Kurs Bio144:
Datenanalyse in der Biologie

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Lecture 5: ANOVA

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Overview (todo: check)

- 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)

Course material covered today

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

- Chapter 12 from Stahel book "Statistische Datenenalyse"
- "The new Statistics with R" chapter 2 (ANOVA)
- "Getting Started with R" chapters 5.6 and 6.2

Recap of the linear regression model

to do

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.

Questions of AN(C)OVA:

- Generally: Are the means of twor or more groups different?
- Example: Are different plant breeds different in important aspects (e.g., yiels / Ertrag)?
- Example: What is the influence of different treatments on plants (Biology) or patients (Medicine)?

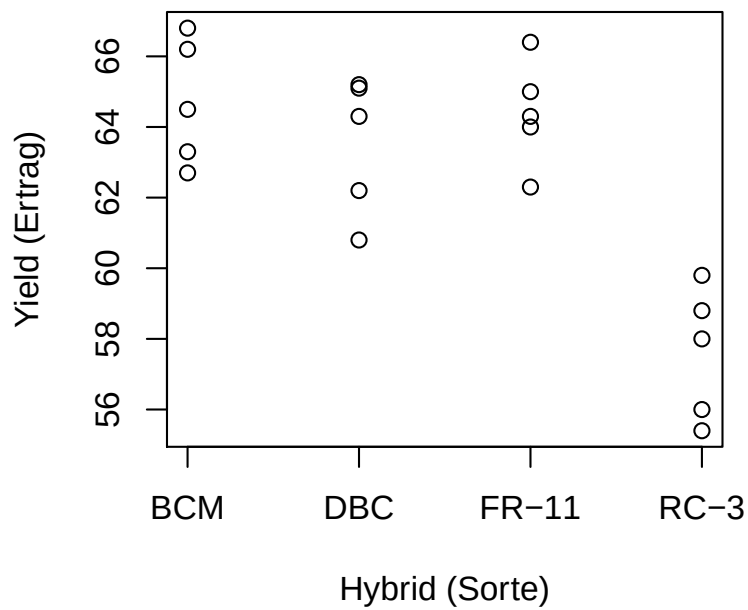
Beispiel: Ertragspotential bei Hybrid-Mais mit erhöhter Pilzbrand-Resistenz

(Source: W. Blanckenhorn, UZH)

Es wurden 4 Hybrid-Mais-Sorten angebaut und ihr Körnerertrag ermittelt. Jede Sorte wurde an 5 Orten angepflanzt.

Frage: Unterscheiden sich die Hybrid-Mais-Sorten im Ertrag?

Achtung: Die Frage bezieht sich auf *irgendeinen* Unterschied. Präziser könnte man fragen, ob sich irgendeine der Sorten von den anderen unterscheidet?



Naive idea: To carry out pairwise t-tests between any two groups.

1. How many tests would this imply?
2. Why is this not a very clever idea?

Naive idea: To carry out pairwise t-tests between any two groups.

1. How many tests would this imply?
2. Why is this not a very clever idea?

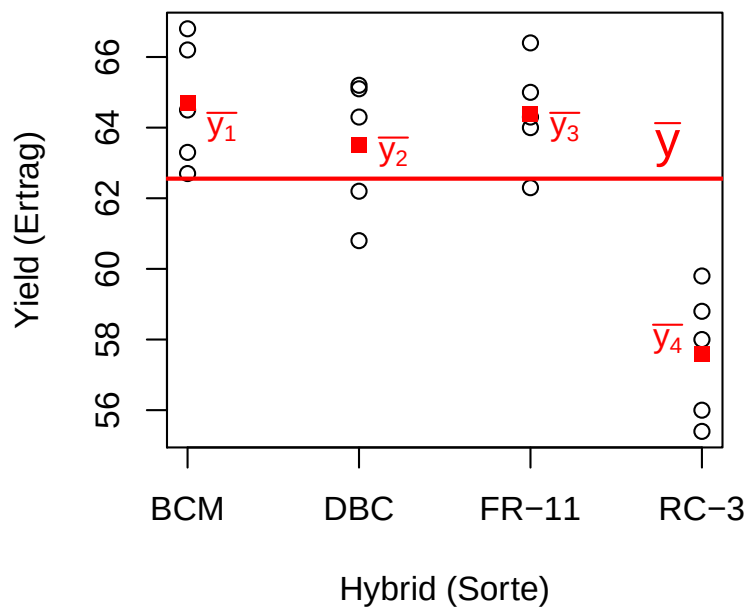
(Generally, the number of pairwise tests can be calculated by $g(g-1)/2$, where g=number of groups.)

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 ANOVA analysis:
Compare the variability within groups (MS_E) to the variability between the group means (MS_G).



We formulate a model as follows:

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

where

- y_{ij}= "Ertrag der j-ten Pflanze der Sorte i"
- μ_i="Mittlerer Ertrag der Sorte i"
- $e_{ij} \sim \mathrm{N}(0, \sigma_e^2)$ is an independent error term.

Typically, this is rewritten as

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

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

Single factor ANOVA (Einfaktorielle Varianzanalyse)

More generally, this leads us to the single factor ANOVA:

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

$$\begin{aligned} y_{ij} = \mu + \beta_i + e_{ij} \quad \text{for} \quad i &= 1, \ldots, g \ , \\ j &= 1, \ldots, n_i, \\ e_{ij} &\sim \mathrm{N}(0, \sigma_e^2) \quad i.i.d. \end{aligned} \tag{1}$$

- μ plays the role of the intercept β_0 in standard regression models.
- The estimation of μ, β_2, $\ldots$, β_g is again done by least squares minimization.
- The $e_{ij} \sim \mathrm{N}(0, \sigma_e^2)$ $i.i.d.$ assumption is again crucial, so model checking will be needed again.

Attention: Model (1) is overparameterized, thus an additional constraint is needed! Most popular:

- $\beta_1 = 0$ (treatment contrast; default in R).
- $\sum_i \beta_i = 0$ (sum-to-zero contrast).

To do: move the actual analysis here, only then continue to explain the F-test etc.!

The ANOVA test: The F-test

Test now *globally* if the groups differ. That is:

H_0 : $\mu_1 = \mu_2 = \ldots = \mu_g$ or, equivalently $\beta_1 = \beta_2 = \ldots = \beta_g$

H_1 : At least two groups are different

To test if more than one parameter in a regression model is $=0$ at the same time, we have used the F-**test** in linear regression – and we also need the F-test here!

To derive the ingredients of the F-test, we again look at the decomposition of variance:

Variance decomposition

(Remember this idea from week 3, slide 24, and replace $\hat{y}_{ij}$ by $\overline{y}_{.i}$)

$$\begin{aligned} SS_{total} &= SS_{\text{between groups}} + SS_{\text{within groups}} \\ \sum_{i=1}^{g}\sum_{j=1}^{n_i}(y_{ij}-\overline{y})^2 &= \sum_{i=1}^{g} n_i(\overline{y}_{.i}-\overline{y})^2 + \sum_{i=1}^{g}\sum_{j=1}^{n_i}(y_{ij}-\overline{y}_{.i})^2 \end{aligned}$$

Degrees of freedom:

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

From this:

$$\left.\begin{aligned} MS_G &= \tfrac{SS_{\text{between}}}{g-1} \\ MS_E &= \tfrac{SS_{\text{within}}}{n-g} \end{aligned}\right\} \Rightarrow F = \frac{MS_G}{MS_E} \quad \text{is } \sim F_{g-1,n-g} \text{ distributed.}$$

Interpretation of the F statistic

- MS_G: Quantifies the variability **between** groups.
- MS_E: Quantifies the variability **within** groups.

Thus $F = \frac{MS_G}{MS_E}$ is large when MS_G is "large" with respect to MS_E. The larger F, the more likely that H_0 is false.

- F increases
  - when the group means become more different, or
  - when the variability within groups decreases.
- On the other hand, Fdecreases
  - when the group means become more similar, or
  - when the variability within groups increases.

▸ ANOVA App

The ANOVA table

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

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

Hybrid-Mais example – Data

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

HYBRID	LOCATION	YIELD
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

```
> str(d.mais)

'data.frame':        20 obs. of  3 variables:
 $ HYBRID  : Factor w/ 4 levels "BCM","DBC","FR-11",..: 3 3 3 3 3 1 1 1 1 1 ...
 $ LOCATION: Factor w/ 5 levels "C","NE","NW",..: 3 2 1 4 5 3 2 1 4 5 ...
 $ YIELD   : num  62.3 64 64.3 65 66.4 63.3 62.7 66.2 66.8 64.5 ...
```

Hybrid-Mais example – Estimation

Using the `aov()` function in R directly estimates the ANOVA table:

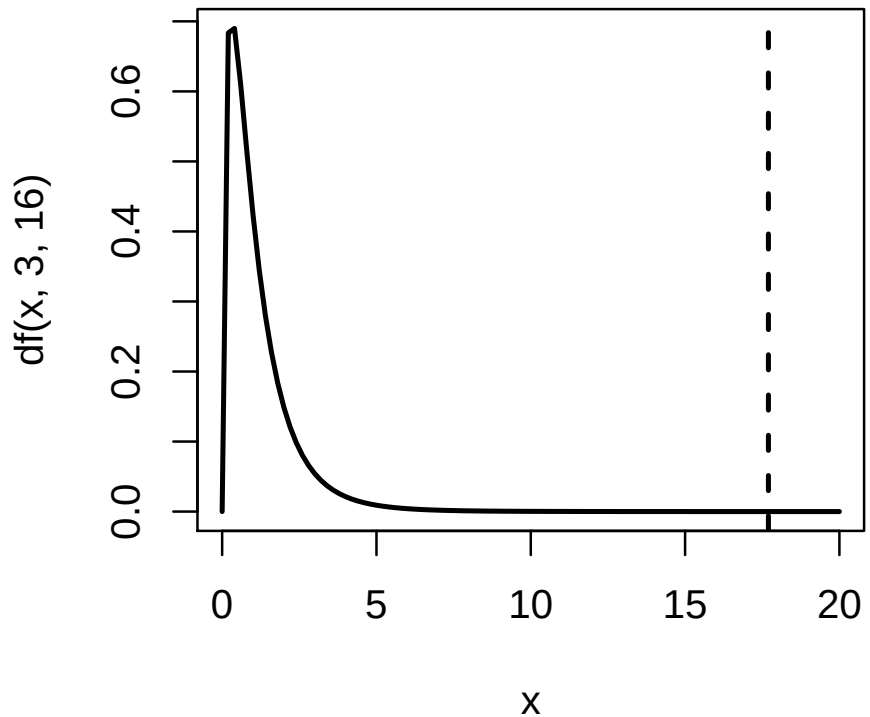
```
> r.mais <- aov(YIELD ~ HYBRID, d.mais)
> summary(r.mais)

            Df Sum Sq Mean Sq F value   Pr(>F)
HYBRID       3 167.44   55.81   17.68 2.47e-05 ***
Residuals   16  50.51    3.16
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Here: $F = 17.68$ is F-distributed with 3 and 16 degrees of freedom, and the p-value of the test "$\beta_1 = \beta_2 = \beta_3 = \beta_4$?" is < 0.0001.

Conclusion: the four groups are clearly different!

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

The F-distribution with 3 and 16 degrees of freedom, as well as the estimated value F=17.68:



ANOVA as a special case of a linear model

The clou is: Model (1) is identical to the regression model with a factor covariate, see slides 36/37 from week 3.

Interpretation: The levels of the factor are now the different group memberships.

Thus (assuming $\beta_1 = 0$):

$$y_{ij} = \begin{cases} \mu + e_{ij}, & \text{for group 1} \\ \mu + \beta_2 + e_{ij}, & \text{for group 2} \\ \ldots & \\ \mu + \beta_g + e_{ij}, & \text{for group } g \ , \end{cases}$$

and $\hat{y}_{ij} = \overline{y}_{.i} = \mu + \beta_i$ can be interpreted as the predicted value.

It is therefore possible to perform an ANOVA with the `lm()` function in R and obtain the same results, either by looking at the summary table:

```
> r.lm <- lm(YIELD~HYBRID,d.mais)
> summary(r.lm)

Call:
lm(formula = YIELD ~ HYBRID, data = d.mais)

Residuals:
   Min     1Q Median     3Q    Max
 -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
```

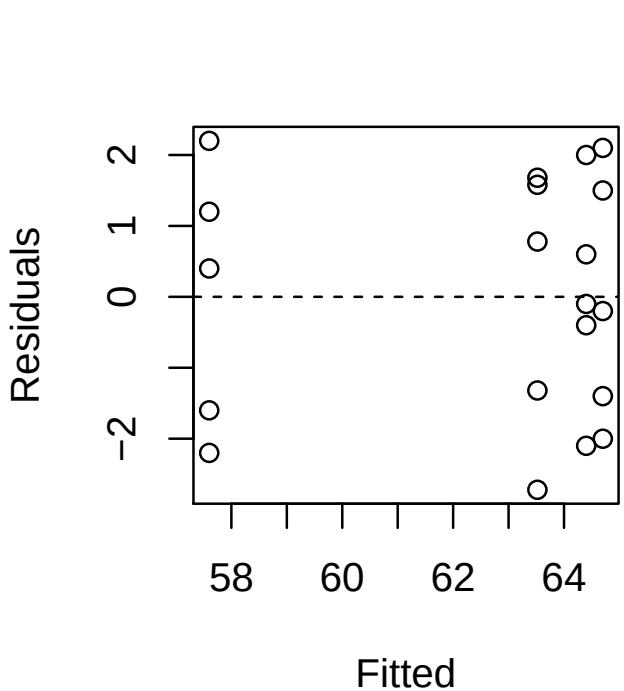
...or by generating the ANOVA table for an lm object:

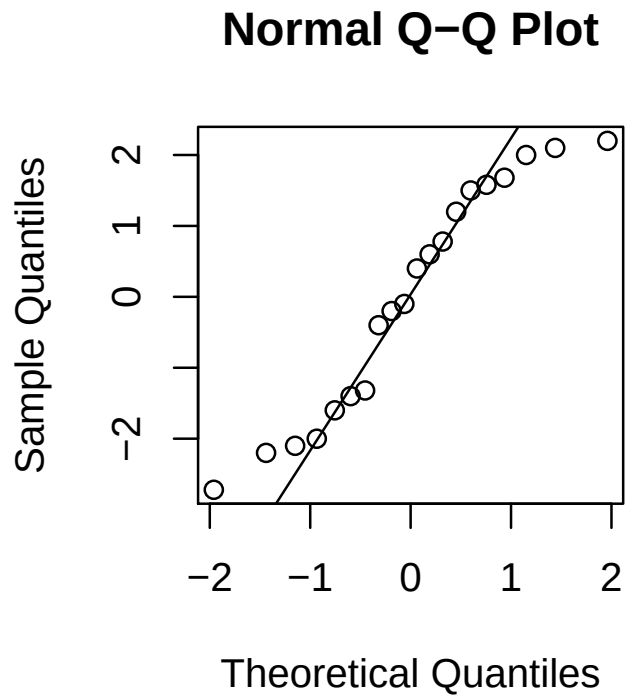
```
> anova(r.lm)

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 ***
Residuals 16  50.508   3.157
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Compare the output with the previous slide and slide 18 for a while....

Testing modelling assumptions

Tukey-Anscombe (TA) and QQ plots are useful again:





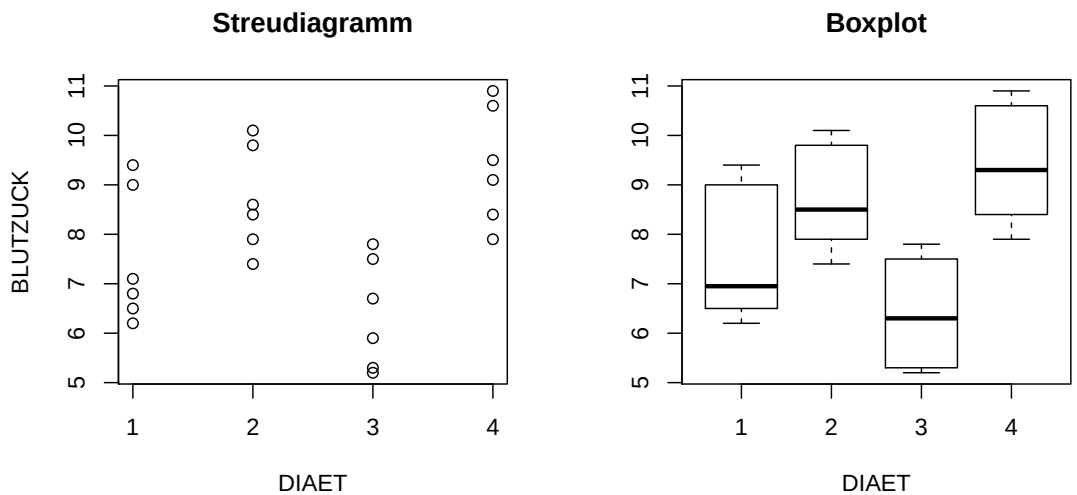
Exercise: Ernährung und Blutzucker

Remember example 3 from the first week:

24 Personen werden in 4 Gruppen unterteilt. Jede Gruppe erhält eine andere Diät (DIAET). Es werden zu Beginn und am Ende (nach 2 Wochen) die Blutzuckerwerte gemessen. Die Differenz wird gespeichert (BLUTZUCK).

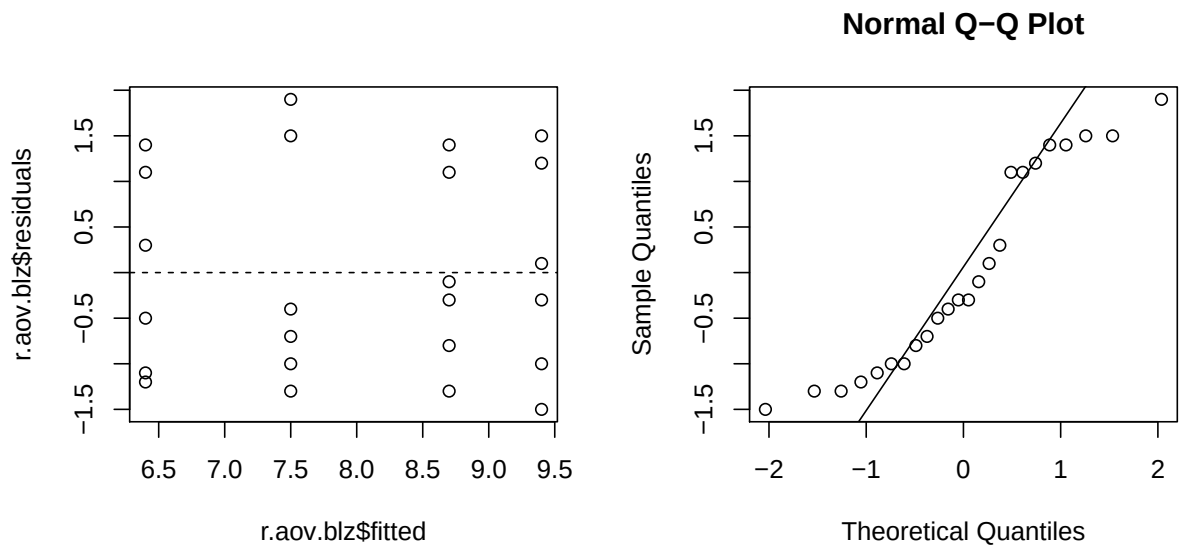
Frage: Unterscheiden sich die Gruppen in der Veränderung der Blutzuckerwerte?

```
> par(mfrow=c(1,2))
> plot(BLUTZUCK ~ DIAET,d.blz,xaxt="n",main="Streudiagramm")
> axis(1,1:4)
> boxplot(BLUTZUCK ~ DIAET,d.blz,xaxt="n",xlab="DIAET",main="Boxplot")
> axis(1,1:4)
```



Interpret the results and the residual plots:

```
> d.blz$DIAET <- as.factor(d.blz$DIAET)
> summary(aov(BLUTZUCK ~ DIAET,d.blz))

            Df Sum Sq Mean Sq F value  Pr(>F)
DIAET        3  31.56   10.52   7.514 0.00148 **
Residuals   20  28.00    1.40
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```



Multiple comparisons, multiple tests

To remember:

- The F-Test is used to check whether any two group means differ.
- Using pairwise tests is not a very good idea (see slide 7), because this leads to a multiple testing problem:

  When many tests are carried out, the probability to find a "significant" result **by chance** increases.

  For instance, for four groups there are $4 \cdot 3/2 = 6$ pairwise combinations that could be tested. The probability to find *at least one result by chance* is much higher than the 5% error level!!!

Post-hoc tests

Still: If the test $\beta_1 = \beta_2 = \ldots = \beta_g = 0$ is rejected, one is 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 **h**onest **s**ignificant **d**ifferences (HSD) approach
- Fisher **l**east **s**ignificant **d**ifferences (LSD) approach

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).

Calculate the pairwise differences and tests with adjustments for the "Blutzucker" example:

Differences:

	1	2	3
2	1.2		
3	-1.1	-2.3	
4	1.9	0.7	3.0

Tukey HSD p-values:

	1	2	3
2	0.32		
3	0.40	0.01	
4	0.05	0.74	0.001

Bonferroni p-values:

	1	2	3
2	0.57		
3	0.74	0.02	
4	0.07	1.00	0.002

Fisher p-values:

	1	2	3
2	0.09		
3	0.12	0.003	
4	0.01	0.32	3e-04

- Bonferroni p-values are the most conservative (largest p).
- Fisher p-values are the least conservative (smallest p).

Other contrasts

Sometimes additional comparisons are of interest. For example, a new diet is to be compared to other, existing diets.

In the "Blutzucker" example, this could be, for intance: "Is diet 1 different from diets 2-4?"

(Check also chapter 5.6.5 in GSWR, 2nd edition)

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 weightgain?

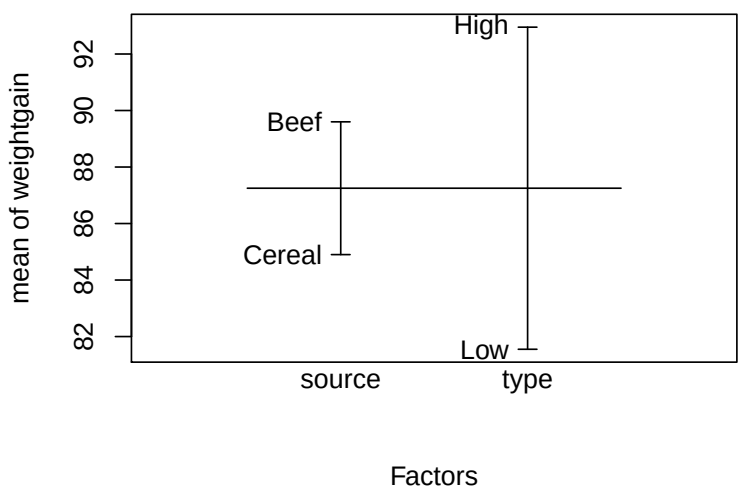
Complication: This is a factorial design (gekreuzte Faktoren), because each combination of protein source (beef/cereal) $\times$ level (high/low) is present (2×2 groups).
Design:

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

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

```
> tapply(weightgain$weightgain,list(weightgain$source,weightgain$type),FUN=mean)

        High  Low
Beef   100.0 79.2
Cereal  85.9 83.9

> tapply(weightgain$weightgain,list(weightgain$source,weightgain$type),FUN=sd)

           High      Low
Beef   15.13642 13.88684
Cereal 15.02184 15.70881
```

```
> plot.design(weightgain)
```



- Protein source (beef/cereal) seems less important than the amount (high/low).
- Variances seem to be equal in the four groups.

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_j (here $j = 1, 2$) for the ith level of the first factor and the jth level of the second factor:

Assume we have a factorial design with two factors β_i and γ_j, then the kth outcome in the group of i and j, y_{ijk} is modelled as

$$y_{ijk} = \mu + \beta_i + \gamma_j + e_{ijk} \quad \text{with} \quad e_{ijk} \sim \mathrm{N}(0, \sigma_e^2) \quad i.i.d.$$

Again, additionl constraints are needed!

- $\beta_1 = \gamma_1 = 0$ (treatment contrast; default in R).
- $\sum_i \beta_i = \sum_i \gamma_i = 0$ (sum-to-zero contrast).

Two-way ANOVA in R

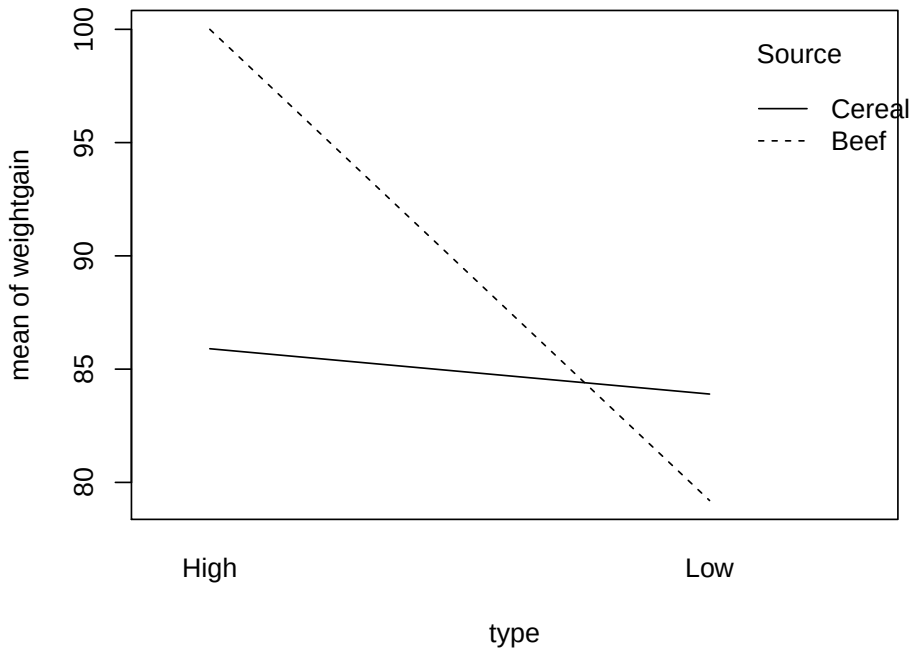
In R, a two-way ANOVA is as simple as one-way ANOVA, just add another variable:

```
> r.aov <- aov(weightgain ~ source + type,weightgain)
> summary(r.aov)

            Df Sum Sq Mean Sq F value Pr(>F)
source       1    221   220.9   0.915  0.345
type         1   1300  1299.6   5.383  0.026 *
Residuals   37   8933   241.4
---
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.

However: what if the additive model does not hold?

A so-called interaction plot (`interaction.plot()` in R) helps to understand if the additive model is reasonable:



The lines are not parallel, indicating that there is an interaction between type and source!

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

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:

$$y_{ijk} = \mu + \beta_i + \gamma_j + (\beta\gamma)_{ij} + e_{ijk} \quad \text{with} \quad e_{ijk} \sim \mathrm{N}(0, \sigma_e^2) \quad i.i.d.$$

- 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!

Two-way ANOVA in R – Including an interaction

Again the rats example, this time including the interaction term:

```
> r.aov <- aov(weightgain ~ source * type,weightgain)
> summary(r.aov)

            Df Sum Sq Mean Sq F value Pr(>F)
source       1    221   220.9   0.988 0.3269
type         1   1300  1299.6   5.812 0.0211 *
source:type  1    884   883.6   3.952 0.0545 .
Residuals   36   8049   223.6
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

The coefficient estimates can be obtained as follows:

```
> r.lm <- lm(weightgain ~ source * type,weightgain)
> summary(r.lm)$coef

                    Estimate Std. Error   t value     Pr(>|t|)
(Intercept)            100.0   4.728577 21.148009 6.842420e-22
sourceCereal           -14.1   6.687218 -2.108500 4.201233e-02
typeLow                -20.8   6.687218 -3.110411 3.644273e-03
sourceCereal:typeLow    18.8   9.457155  1.987913 5.446757e-02
```

Interpretation of the coefficients

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]:

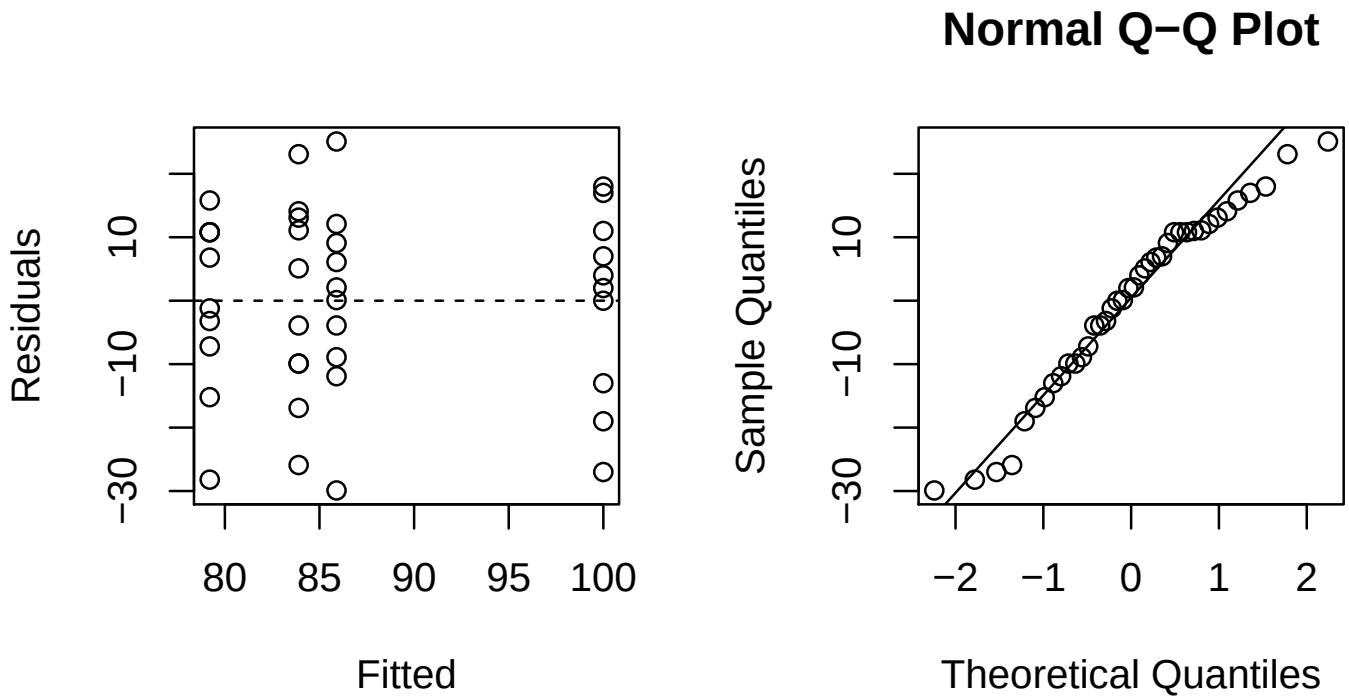
$\hat{\beta}_{beef} = 0$, $\hat{\beta}_{cereal} = -14.1$,
$\hat{\gamma}_{high} = 0$, $\hat{\gamma}_{low} = -20.8$,
$(\hat{\beta\gamma})_{cereal\,/\,low} = 18.8$, $(\hat{\beta\gamma})_{beef\,/\,high} = (\hat{\beta\gamma})_{beef\,/\,low} = \hat{(}\beta\gamma)_{cereal\,/\,high} = 0$.

Therefore:

Group 1:	beef / high	$\hat{y}_{beef,high} = 100 + 0 + 0 + 0 = 100$
Group 2:	cereal / high	$\hat{y}_{cereal,high} = 100 + (-14.1) + 0 + 0 = 85.9$
Group 3:	beef / low	$\hat{y}_{beef,low} = 100 + 0 + (-20.8) + 0 = 79.2$
Group 4:	cereal / low	$\hat{y}_{cereal,low} = 100 + (-14.1) + (-20.8) + 18.8 = 83.9$

Compare these values to slide 32!

And finally, again, checking some modelling assumptions:



Exercise:

In an experiment the influence of four levels of fertilizer (DUENGER) on the yield (ERTRAG) on 5 species (SORTE) of crops was investigated. The data contains the following colums:

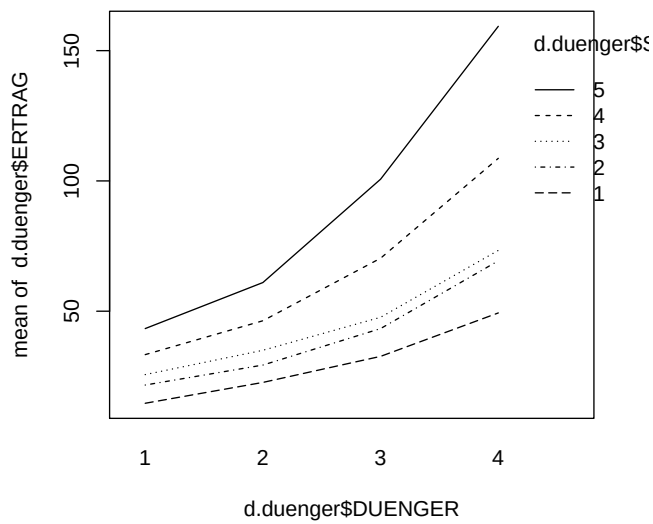
DUENGER (4 levels) SORTE (5 levels) ERTRAG

The first 10 rows of the data:

```
> d.duenger[1:10,]
```

	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

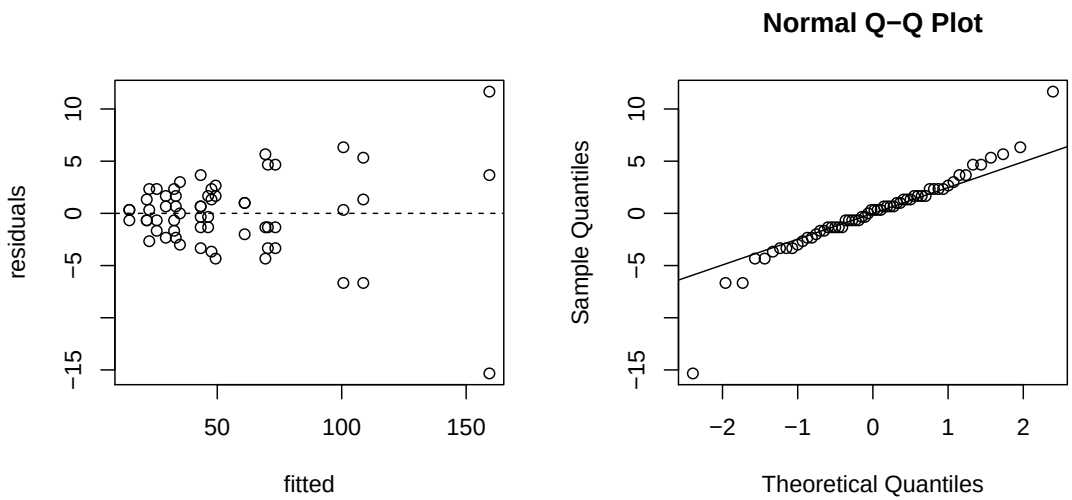
And the interaction plot:



The interaction plot indicates that an interaction between SORTE and DUENGER is needed in the analysis. The results and residal plots are given as follows:

```
> d.duenger$SORTE <- as.factor(d.duenger$SORTE)
> d.duenger$DUENGER <- as.factor(d.duenger$DUENGER)
> r.duenger <- aov(ERTRAG ~ DUENGER*SORTE,d.duenger)
> summary(r.duenger)
```

```
              Df Sum Sq Mean Sq F value Pr(>F)
DUENGER        3  35801   11934  580.71 <2e-16 ***
SORTE          4  27805    6951  338.26 <2e-16 ***
DUENGER:SORTE 12   7674     640   31.12 <2e-16 ***
Residuals     40    822      21
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

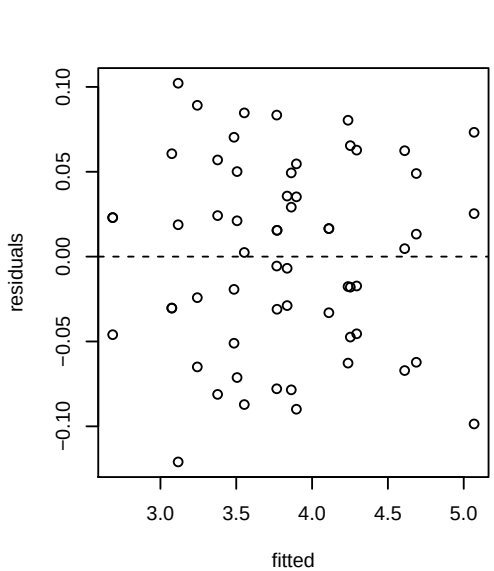


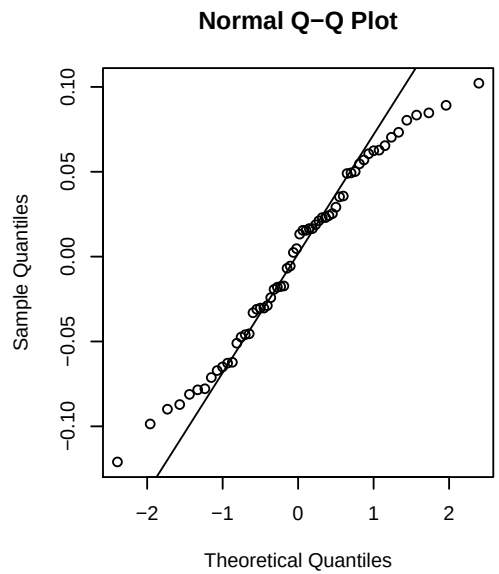
Interpretation? What is here the problem (look at the TA plot)? Ideas?

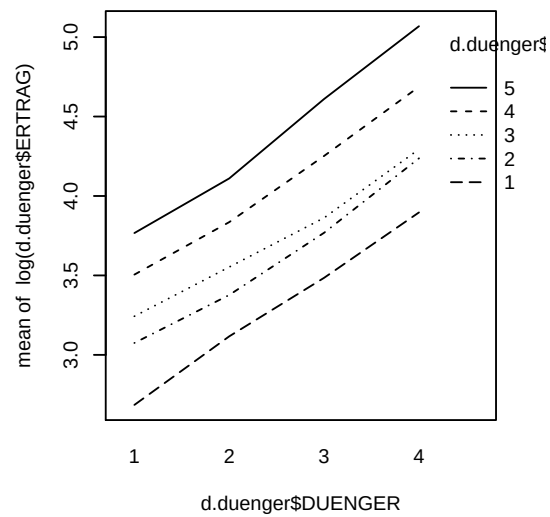
Todo: don't show this slide previously!

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

```
> r.duenger2 <- aov(log(ERTRAG) ~ DUENGER*SORTE,d.duenger)
> summary(r.duenger2)

             Df Sum Sq Mean Sq F value Pr(>F)
DUENGER       3 11.692   3.897 854.050 <2e-16 ***
SORTE         4  8.520   2.130 466.785 <2e-16 ***
DUENGER:SORTE 12  0.093   0.008   1.696  0.105
Residuals     40  0.183   0.005
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```







Some remarks

- The t-test to compare the mean of two gropus is a special case of ANOVA.
- ANOVA is a special cases 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.

Summary