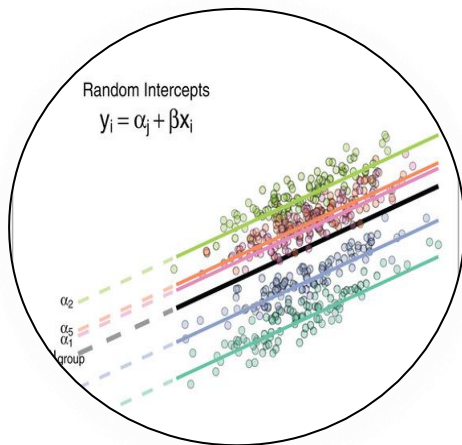


# PhD course Mixed Linear Models

## Session 4: Estimation and testing in a mixed model

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# An example with a crop experiment



Nine trial fields, each field divided into plots

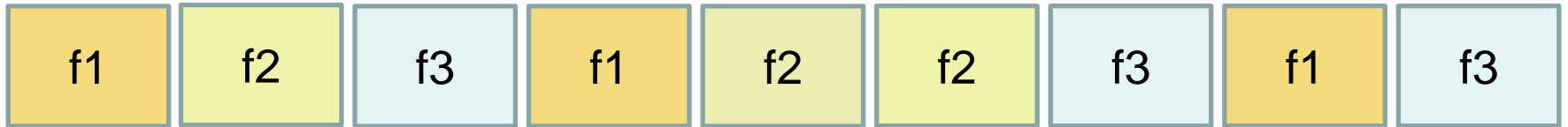
Response variable :  $y$  = yield of oats per plot

Three types of fertilizer  $f_1$ ,  $f_2$ ,  $f_3$

Four varieties of oats  $v_1$ ,  $v_2$ ,  $v_3$ ,  $v_4$



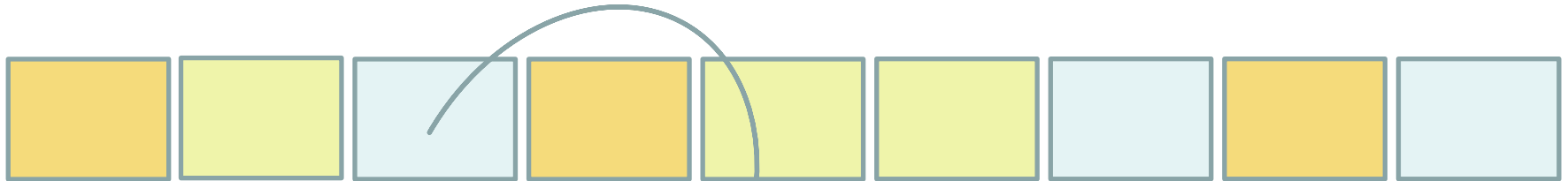
# Assigning fertilizers to fields



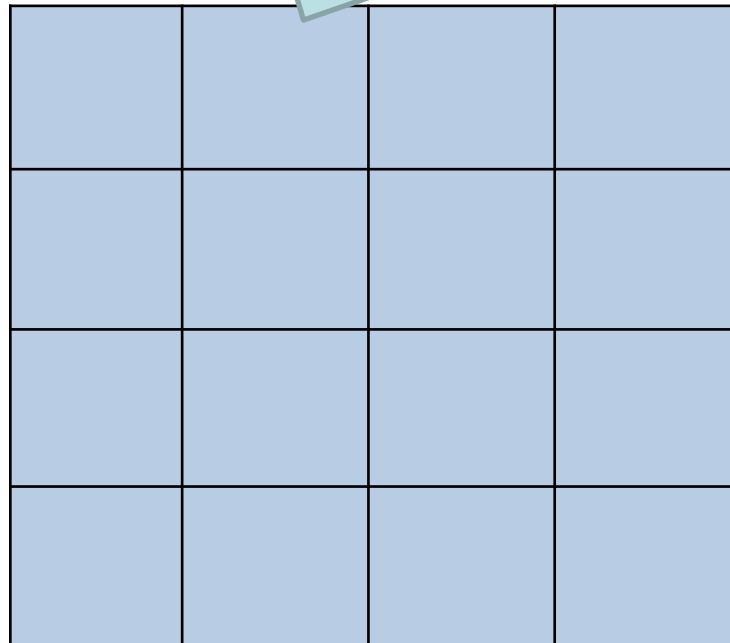
For practical reasons fertilizers are assigned to whole fields.

Randomly three fields are assigned to each type of fertilizer f1, f2, or f3.

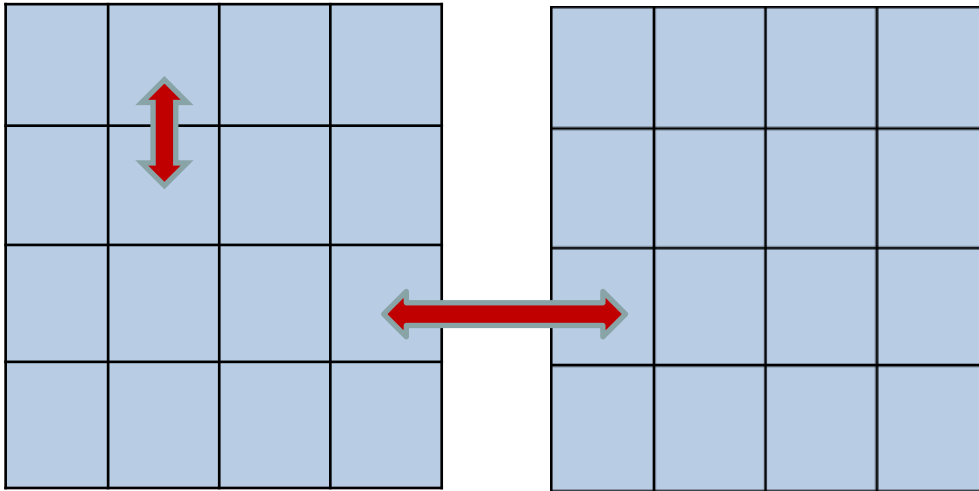
# 16 plots in a field



Each field consists of 16 plots in four rows and four columns.



# Differences among fields



Fields will differ in e.g. amount of organic matter in the soil, soil water content, presence of weeds, ...

Therefore, two plots in the same field can be expected to be more alike than two plots in different fields.

We need random field effects.

# Assigning varieties to plots in each field


Within each field the four varieties will be randomly assigned to the 16 plots.

# Assigning varieties to plots in each field

V1	V4	V2	V3
V3	V2	V3	V4
V1	V4	V2	V1
V4	V1	V3	V2

Here are the results for one of the fields.

Per plot we observe the yield  $y$ .

# The model

The mixed linear model looks like this:

$$y_{ijkl} = \mu + f_i + v_j + tv_{ij} + F_k + \epsilon_{ijkl}$$

We will assume that the **random effects** are independent and from two normal distributions:

$$F_k \sim N(0, \sigma_F^2) \quad \text{and} \quad \epsilon_{ijkl} \sim N(0, \sigma^2)$$

This is also an example of a **split-plot model**.



# Compare with the food ration example

Fields

Fertilizer levels over fields

Varieties within fields  
Randomly assigned  
to plots within fields

Variation between plots  
within fields  
= error variation

Animals

Sexes over animals

Feed rations within animals  
Randomly assigned  
to moments within animals  
(random order of feed rations)

Variation between moments  
within animals  
= error variation

# ANOVA, REML & R - 1

```
# reading the data
# Field, T (type of fertilizer), V (Variety), y (yield)

setwd("...")
Mydata <- read.table('Data_type_variety.txt', header=T)
summary(mydata)
attach(mydata)

# making experimental factors
field <- factor(Field)
t      <- factor(T, labels=c('T1', 'T2', 'T3'))
v      <- factor(V, labels=c('V1', 'V2', 'V3', 'V4'))

# split-plot, ANOVA method
lmmanova <- aov(y ~ t + v + t:v + Error(field),
               data=mydata)
summary(lmmanova)
```

Split-plot  
arrangement with  
fields as whole plots  
and plots within fields  
as sub-plots.

Fertilizer as whole  
plot treatment.

Variety as subplot  
treatment.

# ANOVA, REML & R - 2

```
# split-plot, REML
library(lme4)
lmmanova2 <- lmer(y ~ t + v + t:v + (1|field), data=mydata)

#follow-up with pairwise comparisons
library(emmeans)
emmeans(lmmanova2, pairwise ~ t, adjust="none")
emmeans(lmmanova2, pairwise ~ v, adjust="none")
emmeans(lmmanova2, pairwise ~ t:v, adjust="none")

# Kenward-Roger approximate F-test for
# interaction T x V
library(pbkrtest)
lmmA <- lmer(y ~ t + v + t:v + (1|field), data=mydata)
lmmB <- lmer(y ~ t + v + (1|field), data=mydata)
Fint <- KRmodcomp(lmmA, lmmB)
summary(Fint)
```

To be discussed in this part about testing:

(approximate) F-tests for fixed effects

pairwise comparisons for fixed effects

likelihood ratio test for components of variance

# ANOVA, REML & R - 3

```
# Likelihood ratio test for variance component of fields  
# lmer does not work without random effects  
# therefore fit reduced model with routine lm and REML is true
```

```
lmmC <- lmer(y ~ t + v + t:v + (1|field), data=mydata)  
lmmD <- lm(y ~ t + v + t:v, data=mydata)
```

```
LRT <- as.numeric(2*(logLik(lmmC) - logLik(lmmD, REML=T)))
```

```
PvalueFields <- pchisq(LRT,1,lower=F)/2
```

```
LRT
```

```
PvalueFields
```

Likelihood ratio test in mixed model to be discussed in detail later on.

# Testing in a balanced mixed model

## F-tests

Testing for fixed effects has already been discussed: F tests are constructed by taking ratio's of appropriate mean squares.

For testing a variance component often a similar approach may be followed, e.g. for  $H_0: \sigma_u^2 = 0$  the ratio  $MS_{animals} / MSE$  can be used as a test statistic in the feed ration example.

# F-test for $H_0: \sigma_u^2 = 0$ in feed ration data

## Analysis of variance

Variate: y

Source of variation	df	SS	MS	F-stat.	P-value
<b>Animal stratum</b>					
Sex	1	83.36554	83.36554	10.97	0.008
Residual (= u-terms)	10	75.98408	7.59841	118.57	
<b>Animal.*Units* stratum</b>					
Treat	1	0.56120	0.56120	8.76	0.014
Treat.Sex	1	0.03760	0.03760	0.59	0.461
Residual (= e-terms)	10	0.64084	0.06408		
Total	23	160.58926			

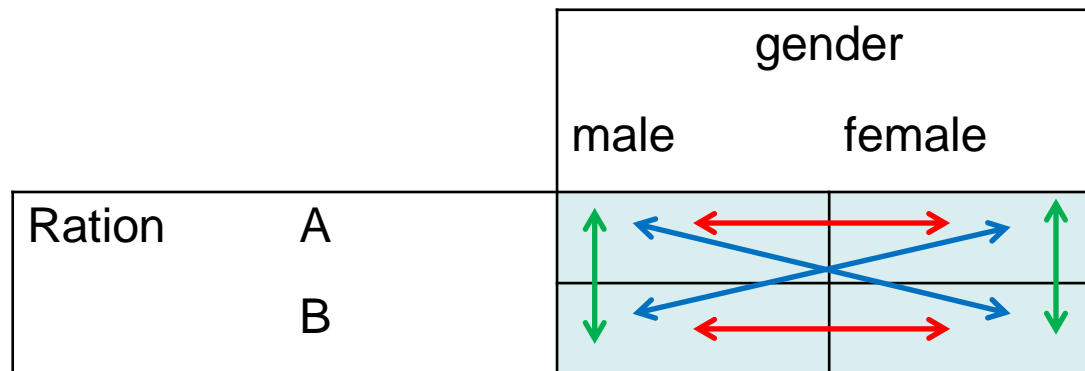
Test for  $H_0: \sigma_u^2 = 0$ :  $F = 7.59841 / 0.06408 = 118.57$ ,  
compare with F-distr. df1 =10, df2 =10, very significant:  $P < 0.001$ .

## Follow-up by pairwise comparisons – significant interaction

Suppose interaction was significant in the split-plot model for the feed ration example (it actually was not, but we pretend it was).

As a follow-up the means of the four combinations of gender and ration (male/A, female/A, male/B, female/B) are compared pairwise.

That may amount to six comparisons.



# Comparing feed rations within sexes - 1

For comparing A and B within the same gender, e.g. means male/A & male/B, differences within individuals can be used.

So, animal random effects cancel out, only error terms  $e$  are involved.

Two separate t-tests can be used (one for each sex), with the degrees of freedom of the error (residual) mean square.



# Comparing feed rations within sexes - R

```
> library(emmeans)
> emmeans(lmmanova, pairwise ~ Ration:Gender, adjust="none")
```

\$lsmeans

Ration	Gender	lsmean	SE	df	lower.CL	upper.CL
1	1	33.42833	0.7990876	10.17	31.65186	35.20481
2	1	33.20167	0.7990876	10.17	31.42519	34.97814
1	2	29.78000	0.7990876	10.17	28.00352	31.55648
2	2	29.39500	0.7990876	10.17	27.61852	31.17148

Degrees-of-freedom method: **satterthwaite**

Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
1,1 - 2,1	0.2266667	<b>0.1461554</b>	<b>10.00</b>	1.551	<b>0.1520</b>
1,1 - 1,2	3.6483333	1.1300805	10.17	3.228	0.0089
1,1 - 2,2	4.0333333	1.1300805	10.17	3.569	0.0050
2,1 - 1,2	3.4216667	1.1300805	10.17	3.028	0.0125
2,1 - 2,2	3.8066667	1.1300805	10.17	3.368	0.0070
1,2 - 2,2	0.3850000	0.1461554	10.00	2.634	0.0250

sed and degrees of freedom 10  
P-value = 0.15 from t-distr. df = 10

## Pairwise comparisons – broken degrees of freedom

For comparing means that are not within sexes, e.g. means male/A and female/A, there is no exact t-test available.

An approximate t-test can be used, with (broken) degrees of freedom derived from the so-called **Satterthwaite approximation**.

Even in a balanced lay-out not all significance tests are exact.

# Comparing sexes within rations - R

```
> library(emmeans)
> emmeans(lmmanova, pairwise ~ Ration:Gender, adjust="none")
```

\$lsmeans

Ration	Gender	lsmean	SE	df	lower.CL	upper.CL
1	1	33.42833	0.7990876	10.17	31.65186	35.20481
2	1	33.20167	0.7990876	10.17	31.42519	34.97814
1	2	29.78000	0.7990876	10.17	28.00352	31.55648
2	2	29.39500	0.7990876	10.17	27.61852	31.17148

Degrees-of-freedom method: **satterthwaite**

Confidence level used: 0.95

\$contrasts

contrast	estimate	SE	df	t.ratio	p.value
1,1 - 2,1	0.2266667	0.1461554	10.00	1.551	0.1520
1,1 - 1,2	3.6483333	1.1300805	10.17	3.228	0.0089
1,1 - 2,2	4.0333333	1.1300805	10.17	3.569	0.0050
2,1 - 1,2	3.4216667	1.1300805	10.17	3.028	0.0125
2,1 - 2,2	3.8066667	1.1300805	10.17	3.368	0.0070
1,2 - 2,2	0.3850000	0.1461554	10.00	2.634	0.0250

sed and broken degrees of freedom 10.17  
P-value from t-distr.  $df = 10.17 \approx 10$

# Pairwise comparisons when interaction is not significant

Genstat output

Grand mean 31.451

Ration	1	2	
	31.604	31.298	
Sex	male	fem	
	33.315	29.587	
Ration	Sex	male	fem
1		33.428	29.780
2		33.202	29.395

Standard errors of differences of means

Table	Ration	Sex	Ration Sex
rep.	12	12	6
s.e.d.	0.1033	1.1253	1.1301
d.f.	10	10	10.17
Except when comparing means with the same level(s) of Sex			
			0.1462
d.f.			10

look at e.g. ration means  
Discussed yesterday where  
we derived the sed = 0.1033

# Estimation of fixed effects in a mixed model - 1

For **balanced data**, in a factorial experiment, like the feed ration example, estimators  $\hat{\beta}$  of fixed effects are differences between sample means. These estimators do not depend upon the unknown components of variance and are unbiased.

For **unbalanced data**, estimators  $\hat{\beta}$  of fixed effects are generalized least squares estimators and generally depend upon the ratio of the components of variance and the error (or residual) variance. Estimators  $\hat{\beta}$  are unbiased when these ratios of components are known.

Otherwise, when variance components are replaced by their estimated values from REML, results from e.g. Kackar & Harville (1984) suggest that for all practical purposes  $\hat{\beta}$  will still be unbiased.

## Estimation of fixed effects in a mixed model - 2

The estimated covariance matrix of  $\hat{\beta}$  is: 
$$Cov(\hat{\beta}) \approx (X'V^{-1}X)^{-1}$$

This is an exact result when the components of variance in the covariance matrix  $V$  of the observations are known.

When variance components are estimated, standard errors will not include the associated additional variation.

Therefore, standard errors commonly presented in software tend to be somewhat too small.

Inflated standard errors (Kackar & Harville, 1984), to account for the additional variation in  $\hat{\beta}$ , are available, but not generally used.

# Testing in an unbalanced mixed model

## Approximate F-test & Wald test for fixed effects

Approximate F tests (Kenward & Roger) are available for fixed effects.

Alternatively, the classic Wald test may be used for fixed effects.

Often when the numerical load for the approximate F-test is too high.

The Wald test ignores that components of variance are estimated.

The Wald test statistic is referred to a Chi-square distribution.

(Approximate) F test has degrees of freedom  $df_1$  and  $df_2$ , while classic Wald test has only degrees of freedom  $df_1$ .

P-values of the classic Wald test tend to be too small: Wald test is liberal.

So, when you can, use the approximate F-test.

# Approximate F-tests for the carcass data

variable Cooking loss – Genstat output

## Tests for fixed effects

### Sequentially adding terms to fixed model

Analogous to Type I (often not very relevant)

Fixed term	Wald statistic	n.d.f. (df1)	F statistic	d.d.f. (df2)	P-value
Lr	0.57	1	0.57	4.0	0.494
Susp	4.05	1	4.05	3.8	0.119
Temp	1325.38	4	331.35	30.0	<0.001
Loc	5.70	5	1.14	31.3	0.360
Susp.Temp	4.96	4	1.24	30.0	0.315
Susp.Loc	1.78	5	0.36	31.3	0.874

### Dropping individual terms from full fixed model

Analogous to Type II (usually most relevant)

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	P-value
Lr	0.59	1	0.59	4.0	0.486
Susp.Temp	4.96	4	1.24	30.0	0.315
Susp.Loc	1.78	5	0.36	31.3	0.874

denominator degrees of freedom, again a broken number



## Unbalanced mixed model- Likelihood ratio test for dispersion parameters

For testing dispersion parameters, e.g.

$$H_0 : \sigma_u^2 = 0, \quad H_0 : \sigma_1^2 = \sigma_2^2, \quad H_0 : \rho = 0.$$

generally there is no approximate F-test available.

The Wald test often performs poorly: it is based on approximate normality and estimators of dispersion parameters tend to have seriously skewed distributions for realistic sample sizes.

The **likelihood ratio test** (LRT) is often used for hypotheses about dispersion parameters.

# Intermezzo about the likelihood ratio test - 1



Egon Sharpe Pearson

The LRT compares the log likelihood  $L$  of two  
nested models:



Jerzy Neyman

- a larger model, usually the **current model** fitted to the data
- a smaller model, obtained from the larger model by **imposing a null hypothesis**, e.g.  $\sigma_u^2 = 0$ .

Larger model

Smaller model  
under  $H_0$

Formal tests are  
only available for  
nested models.

# The likelihood ratio test - 2

When the respective log likelihoods are  $L_1$  and  $L_2$ , the test statistic

$$-2\log(\text{lik}_2 / \text{lik}_1) = (-2L_2) - (-2L_1) = D_2 - D_1$$

$D$  is called the deviance  
can be read from the output

measures how much less likely the smaller model  $D_2$  is compared to the larger model  $D_1$ .

Outcome referred to **Chi-square distribution** with degrees of freedom:

$d$  = difference in parameters between the larger and smaller model.

Large values of the test statistic are significant.

# The likelihood ratio test – the deviance

- The deviance is a measure of the fit of the model.
- The smaller the deviance the better.
- A difference between deviances of two nested models equals the test statistic of the likelihood ratio test for comparing these models.
- When the difference is too large, we reject the smaller model in favour of the larger model.
- This means that we reject the null hypothesis, e.g.  $H_0: \sigma_u^2 = 0$ , that restricted the larger model to the smaller model.
- In an ordinary linear model the deviance will be the error sum of squares *SSE*.

# The likelihood ratio test for REML - 1

Often matrix  $K$  for error contrasts  $Ky$  is not unique.

Consequently, the log likelihood  $L$  in REML is not unique.

The log likelihood of REML is unique up to an arbitrary constant.

Therefore, the REML deviance  $D = -2L$  is not unique either.

The REML deviance is unique up to an arbitrary constant too.

So, you need to be careful when you use the REML deviance.

# The likelihood ratio test for REML - 2

When the fixed part of the model remains the same, the same error contrasts will be used in both models.

In that case, the difference between two deviances based on the same error contrasts is unique, because the arbitrary constant is the same and cancels.

So, the test statistic of the LRT in REML, for a dispersion parameter, as a difference between deviances, does not depend on the choice of error contrasts, and can be used.

# Boundary problems - 1

There is a **boundary problem** when the null hypothesis is on the edge of parameter space, e.g. in the feed ration example:

$$H_0 : \sigma_u^2 = 0$$

Do not compare the test statistic with a chi-square distribution with  $df = 1$ , but with a 50 / 50 mixture of a Chi-square distribution with  $df = 0$  and  $df = 1$  degrees of freedom.

This implies that the P-value derived from chi-square distribution with  $df = 1$  degrees of freedom **has to be divided by 2**.

## Boundary problems - 2

$$H_0: \sigma_u^2 = 0$$

An equivalent hypothesis is:

$$H_0: \rho = 0$$

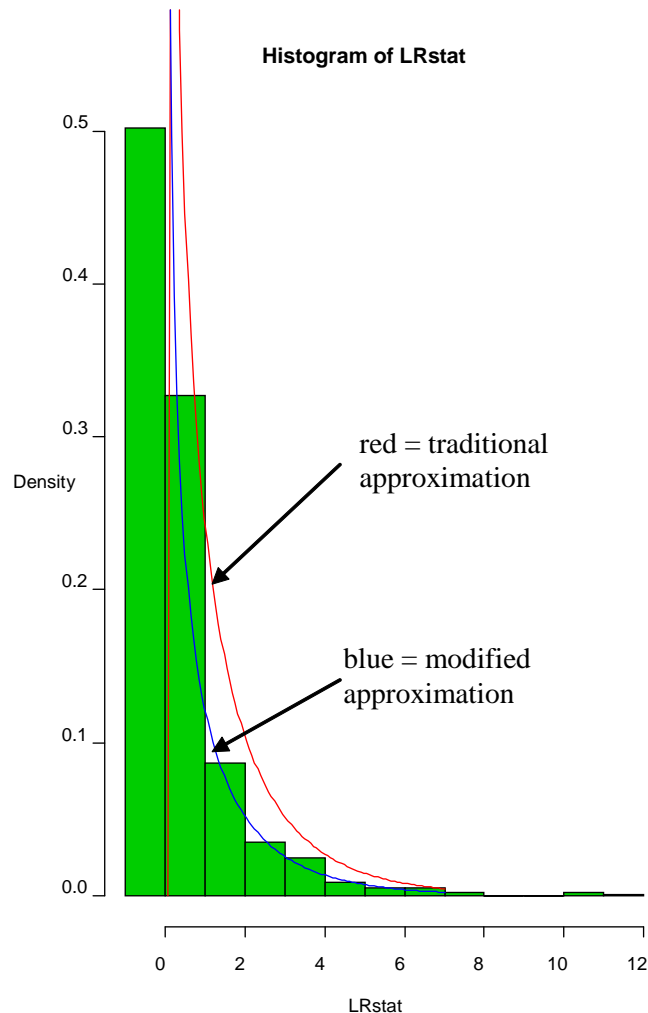
where  $\rho$  is the correlation between observations on the same animal.

When  $\rho < 0$  biologically makes sense, the boundary problem vanishes.

In that case you do not divide the P-value by 2.



# Boundary problems – feed ration data



Histogram of values of the likelihood ratio statistic from 1000 simulations (using estimates from the real data to simulate 'new' observations), for the null hypothesis:

$$H_0 : \sigma_u^2 = 0$$

In **red** the traditional chi-square approximation with  $df = 1$ .

In **blue** the modified approximation with the 50/50 mixture.

# LRT for $H_0: \sigma_u^2 = 0$ in the feed ration data

## REML variance components analysis

## Genstat output

Response variate: y  
Fixed model: Constant + Treat + Sex + Treat.Sex  
Random model: Animal  
Number of units: 24

Deviance: -2\*Log-Likelihood

Deviance	d.f.	(large model)
19.97	18	

Response variate: y  
Fixed model: Constant + Treat + Sex + Treat.Sex  
Number of units: 24

Deviance: -2\*Log-Likelihood

Deviance	d.f.	(small model)
54.03	19	

## Likelihood ratio test:

$$LR = 54.03 - 19.97 = 34.1,$$

chi-square distr., df = 1  
(because we test one parameter)  
divide P-value by 2

## F test was:

$$F = 118.57$$

F-distr., df1 = 10, df2 = 10

Both yield very low P-values.

## LRT for $H_0: \sigma_u^2 = 0$ in the feed ration data with R

```
> # Likelihood ratio test component of variance for animals  
> # lmer does not work without random effects  
> # therefore fit reduced model with routine lm and REML is true
```

```
> lmmC <- lmer(y ~ Ration + Gender + Ration:Gender + (1|Animal))  
> lmmD <- lm(y ~ Ration + Gender + Ration:Gender)
```

```
> LRT <- as.numeric(2*(logLik(lmmC)-logLik(lmmD, REML=T)))
```

```
> PvalueAnimals <- pchisq(LRT,1,lower=F)/2
```

calculating the test statistic (34.1)

```
> LRT  
[1] 34.05999
```

```
> PvalueAnimals  
[1] 2.671937e-09
```

boundary correction,  
i.e. division by 2

P-value is quite small indeed:  
0.00000000267...

# REML likelihood ratio test and fixed effects

Do not use LRT for fixed effects in combination with REML!

Error contrasts for larger and smaller model are not the same and LRT will not make sense.

- use ML rather than REML to derive deviances
- or better
- use approximate F tests for fixed effects (recommended)

# F- test in feed ration data – R - 1

Approximate F-test is actually exact here (because data are balanced).

Here are two routines to produce the F-tests: KRmodcomp & Anova.

```
> # Kenward-Roger F-test for interaction
> library(pbkrtest)
> lmmA <- lmer(y ~ Ration + Gender + Ration:Gender + (1|Animal))
> lmmB <- lmer(y ~ Ration + Gender + (1|Animal))
> Fint <- KRmodcomp(lmmA, lmmB)
> summary(Fint)
```

F-test with Kenward-Roger approximation; computing time: 0.36 sec.

large : y ~ Ration + Gender + Ration:Gender + (1 | Animal)

small : y ~ Ration + Gender + (1 | Animal)

	stat	ndf	ddf	F.scaling	p.value
Ftest	0.5868	1.0000	10.0000	1	0.4614
FtestU	0.5868	1.0000	10.0000		0.4614

# F- test in feed ration data – R - 2

```
> library(car)
> Anova(lmmE, type="III", test.statistic="F")
```

Analysis of Deviance Table (Type III Wald F tests with Kenward-Roger df)

Response: y

	F	Df	Df.res	Pr(>F)	
(Intercept)	1750.0107	1	10.169	1.02e-12	***
Ration	2.4052	1	10.000	0.151980	
Gender	10.4225	1	10.169	0.008858	**
Ration:Gender	0.5868	1	10.000	0.461361	

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Take care: tests for main effects are not correct.

These are connected to the cornerstone representation.

Either ask for type II in Anova:

```
Anova(lmmE, type="II", test.statistic="F")
```

or ask for sum-to-zero condition in preceding lmer:

```
lmmE <- lmer(y ~ Ration + Gender + Ration:Gender + (1|Animal),
             contrasts=list(Ration=contr.sum, Gender=contr.sum))
```

# Testing - summary

Balanced data ..... ANOVA method ..... F-tests based on MSs from ANOVA table

Unbalanced data...REML method ..... approximate F-tests and LRT

fixed effects ..... approximate F-test, F-distr.  
o.w. classic Wald test, chi square distr.

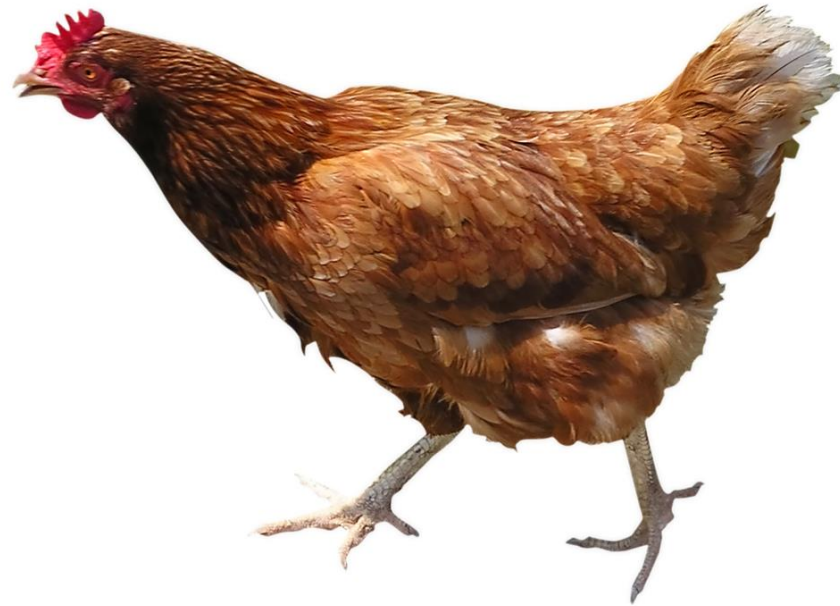
dispersion  
parameters ..... LRT, chi-square distr.  
modification for boundary problems  
(half of P-value from chi square distr.)

- LRT = difference between deviances of two nested models
- do not use LRT with REML for fixed effects

# Bacterial contamination

## a case study

plenary discussion





# Structure of the data

Is there a difference in bacterial contamination (response  $y$ ) between neck and breast area of chickens?

Experiment:

ten batches (different farms) of chickens,  
about 10 chickens per batch,  
two observations at two positions (neck & breast) per chicken.

Provide:

- significance test for position
- estimated mean difference, se, confidence interval
- range of possible differences over chickens
- impact of batch effects...

# First moves

## Fixed & random effects

### Fixed:

position (neck or breast area), factor with two levels

### Random:

Batch, Batch.Animal, Batch.Animal. Position (= error  $e$ ),

three components of variance.

# Further considerations - 1

Take a difference between positions within each animal.

Random batch and animal effects will cancel.

So:

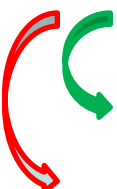
- differences are independent
- differences are tested against the error variance
- test will be an ordinary t-test on differences

Is this reasonable?

## Further considerations - 2

Not unreasonable to assume that differences of the same batch are dependent.

In order to achieve this, add random interaction between Batch and Position.



General level	1	
<u>Batch</u>	9	
<u>Batch.Animal</u>	90	together 100 animals
Position	1	
<u>Batch.Position</u>	9	
<u>Batch. Animal. Position (residual)</u>	90	
Total	200	observations

# Test for component for Batch.Position

When random interaction is negligible, the model that includes the interaction involves a marked loss in degrees of freedom in the denominator of the F-test for Position.

Choose between models on the basis of a significance test to see whether the component of variance for interaction between Batch and Position differs significantly from 0.

1. F-test from ANOVA
2. LRT, use 50-50 mixture of chi-square with 0 and 1 df  
i.e. P-value from traditional chi-square  $df = 1$  is halved.

## F-test - 1

$$\text{EMS}_{\text{Batch.Position}} = 10 \sigma_{BP}^2 + \sigma^2$$

$$\text{EMS}_{\text{Batch.Animal.Position}} = \sigma^2$$

$$F = \text{MS}_{\text{Batch.Position}} / \text{MS}_{\text{Batch.Animal.Position}}$$

Under null hypothesis  $H_0: \sigma_{BP}^2 = 0$ , F-distribution, df1 = 9, df2 = 87.

# F-test-2

## Analysis of variance

Variate: y

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Batch stratum 9		185.7327	20.6370			
Batch.Animal stratum	90		73.5761	0.8175	2.98	
Batch.Position stratum						
Position	1		25.5975	25.5975	15.05	0.004
Residual	9		15.3076	1.7008	6.20	
Batch.Animal.Position stratum						
	87	(3)	23.8625	0.2743		
Total	196	(3)	319.1540			

Involves a little imputation  
because a few observations  
are missing

$$F = 1.7008 / 0.2743 = 6.2, \text{ F-distr. } df1 = 9, df2 = 87, P\text{-value} < 0.001$$

# Further analysis with REML

## Estimated variance components

Random term	component	s.e.
Batch	0.9179	0.4883
Batch.Animal	0.2716	0.0646
Batch.Position	0.1462	0.0821

← note that component was significant, despite relatively high se

## Residual variance model

Term	Factor	Model(order)	Parameter	Estimate	s.e.	
Batch.Animal.Position		Identity		Sigma2	0.273	0.0412

## Tests for fixed effects

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Position	14.99	1	14.99	9.0	0.004

## Table of effects for Position

Position	neck	breast	
	0.0000	-0.7228	se = 0.1867

## Table of predicted means for Position

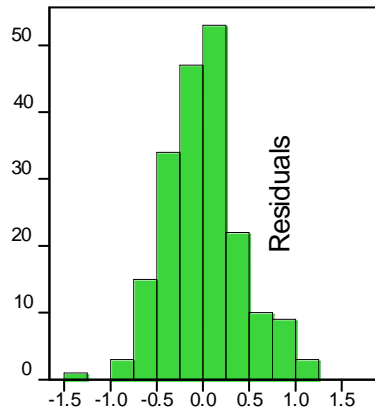
Position	neck	breast	
	2.042	1.319	se = 0.335



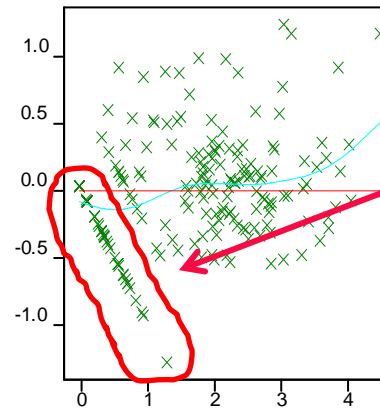
# Diagnostic plots of residuals

y

Histogram of residuals

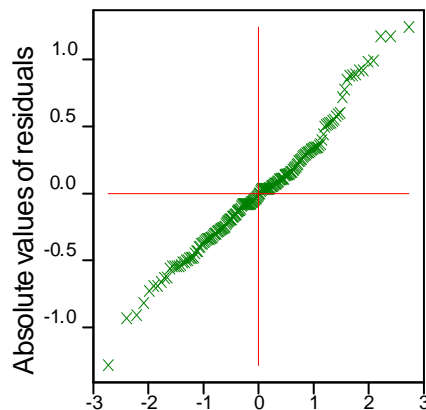


Fitted-value plot



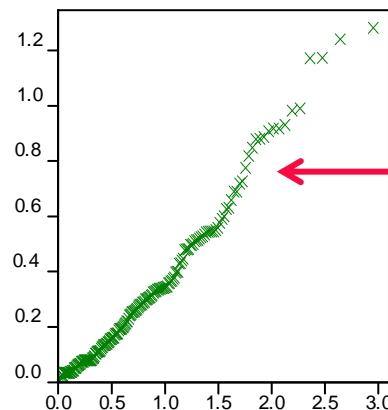
lower bound because of detection limit, deserves extra attention (skipped here)

Normal plot



Expected Normal quantiles

Half-Normal plot



Expected Normal quantiles

inference for position effect is not very sensitive to departures from normality  
this plot looks OK