```
MTB > glm 'seed' = 'root' 'grazing' 'root'*'grazing';
SUBC> covariate 'root'.
```

Analysis of Variance for seed

Source	DF	Seq SS	Adj SS	Adj MS	F	P
root	1	16800.4	18791.6	18791.6	402.57	0.000
grazing	1	5266.7	157.1	157.1	3.37	0.075
grazing*root	1	4.6	4.6	4.6	0.10	0.754
Error	36	1680.5	1680.5	46.7		
Total	39	23752.2				

Root is listed first, which controls for this covariate in the sequential analysis.

The Minitab package uses the adjusted SS to calculate F-ratios.

## 7. ANOVA

The default in the R package is the sequential SS.

```
SeedMod<-lm(Mass~RootDiameter+Grazed+Root*Grazed,data=CrwTb9_1)
anova(SeedMod)
```

		SS	MS	Fratio	
Root	1	16800.4	16800.4	359.912	< 2.2e-16
Grazed	1	5266.7	5266.7	112.8286	1.21E-12
Root:Grazed	1	4.6	4.6	0.0994	0.7544
Residuals	36	1680.5	46.7		

First, the interaction term.

$$LR = ((4.6/1680.5)+1)^{40/2} = 1.1 \text{ from sums of squares}$$

$$LR = ((1/36)(0.10)+1)^{40/2} = 1.1 \text{ from F and degrees of freedom}$$

There is no evidence of an interactive effect. The slopes are parallel.

Next, the grazing effect.

$$LR = ((157.1/1680.5)+1)^{40/2} = 2.1 \times 10^{-12} \text{ from sums of squares}$$

There is strong evidence for a grazing effect in the sequential analysis.

Looking at the adjusted SS table we see a much reduced SS and hence much reduced evidence for a grazing effect.

$$LR = ((157/1680.5)+1)^{40/2} = 6 \text{ from sums of squares.}$$

There is inadequate evidence ( $LR < 10$ ) for a grazing effect.

## 8. When assumptions are not met, decide whether to re-compute likelihood ratio.

Likelihood ratios and the F-statistics calculated from them depend on the error model. We already have seen that the residuals plotted against the regression variable show a pattern. As we saw when we sketched the graphical model, the slope of the straight line is biased downward by the predominance of ungrazed plants at small root sizes, and the predominance of grazed plants at large root sizes. Rather than revising the statistical model, we can check our results against a completely different tactic, that of taking the data over the limited range of root sizes where both grazed and ungrazed plants occur. This occurs in the middle of the graph, at root sizes from 6.225 to 7.69 mm. This removes the confounding effect of difference in root size between the grazed and ungrazed plants. Instead of revising the statistical model, we control for root size by executing the same ANCOVA model with data where the confounding effect is removed.

## 9. Summary of results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Root	1	850.7	850.7	29.7567	0.0001465	***
Grazed	1	3603.6	3603.6	126.0551	1.011e-07	***
Root:Grazed	1	159.9	159.9	5.5919	0.0357427	*
Residuals	12	343.1	28.6			

$$LR = ((1/12)(5.59)+1)^{40/2} = 2097 \quad \text{from F and degrees of freedom}$$

With unconfounded analysis we have evidence for a difference in regression lines for grazed and ungrazed plants.

For the unconfounded analysis, the interactive effect is now evident.

	Estimate	Std. Error	t value	Pr(> t )
(Intercept)	-57.177	23.778	-2.405	0.033234
Root	18.563	3.508	5.291	0.000191
Grazed yes	-124.799	39.082	-3.193	0.007729
Root:Grazed yes	13.237	5.598	2.365	0.035743

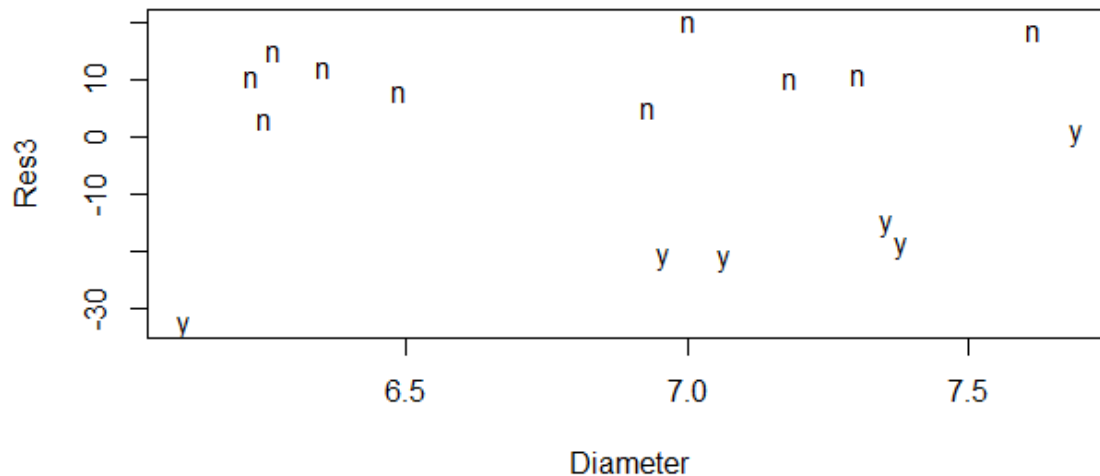
In the presence of an interactive effect we cannot interpret the main effect, grazing.

The slope estimate for ungrazed is 18.5 mg/mm

The slope estimate for grazed is  $18.5 + 13.2 = 31.7$  mg/mm.

The relation of fruit size to root size is greater for grazed than ungrazed plants. This can be attributed to large plants having greater capacity to withstand grazing than smaller plants.

The difference in fruit size is evident in the graph of the residuals from regression for the balanced data.



The residuals for the unconfounded analysis show far less pattern than those for unbalanced data.

The residuals show clear separation of grazed and ungrazed plants, with far lower fruit biomass for grazed than ungrazed plants.



## 10. Conclusion

When slopes are homogeneous, a convenient way to compare effects of grazed and ungrazed plants is to use the points at which  $x = \text{zero}$  (the y-intercepts). When the slopes are heterogeneous, as with the unconfounded analysis, divergence in slopes amplifies the difference in y-intercepts.

When slopes are heterogeneous, an alternative to comparing the y-intercepts is to compare the residuals from regression for grazed and ungrazed plants. For the balanced data, the residuals from regression on root diameter were negative for grazed, positive for ungrazed.

Ungrazed average = 11.04 mg    Grazed average = -40.5 mg

Ungrazed - Grazed = -29.44 mg

The reduction due to grazing is substantial, compared to the average biomass of all ungrazed plots = 69.4 mg

-29/69    # = -42 %    Balanced data (Residuals from regression)

-36/69    # = -52 %    Unbalanced data (Comparison of y-intercepts)

The parameter estimates differ by a ratio of  $36/29 = 1.24$ . The reduction in fruit biomass estimated from unconfounded analysis is high, compared to that estimated from the confounded analysis.

## Estimate of grazing effect based on comparison of Y-intercepts, unbalanced data.

The slopes are parallel.  $\beta_{root*Gr=0} = \beta_{root*Gr=1}$

Because of the offset, we report the rate in each group, not the overall rate.

### 9. Analysis of parameters of biological interest.

	grazing	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN
fruit	0	20	50.88	54.24	50.84	21.76	4.87
	1	20	67.94	70.85	68.21	24.97	5.58

When root size is not taken into account, the fruit biomass appears to be less for ungrazed than for grazed.

Ungrazed      50.88 mg

Grazed      -67.94 mg

Difference      -17.06 mg

This is because initially the grazed plants were larger than the ungrazed plants.

To compare grazed vs ungrazed, controlled for size, we calculate the vertical separation between the two regression lines. A convenient point at which to do this is the point at which  $x = \text{zero}$  (the y-intercepts).

$\hat{a}$	=	$\beta_o$	-	$\beta_{root}$	*	mean(X)
$\hat{a}_{Gr=no}$	=	Mean( $M_{Gr=no}$ )	-	$\beta_{root}$	*	Mean( $root_{GR=no}$ )
	=	50.88	-	23.6	*	6.053) = -91.729 mg
$\hat{a}_{Gr=yes}$	=	Mean( $M_{Gr=Yes}$ )	-	$\beta_{root}$	*	Mean( $root_{GR=Yes}$ )
	=	67.94	-	23.6	*	8.309) = -127.82 mg

The intercept for grazed lies below that for ungrazed.

The vertical separation between the two regression intercepts is:

Grazed      -127.820) mg

Ungrazed      - (-91.729) mg

Difference      - 36.091 mg

By this accounting, the fruit biomass for grazed plants was less by 36 mg. This accounting is based on a regression line extrapolated beyond the data, from unbalanced data that distorts the regression. The regression slope for all data differs from the regression slope in each group, which are nearly the same.

### **Estimate of grazing effect based on residuals from regression in each group.**

When grazing effects are controlled by sequential analysis, the covariate appears first, with grazing second. This is equivalent to taking the residuals from the covariate, then comparing the means of the residuals from the two groups. The analysis above used sequential sums of square.

The use of sequential SS suggests a different approach, which is to estimate the regression in each group, calculate the residuals from that regression in each group, and then compare the residuals, which are now controlled for initial size.

The tactic fails for all data (ANCOVA on unbalanced design)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Grazed	1	0.0	0.000	0	1
Residuals	38	1680.5	44.222		

This tactic eliminates all of the differences between the two groups. In retrospect the regression equation in each group estimates 4 parameters that together eliminate the intercept differences as well as the slope effects.