

BIO144 Data Analysis in Biology

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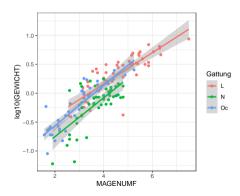
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Recap of muddiest point from last week



Main topic: Fitting and interpreting models with interactions.

Let's go back to the earthworm example, and fit a model that allows species-specific intercepts and slopes:



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```
##
## Call:
## lm(formula = log10(GEWICHT) ~ MAGENUMF * Gattung, data = d.wurm)
##
## Residuals:
       Min
                 10 Median
                                          Max
## -0 75318 -0 12834 0 01742 0 12268 0 59732
## Coefficients:
                     Estimate Std. Error t value Pr(>|t|)
## (Intercept)
                     -0.92394
                                0.13402 -6.894 1.82e-10 ***
## MAGENUME
                     0.27091
                                0.02816 9.620 < 2e-16 ***
## GattungN
                     -0.49990
                                0.21454 -2.330
                                                  0.0213 *
## GattungOc
                     -0.33921
                                0.17228 -1.969
                                                  0.0510 .
## MAGENUMF: GattungN
                    0.06516
                                0.05289 1.232
                                                  0.2200
## MAGENUMF: GattungOc 0.07894
                                0.04430
                                         1.782
                                                  0.0769 .
## ---
## Signif. codes: 0 '*** 0.001 '** 0.01 '* 0.05 '.' 0.1 ' 1
## Residual standard error: 0.2185 on 137 degrees of freedom
## Multiple R-squared: 0.7652, Adjusted R-squared: 0.7566
## F-statistic: 89.29 on 5 and 137 DF. p-value: < 2.2e-16
```

- Which are the interaction terms?
- ► Interpretation?

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We have now actually fitted three models, one model for each species:

L:
$$\hat{y}_i = -0.92 + -0.92 + 0.27 \cdot MAGENUMF$$

N:
$$\hat{y}_i = -0.92 + -0.50 + 0.27 + 0.07 \cdot MAGENUMF$$

O:
$$\hat{y}_i = -0.92 + -0.34 + 0.27 + 0.08 \cdot MAGENUMF$$

To remember:

- ▶ The "Gattung" terms in the model output are the differences in intercepts with respect to the reference category.
- ▶ The "MAGENUMF: Gattung" terms in the model output are the differences in **slopes** with respect to the reference category.

Testing for an interaction term

If we want to find out if the interaction term for a categorical explanatory variable with more than two categories is relevant, we again need an F-test, that is, use the anova() function:

```
anova(r.lm)
## Analysis of Variance Table
##
## Response: log10(GEWICHT)
##
                   Df Sum Sq Mean Sq F value Pr(>F)
                    1 19.7790 19.7790 414.4743 < 2.2e-16 ***
## MAGENUME
## Gattung
                    2 1.3537 0.6768 14.1835 2.521e-06 ***
## MAGENUMF:Gattung 2 0.1729 0.0864 1.8112
                                                 0.1673
## Residuals
                  137 6.5377 0.0477
## ---
## Signif. codes: 0 '*** 0.001 '** 0.01 '* 0.05 '.' 0.1 ' 1
```

Here, p = 0.167, thus there is not much evidence that the three species differ in their

Overview for today



- One-way ANOVA
- ► Post-hoc tests and contrasts
- Two-way ANOVA
- ANOVA as special cases of a linear model

Note:

ANOVA = ANalysis Of VAriance (Varianzanalyse)

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Course material covered today



The lecture material of today is based on the following literature:

- Chapter 12 from Stahel book "Statistische Datenenalyse"
- ▶ "Getting Started with R" chapters 5.6 and 6.2

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ANOVA and ANCOVA



ANOVA = Varianzanalyse

ANCOVA = Kovarianzanalyse

Introduction by Sir R. A. Fisher (1890-1962). He worked at the agricultural research station in Rothamstead (England). AN(C)OVA are/were therefore traditionally used to analyze agricultural experiments.

Central question of AN(C)OVA:

Are the means of two or more groups different?

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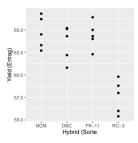
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"Pilzbrand"

(Source: W. Blanckenhorn, UZH)

Four different hybrid Mais breeds were grown to asses their yield. Each breed was grown at 5 different locations.

Questions: Are there differences in yield among the four hybrids?



We can test with ANOVA whether there are differences between the four breeds.

One idea

To carry out pairwise *t*-tests between any two groups.

- ► How many tests would this imply?
- ▶ Why is this not a very clever idea?

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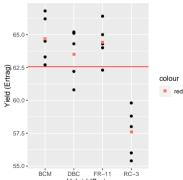
Better idea



Formulate a model that is able to test simultaneously whether there is an **overall** difference between the groups. That is, ask only **one question!**

This leads us to the

Idea of the anlysis of variance (ANOVA): Compare the variability within groups (MS_E) to the variability between the group means (MS_G) .



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$$y_{ij} = \mu_i + \epsilon_{ij}$$
,

where

- \triangleright y_{ii} = "Yield of the j^{th} plant of hybrid i"
- $\triangleright \mu_i$ ="Mean yield of hybrid i"
- $ightharpoonup \epsilon_{ij} \sim (0, \sigma^2)$ is an independent error term.

Typically, this is rewritten as

$$y_{ij} = \mu + \beta_i + \epsilon_{ij}$$
,

where $\mu + \beta_i = \mu_i$ from above, thus the **group mean** of group *i*.

One-way ANOVA (Einfaktorielle Varianzanalyse)



More generally, this leads us to the One-way ANOVA:

Assume we have g groups and in each group i there are n_i measurements of some variable of interest, denoted as y_{ii} . The model is then given as

$$y_{ij} = \mu + \beta_i + \epsilon_{ij}$$
 for $i = 1, \dots, g$,
 $j = 1, \dots, n_i$,
 $\epsilon_{ij} \sim (0, \sigma^2)$ i.i.d. (1)

- \triangleright μ plays the role of the intercept β_0 in standard regression models.
- The estimation of μ , and the β coefficients is again done by least squares minimization.
- ▶ The $\epsilon_{ii} \sim (0, \sigma^2)$ i.i.d. assumption is again crucial, so model checking will be needed again.

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$$ightharpoonup eta_1=0$$
 (treatment contrast; default in R). (such that $\mu=\mu_1$)

Interpretation: Group 1 is usually chosen such that it is some sort of reference group or reference category, for example a standard diet, while groups 2, 3, etc. correspond to novel diets whose effect is tested in an experiment.

$$ightharpoonup \sum_i \beta_i = 0$$
 (sum-to-zero contrast).

Interpretation: The effects β_1 , β_2 etc give the deviation from the population averaged effect.

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ANOVA as a special case of a linear model



Model (1) is identical to the regression model with a categorical explanatory variable, see lecture 4.

Interpretation: The categories are the different group memberships.

Thus (assuming $\beta_1 = 0$):

$$y_{ij} = \left\{ egin{array}{ll} \mu + \epsilon_{ij}, & ext{for group 1} \ \mu + eta_2 + \epsilon_{ij}, & ext{for group 2} \ \dots \ \mu + eta_{m{g}} + \epsilon_{ij}, & ext{for group } m{g} \ . \end{array}
ight.$$

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The ANOVA test: The *F*-test



Aim of ANOVA: to test *globally* if the groups differ. That is:

$$H_0$$
 : $\mu_1=\mu_2=\ldots=\mu_g$ or, equivalently $\beta_2=\ldots=\beta_g=0$

 H_1 : At least two groups are different

Remember lectures 4 and 5: We have already used the F-test for categorical variables (see F-test for the earthworms in lecture 4 or cooking rule on slide 6 in lecture 5). This was equivalent to testing if all β s that belong to a categorical variable are =0 at the same time.

→ Equivalent to testing if the categorical covariate is needed in the model.

This is the very same problem here, thus we need the F-test again!

Variance decomposition



To derive the ingredients of the F-test, we look at the decomposition of variance (Remember this idea from lecture 4, slide 23):

total variability = explained variability + residual variability
$$SS_{total} = SS_{\text{between groups}} + SS_{\text{within groups}}$$

$$\sum_{i=1}^{g} \sum_{j=1}^{n_i} (y_{ij} - \overline{y})^2 = \sum_{i=1}^{g} n_i (\overline{y}_{.i} - \overline{y})^2 + \sum_{i=1}^{g} \sum_{j=1}^{n_i} (y_{ij} - \overline{y}_{.i})^2$$

Degrees of freedom:

$$n-1 = (g-1) + (n-g)$$

From this:

$$\left. egin{align*} MS_G &= rac{SS_{
m between}}{g-1} \ MS_E &= rac{SS_{
m within}}{n-g} \ \end{array}
ight.
ight.$$

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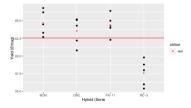
Interpretation of the F statistic



 $ightharpoonup MS_G$: Quantifies the variability **between** groups.

▶ MS_E : Quantifies the variability **within** groups.

Example:



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Interpretation of the F statistic II



- ► F increases
 - when the group means become more different, or
 - when the variability within groups decreases.
- ▶ On the other hand, F decreases
 - when the group means become more similar, or
 - when the variability within groups increases.
- \rightarrow The larger F, the less likely are the data seen under H_0 .

► ANOVA App

https://gallery.shinyapps.io/anova_shiny_rstudio/

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The ANOVA table



An overview of the results is typically given in an ANOVA table (Varianzanalysen-Tabelle):

Variation	df	SS	MS = SS/df	F	p
Between groups	g-1	SS_G	MS_G	$\frac{MS_G}{MS_F}$	$Pr(F_{g-1,n-g} > F))$
Within groups	n-g	SS_E	MS_E		
Total	n-1	SS_{total}			

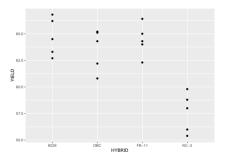
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Our first ANOVA: Hybrid Mais example

HYBRID	LOCATION	YIELD
FR-11	NW	62
FR-11	NE	64
FR-11	C	64
FR-11	SE	65
FR-11	SW	66
BCM	NW	63
BCM	NE	63
BCM	C	66
BCM	SE	67
BCM	SW	64
DBC	NW	61
DBC	NE	64
DBC	C	65
DBC	SE	62
DBC	SW	65
RC-3	NW	55
RC-3	NE	56
RC-3	C	60
RC-3	SE	58
RC-3	SW	59

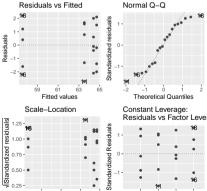
```
glimpse(d.mais)
```



Hybrid-Mais example - Estimation

Using the lm() function in R and then look at the ANOVA table:

Model checking is identical to all we did so far, because we are **still working with linear models!**



 Always when we needed to do an *F*-test and when categorical covariates were involved, the anova() table is required:

```
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```

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```
## Analysis of Variance Table
##
## Response: YIELD
## Df Sum Sq Mean Sq F value Pr(>F)
## HYBRID 3 167.441 55.814 17.681 2.474e-05 ***
```

Signif. codes: 0 '***' 0.001 '**' 0.05 '.' 0.1 ' ' 1

anova(r.mais)

Residuals 16 50.508 3.157

You can see that the value of F=17.68 is F-distributed with 3 and 16 degrees of freedom, and the p-value of the test " $\beta_2=\beta_3=\beta_4=0$ " is <0.0001.

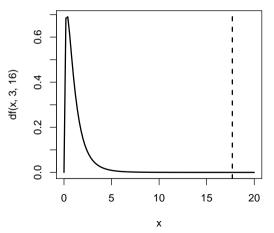
Conclusion: There are differences among the four groups!

 \rightarrow This is equivalent to "The group variable is relevant for the model".

Exercise: Look at the table a bit closer. How are Df, Sum Sq, Mean Sq, F value and Pr(<F) related?

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What happens if you apply summary() to the lm() object?



summary(r.mais)

```
##
## Call:
## lm(formula = YIELD ~ HYBRID, data = d.mais)
## Residuals:
     Min
             10 Median
   -2.72 -1.45 0.15
                        1.52
                                2.20
##
## Coefficients:
              Estimate Std. Error t value Pr(>|t|)
## (Intercept) 64.7000
                           0.7946 81.427 < 2e-16 ***
## HYBRIDDBC
               -1.1800
                           1.1237 -1.050
                                            0.309
## HYBRIDFR-11 -0.3000
                           1.1237 -0.267
                                            0.793
## HYBRIDRC-3 -7.1000
                           1.1237 -6.318 1.02e-05 ***
## ---
## Signif. codes: 0 '*** 0.001 '** 0.01 '* 0.05 '.' 0.1 ' 1
##
## Residual standard error: 1.777 on 16 degrees of freedom
## Multiple R-squared: 0.7683, Adjusted R-squared: 0.7248
## F-statistic: 17.68 on 3 and 16 DF. p-value: 2.474e-05
```

The table contains the estimates of the intercept 64.70 (μ in ANOVA notation, β_0 in regression notation), and estimates for β_2 , β_3 , β_4 (while the reference was set to $\beta_1 = 0$).

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Post-hoc tests



Still: If the test $\beta_2 = \ldots = \beta_g = 0$ is rejected, a researcher is then often interested

- 1. in finding the actual group(s) that deviate(s) from the others.
- 2. in estimates of the pairwise differences.

Several methods to circumvent the problem of too many "significant" test results (type-I error) have been proposed. The most prominent ones are:

- Bonferroni correction
- Tukey honest significant differences (HSD) approach
- Fisher least significant differences (LSD) approach

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Bonferroni correction

Idea: If a total of m tests are carried out, simply divide the type-I error level α_0 (often 5%) such that

$$\alpha = \alpha_0/m$$
.

Tukey HSD approach

Idea: Take into account the distribution of ranges (max-min) and design a new test.

Fisher's LSD approach

Idea: Adjust the idea of a two-sample test, but use a larger variance (namely the pooled variance of all groups).

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Other contrasts



Sometimes additional comparisons are of interest. (Check also chapter 5.6.5 in GSWR)

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Choosing the reference category

Back to the Hybrid Mais example. R orders the categories alphabetically and takes the first level as reference category.

This can be changed manually:

```
levels(d_mais$HYRRID)
## NIII.I.
d.mais <- mutate(d.mais.HYBRID = relevel(as.factor(HYBRID).ref="DBC"))</pre>
anova(lm(YIELD ~ HYBRID, d.mais))
## Analysis of Variance Table
## Response: YIELD
             Df Sum Sq Mean Sq F value Pr(>F)
## HVRRID
             3 167.441 55.814 17.681 2.474e-05 ***
## Residuals 16 50.508 3.157
## ---
## Signif. codes: 0 '*** 0.001 '** 0.01 '* 0.05 '.' 0.1 ' 1
summary(lm(YIELD ~ HYBRID, d.mais))$coef
                                                  Pr(>|t|)
               Estimate Std Error
```

63.52 0.7945754 79.9420714 2.974727e-22

1.18 1.1236992 1.0501030 3.092739e-01

(Intercept)

HYRRIDRCM

Two-way ANOVA (Zweiweg-Varianzanalyse)



Example (from Hand et al. 1994 / Hothorn/Everitt "A Handbook of Statistical Analyses Using R"):

Experiment to study the weight gain of rats, depending on four diets. Protein amounts were either high or low, and the protein source was either beef or cereal. 10 rats for each diet were selected.

Question: How does diet affect weight gain?

Complication: This is a factorial design (gekreuzte Faktoren), because each combination of protein source (beef/cereal) x category (high/low) is present (2 x 2 groups).

Design:

	beef	cereal	
high	group 1	group 2	
low	group 3	group 4	

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Start by looking at means and standard deviations in the groups, as well as at Zurich graphical description of the means:

```
d.weightgain %% group by(source,amount) %% summarise(meanW = mean(weightgain),sdW = sd(weightgain))
     A tibble: 4 x 4
     source amount meanW
            <fct>
                   <dbl> <dbl>
     Reef
            LOW
                    79.2
                          13.9
     Beef
            High
                          15.1
     Cereal Low
                    83.9
                          15.7
## 4 Cereal High
                    85.9
```



- * Protein source (beef/cereal) seems less influential than the amount (high/low).

 * Variances seem to be equal in the four
- * Variances seem to be equal in the four groups.

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Two-way ANOVA – The model



In the presence of a factorial design, the idea is to add separate effects β_i (here i=1,2) and γ_i (here j=1,2) for the ith category of the first categorical variable and the *i*th category of the second explanatory variable:

Assume we have a factorial design with two categories β_i and γ_i , then the kth outcome in the category of i and j, y_{iik} is modelled as

$$y_{ijk} = \mu + \beta_i + \gamma_j + \epsilon_{ijk}$$
 with $\epsilon_{ijk} \sim N(0, \sigma^2)$ i.i.d.

Note: We again need additional constraints. Here we always use the R default ("treatment contrasts")

$$\beta_1 = \gamma_1 = 0.$$

Alternative: $\sum_{i} \beta_{i} = \sum_{i} \gamma_{i} = 0$ (sum-to-zero contrast).

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Two way ANOVA



In R, a two-way ANOVA is as simple as one-way ANOVA, just add another variable:

```
r.weight <- lm(weightgain ~ source + amount, d.weightgain)
anova(r.weight)

## Analysis of Variance Table

## ## Response: weightgain

## Df Sum Sq Mean Sq F value Pr(>F)

## source 1 220.9 220.90 0.9150 0.34501

## amount 1 1299.6 1299.60 5.3829 0.02596 *

## Residuals 37 8933.0 241.43

## ---

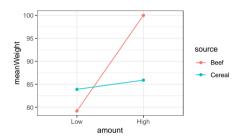
## Signif, codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Interpretation: There seems to be a difference between low and high amounts of protein, but the source (beef/cereal) seems less relevant.

However: what if the additive model does not hold?

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A so-called interaction plot helps to understand if the additive model is reasonable:



Note: if the additive model $\beta_i + \gamma_i$ holds, the lines would be parallel.

However, these lines are not parallel, indicating that there is an interaction between amount and source!

In words: The amount (low/high amount treatment) has a different influence for the Beef and Cereal diets.

Two-way ANOVA with interaction



▶ If the purely additive model is not correct, a more general model with an interaction term $(\beta\gamma)_{ij}$ may be used:

$$y_{ijk} = \mu + \beta_i + \gamma_j + (\beta \gamma)_{ij} + \epsilon_{ijk}$$
 with $\epsilon_{ijk} \sim N(0, \sigma^2)$ i.i.d.

- As in linear regression, interactions allow for an interplay between the variables.
- ▶ In the rats experiment, increasing the amount from low to high has a different effect in the beef than in the cereal diet.
- ► Moreover: The plot on the previous slide shows that for the low amount of proteins case, the cereal diet leads to a larger average weight gain!



Let's include an interaction term in the rats example:

The coefficient estimates can be obtained as follows:

```
## Estimate Std. Error t value Pr(>|t|)
## (Intercept) 79.2 4.728577 16.7492235 1.416943e-18
## sourceCereal 4.7 6.687218 0.7028333 4.866800e-01
## amountHigh 20.8 6.687218 3.1104114 3.644273e-03
## sourceCereal:amountHigh -18.8 9.457155 -1.9879129 5.446757e-02
```

summary(r.weight2)\$coef



This works in the same way as for categorical covariates in regression! To see this, let us estimate the means from the model. From the above output, we have [because of using treatment contrasts]:

$$\begin{split} \hat{\beta}_{beef} &= 0, \ \hat{\beta}_{cereal} = 4.7, \\ \hat{\gamma}_{low} &= 0, \ \hat{\gamma}_{high} = 20.8, \\ (\hat{\beta\gamma})_{cereal/high} &= -18.8 \ (\hat{\beta\gamma})_{beef/high} = (\hat{\beta\gamma})_{beef/low} = \hat{(}\beta\gamma)_{cereal/low} = 0. \end{split}$$

Therefore:

```
Group 1: beef / low \hat{y}_{beef,low} = 79.2 + 0 + 0 + 0 = 79.2

Group 2: cereal / low \hat{y}_{cereal,low} = 79.2 + 4.7 + 0 + 0 = 83.9

Group 3: beef / high \hat{y}_{beef,high} = 79.2 + 0 + 20.8 + 0 = 100

Group 4: cereal / high \hat{y}_{cereal,high} = 79.2 + 4.7 + 20.8 - 18.8 = 85.9
```

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A cautionary note

Be careful: In the presence of interactions, the *p*-values of the main effects can no longer be interpreted as before!

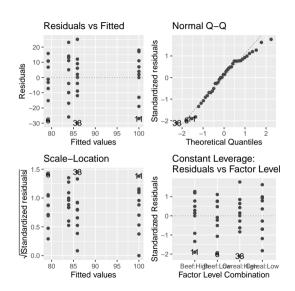
It is then required that separate "stratified" analyses are carried out. For example for "Beef" and "Cereal" protein sources:

```
anova(lm(weightgain ~ amount, subset(d.weightgain, source=="Beef")))
## Analysis of Variance Table
## Response: weightgain
            Df Sum Sq Mean Sq F value Pr(>F)
## amount
          1 2163.2 2163.20 10.253 0.00494 **
## Residuals 18 3797 6 210 98
## Signif. codes: 0 '*** 0.001 '** 0.01 '* 0.05 '.' 0.1 ' ' 1
anova(lm(weightgain ~ amount.subset(d.weightgain.source=="Cereal")))
## Analysis of Variance Table
## Response: weightgain
            Df Sum Sq Mean Sq F value Pr(>F)
                 20 0
                        20.00 0.0847 0.7744
## Residuals 18 4251.8 236.21
```

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And finally, the model diagnostics:





Exercise:



In an experiment the influence of four of fertilizer (DUENGER) on the yield (ERTRAG) on 5 species (SORTE) of crops was investigated. For each DUENGER \times ERTRAG combination, 3 repeats were taken.

The data contain the following columns:

- ► DUENGER (4 levels)
- ► SORTE (5 levels)
- ► ERTRAG (continuous)

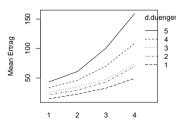
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The first 10 rows of the data:



##		DUENGER	SORTE	ERTRAG
##	1	1	1	14
##	2	1	1	15
##	3	1	1	15
##	4	2	1	20
##	5	2	1	25
##	6	2	1	23
##	7	3	1	35
##	8	3	1	31
##	9	3	1	32
##	10	4	1	52

And the interaction plot:



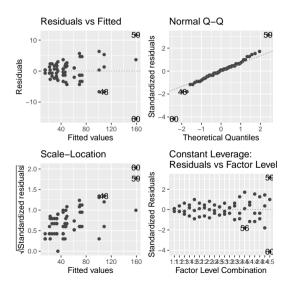
```
d.duenger <- mutate(d.duenger, SORTE=as.factor(SORTE), DUENGER=as.factor(DUENGER))</pre>
r.duenger <- lm(ERTRAG ~ DUENGER*SORTE,d.duenger)
#anova(r.duenger)
```

Look at the TA and the scale-location plots (next slide).

What is the problem?

→ Interpretation? Ideas?

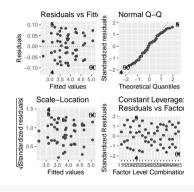




Log-transform the response (ERTRAG) and repeat the analysis:



r.duenger2 <- lm(log(ERTRAG) ~ DUENGER*SORTE,d.duenger)



anova(r.duenger2)

```
## Analysis of Variance Table
        ## Response: log(ERTRAG)
                         Df Sum Sq Mean Sq F value Pr(>F)
                                      3.8972 854.0505 <2e-16 ***
        ## DUENGER
                                      2.1300 466.7851 <2e-16 ***
        ## DUENGER: SORTE 12
                                      0.0077
                                               1.6958 0.1045
Lecture 6: #NQVAduals
```

0.0046

40 0.1825



Btw, the summary table with coefficients looks horrible and the *p*-values are not meaningful! (why?)

	Coefficient	95%-confidence interval	<i>p</i> -value
Intercept	2.69	from 2.61 to 2.76	< 0.0001
DUENGER2	0.43	from 0.32 to 0.54	< 0.0001
DUENGER3	0.80	from 0.69 to 0.91	< 0.0001
DUENGER4	1.21	from 1.10 to 1.32	< 0.0001
SORTE2	0.39	from 0.28 to 0.50	< 0.0001
SORTE3	0.56	from 0.45 to 0.67	< 0.0001
SORTE4	0.82	from 0.71 to 0.93	< 0.0001
SORTE5	1.08	from 0.97 to 1.19	< 0.0001
DUENGER2:SORTE2	-0.13	from -0.29 to 0.03	0.10
DUENGER3:SORTE2	-0.11	from -0.26 to 0.05	0.18
DUENGER4:SORTE2	-0.049	from -0.21 to 0.11	0.53
DUENGER2:SORTE3	-0.12	from -0.28 to 0.04	0.13
DUENGER3:SORTE3	-0.18	from -0.34 to -0.02	0.026
DUENGER4:SORTE3	-0.16	from -0.32 to -0.00	0.046
DUENGER2:SORTE4	-0.10	from -0.26 to 0.06	0.20
DUENGER3:SORTE4	-0.053	from -0.21 to 0.10	0.50
DUENGER4:SORTE4	-0.03	from -0.19 to 0.13	0.71
DUENGER2:SORTE5	-0.088	from -0.25 to 0.07	0.27
DUENGER3:SORTE5	0.044	from -0.11 to 0.20	0.58
DUENGER4:SORTE5	0.09	from -0.07 to 0.25	0.25

Questions: Number of parameters? Degrees of freedom (60 data points)?

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Some summary remarks



- ▶ The *t*-test to compare the mean of two groups is a special case of ANOVA.
- ► ANOVA is a special case of the linear regression model.
- ANOVA is often taught in separate lectures, although it could be integrated in a lecture on linear regression.
- ► ANOVA is traditionally most used to analyze experimental data, though this is changing...

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