Statisitcal Thinking in Biology Research

Terry Neeman and Timothee Bonnet

November 20, 2018

Acknowledgemnts and warning

Key ideas for today

- Statistics in biology is the study of biological variation

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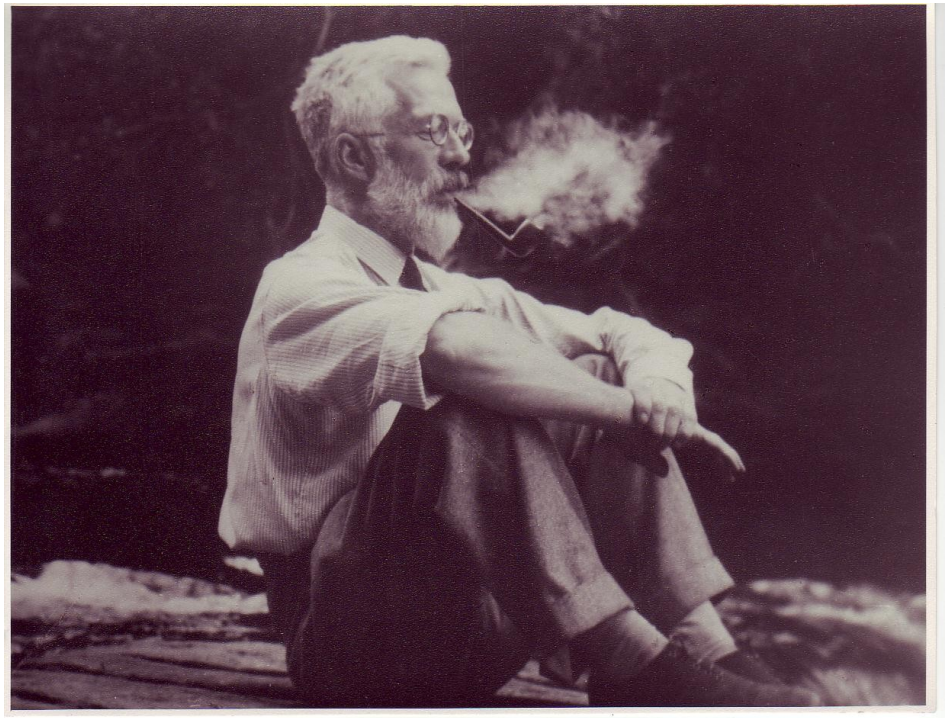
- Statistics in biology is the study of biological variation
- Statistical ideas about biological variation inform the design of experiments
- Statistical ideas about biological variation inform the analysis of experiments
- Statistical thinking is an essential component of scientific thinking

A bit of history of statistical methods

R.A. Fisher: 1890-1962



Statistical Principles for Research Workers (1925)

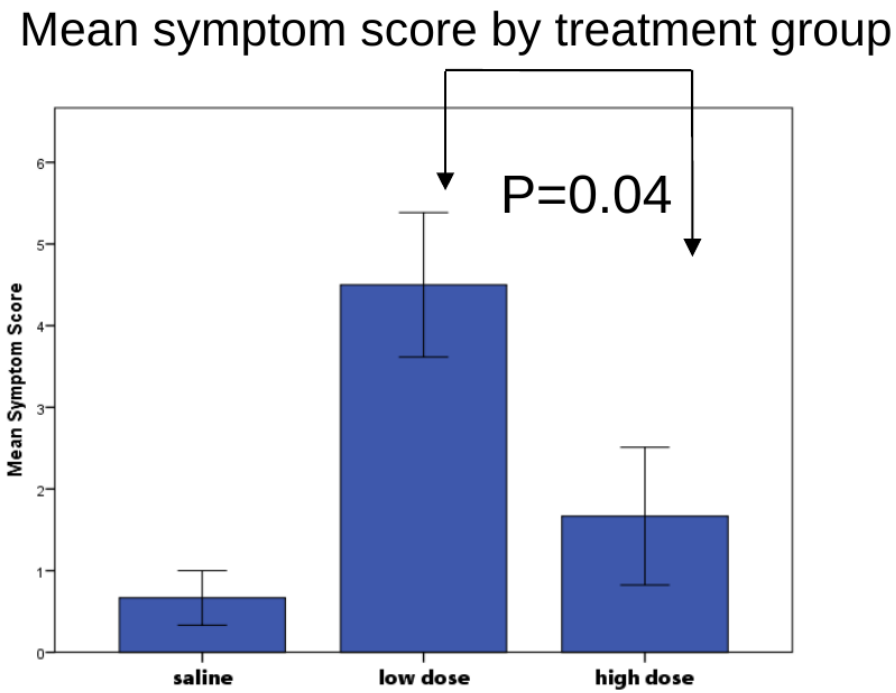
A bit of history of statistical methods

R.A. Fisher: 1890-1962

Statistical Principles for Research Workers (1925)

1. Cautionary tales from the front
2. Introduction to Statistical Modelling
3. Another look at essential steps

Message 1: A small p-value is not always evidence of a treatment effect



Vaccine challenge experiment:

- 6 mice/group (saline/low dose/high dose)
- All mice challenged with Shigella
- Followed for 14 days
- Outcome: Symptom score average Days 2 - 8

One-way ANOVA (post-hoc Bonferroni) p=0.04

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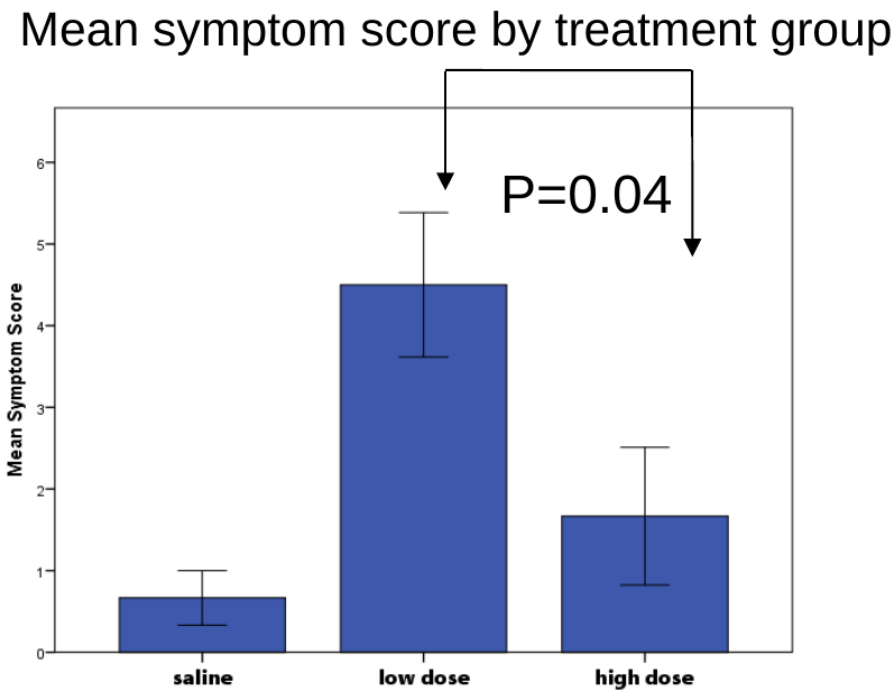

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Do you think the vaccine works? What is strange?

Message 1: A small p-value is not always evidence of a treatment effect

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Experimental design

The observed difference in outcome could be the result of:

- Cage effects
- Mouse strain effects

These effects are CONFOUNDED with treatment effect

Cage 1:
saline

Cage 2:
Low Dose

Cage 3:
High Dose

Message 2: p-values from simple comparisons cannot tell us when differences are “different”

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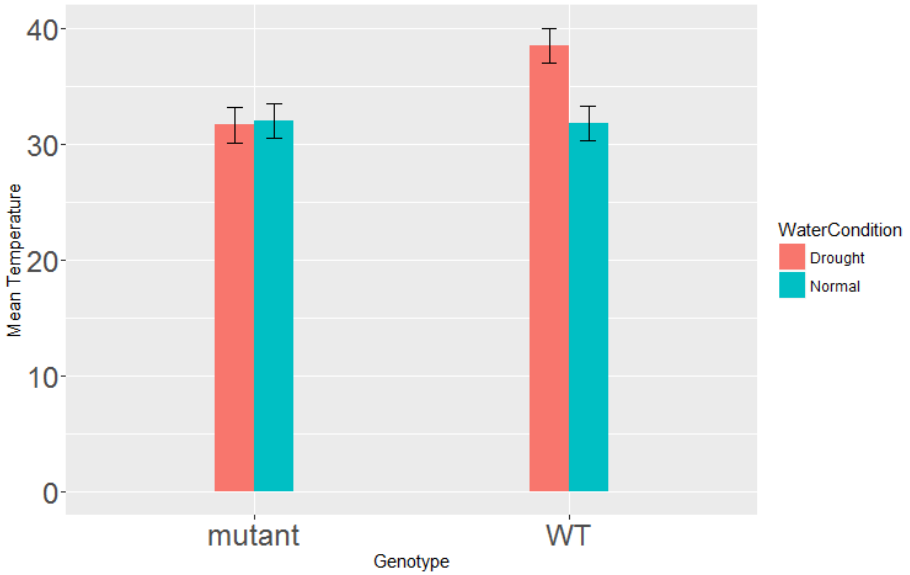

Are temperature mechanisms modified in a genetically modified tomato plant?

- Genotypes: WT/mutant
- Water condition: Normal/Drought
- Leaf temperature measured

Comparisons made using t-tests

Evidence of difference + No evidence of difference $\neq$ Evidence that differences are different.

Message 3: Interpreting experimental results needs more than t-tests

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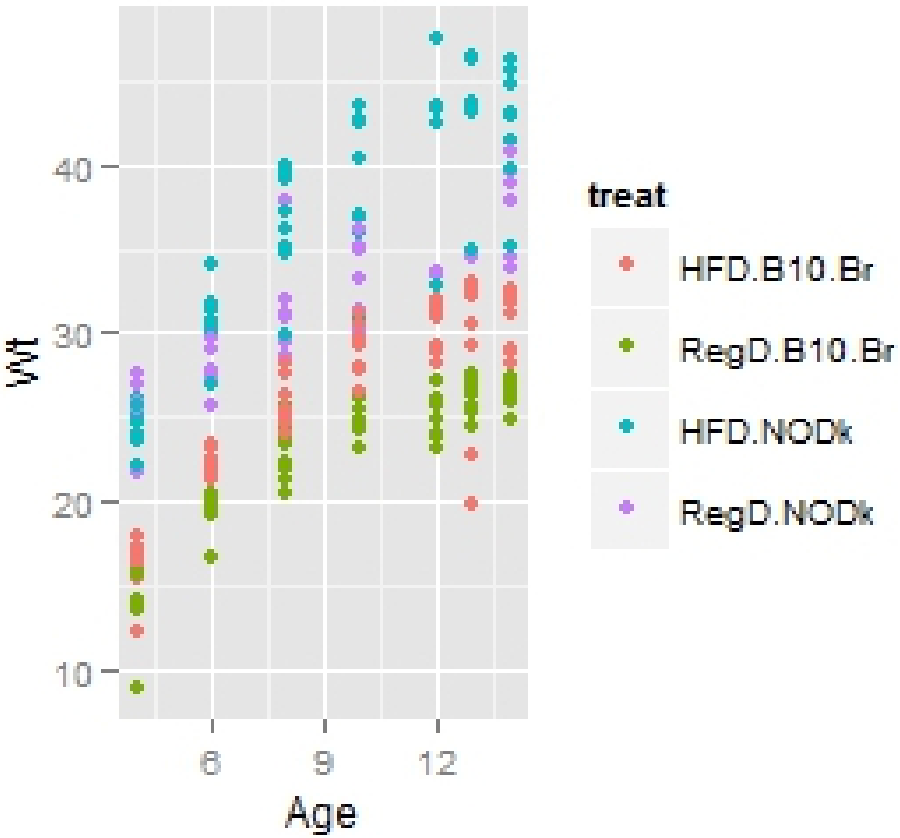
Research question: Are mice susceptible to obesity when exposed to a high fat diet?



Experimental set-up:

- 37 mice: 16 NODk /21 WT
- Randomised to either regular or high fat diet
- Monitored for 14 weeks
- Outcome measure: Body weight (g)
- Experimental factors: Diet (2), Strain (2), Time (8)

Acknowledgements: Ainy Hussain, PhD student 2013

Message 4: Knowing how to combine information across subgroups can improve inference

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Comparing yield in five barley varieties (1930s)
Experimental factors: 5 varieties of barley, 6 locations, 2 time points.
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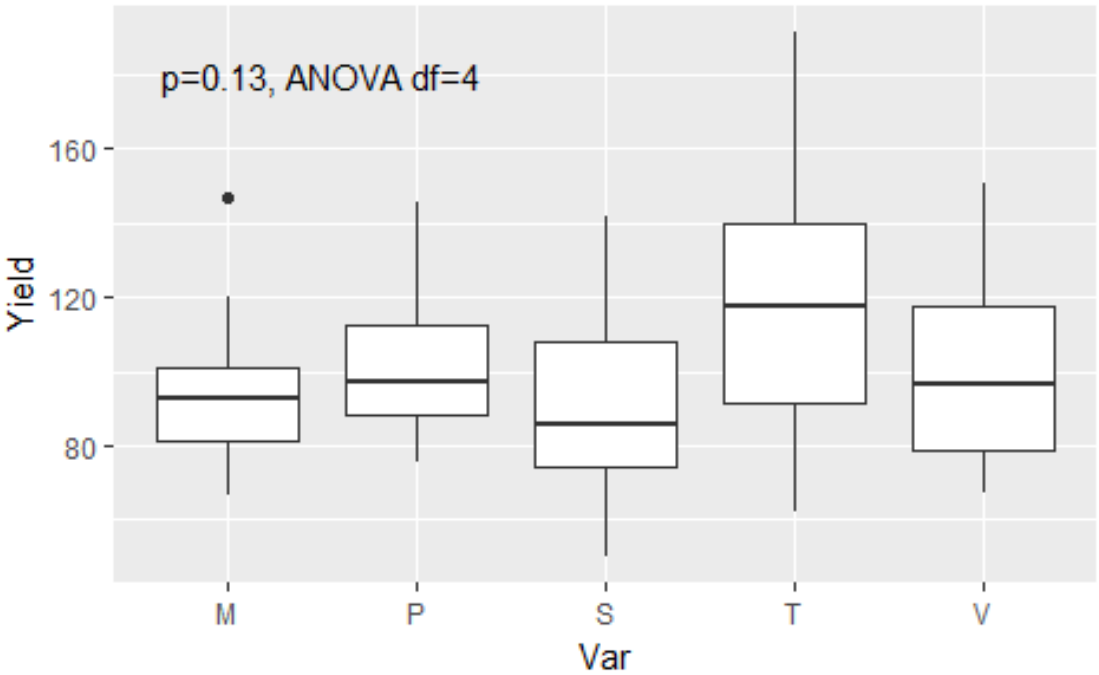

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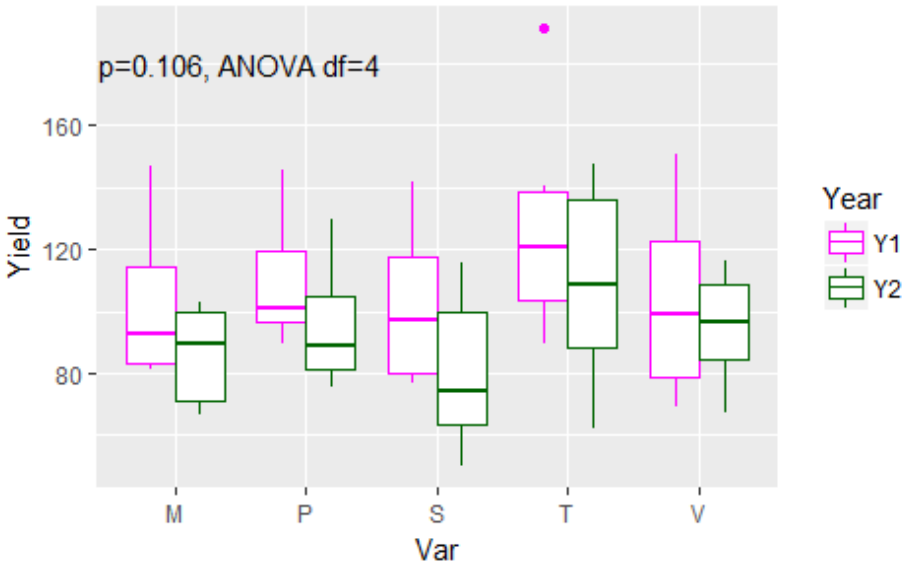

Controlling for other sources of variation:

- Controlling for year = comparing yield WITHIN years and combining these

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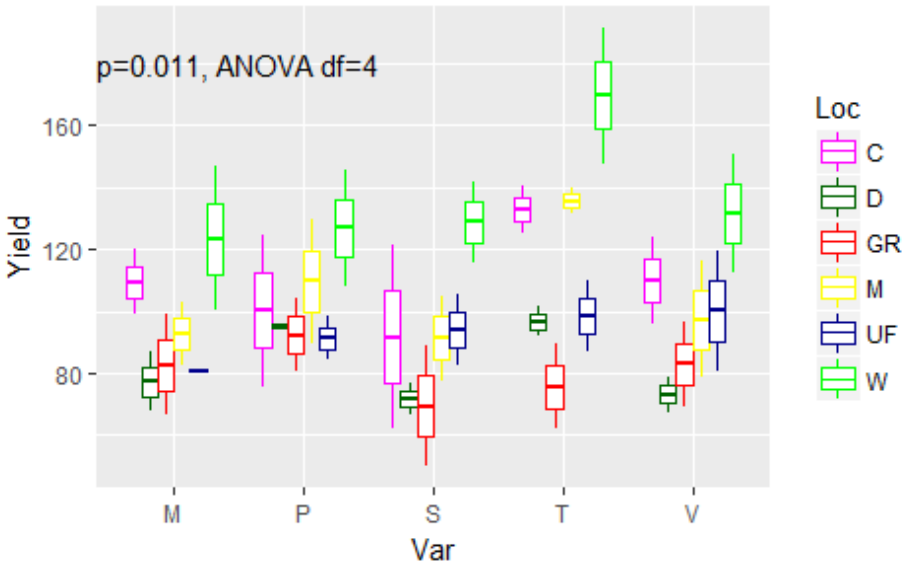

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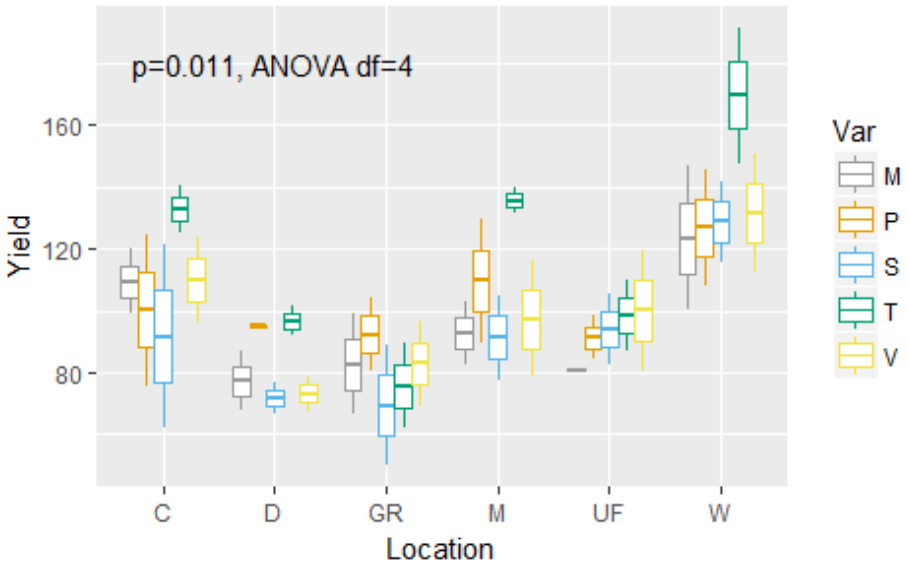

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Research question: How does cold duration impact upon germination in alpine plant A. glacialis?

Experimental set-up:

- Seed collections from alpine region in Australia
- 3 Regions- low/high altitude
- 4 sets of Petri dishes
- 4 cabinet shelves
- Response - % germinated

What other factors are important to consider when comparing cold duration?

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4. Combining information across subgroups can improve inference. **A statistical model enables accumulation of evidence across experiments.**

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1. A small p-value is not always evidence of a treatment effect. **Good experimental design matters.**
2. p-values from simple comparisons cannot tell us when differences are "different". **For each question / comparison, a specific test**
3. Interpreting experimental results needs more than t-tests. **Need a statistical model of the experiment, matching scientific question.**
4. Combining information across subgroups can improve inference. **A statistical model enables accumulation of evidence across experiments.**
5. Knowing what factors contribute to the variation in outcome matters. **A statistical model allows one to incorporate effect of other factors in the analysis.**

1. Cautionary tales from the front
2. Introduction to Statistical Modelling
3. Another look at essential steps

Introduction to Statistical Modelling

- What is a statistical model?
- Modelling outcomes:
  - a summary of data
  - a prediction model
  - an explanatory model
- Model – may take many different functional forms
- Model – a conceptualization of the experiment

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ALWAYS BEGIN WITH A RESEARCH QUESTION

Key components of a statistical model of an experiment

- Outcome measure
  - Response variable
  - Measure of interest
- Experimental factors
  - Conditions that can be manipulated
  - Conditions of interest (e.g. genotype, gender)
  - Main questions: do the conditions impact upon the outcome measure?
- Blocking factors
  - Conditions (not of interest) that may impact upon the outcome measure
  - Sources of variation in the experiment that need to be controlled for
  - Clustering of experimental units

ALWAYS BEGIN WITH A RESEARCH QUESTION

Key Objectives of a statistical model of an experiment

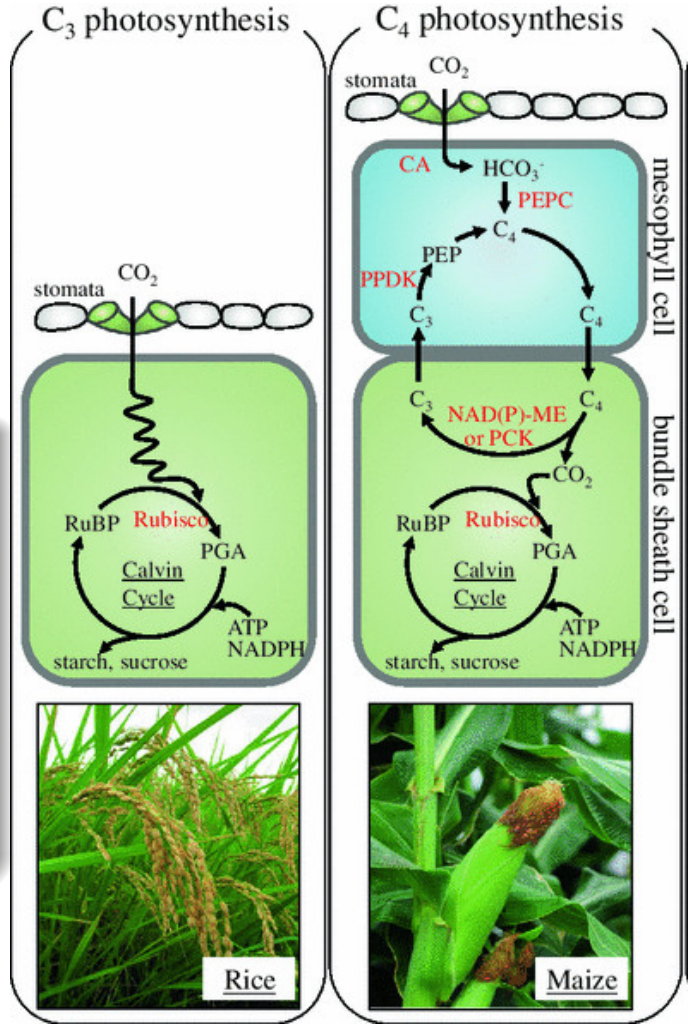
- To compare the mean response of an organism/system to a set of different experimental conditions.
  - Obtain estimate of "Treatment effect"
  - Is this "effect" different in subgroups of interest?
- What are the most important factors influencing the mean response?
- Subsidiary question: how can we design our experiment in future to more efficiently test our hypotheses?

Example 1: Does dark respiration differ between C3 and C4 plants?

Outcome measure: dark respiration
Experimental factor: Plant type (C4/C3)
Data: 6 plants each of C4, C3

Can calculate

- Observed overall mean
- Observed mean C3 plants
- Observed mean C4 plants
- Variation around each mean



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Statistical model

Respiration = Mean for C3 + Difference C4-C3 * (is C4?) + Noise

$response = A + D \times predictor + \epsilon$

A and D are the model PARAMETERS.

We want to infer whether D is different from 0

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- No blocking factor / corrections

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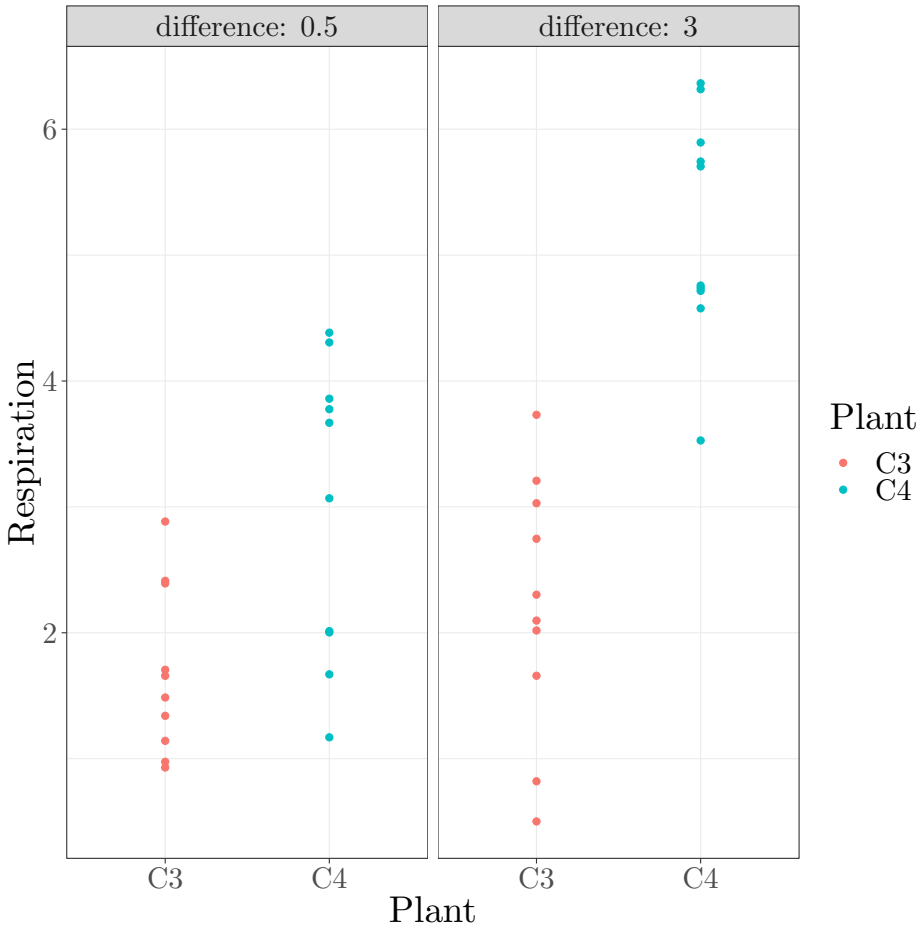
When can we know whether $D \neq 0$?



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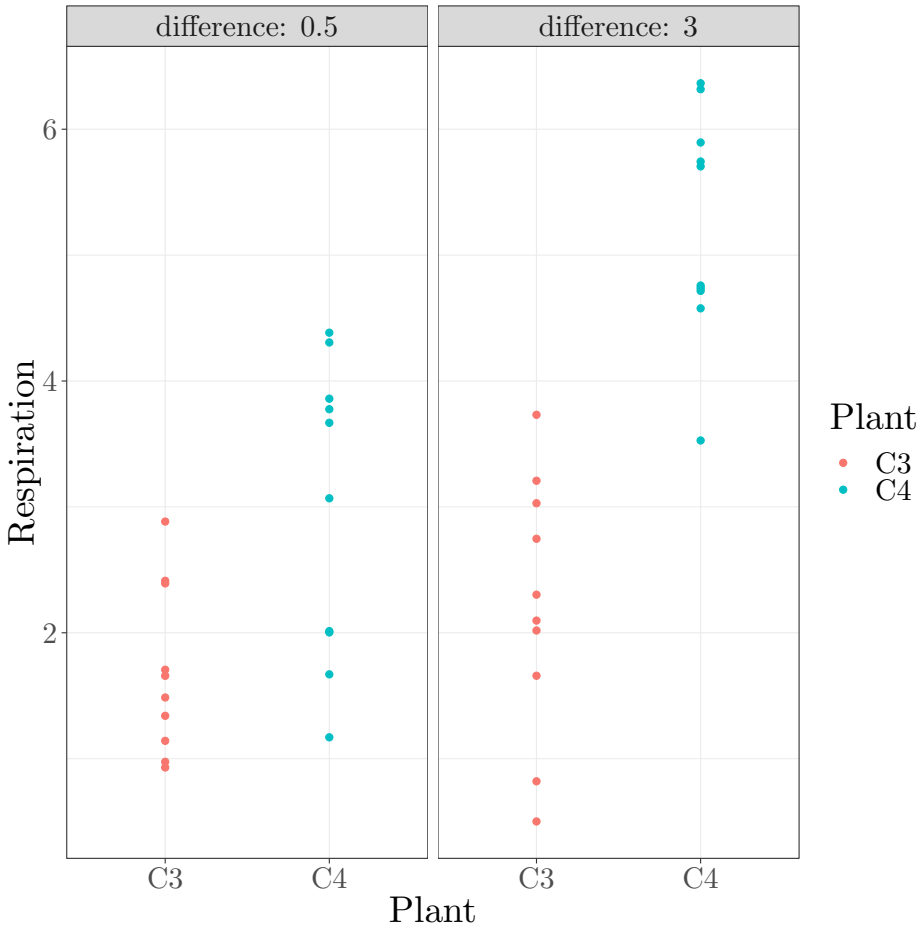

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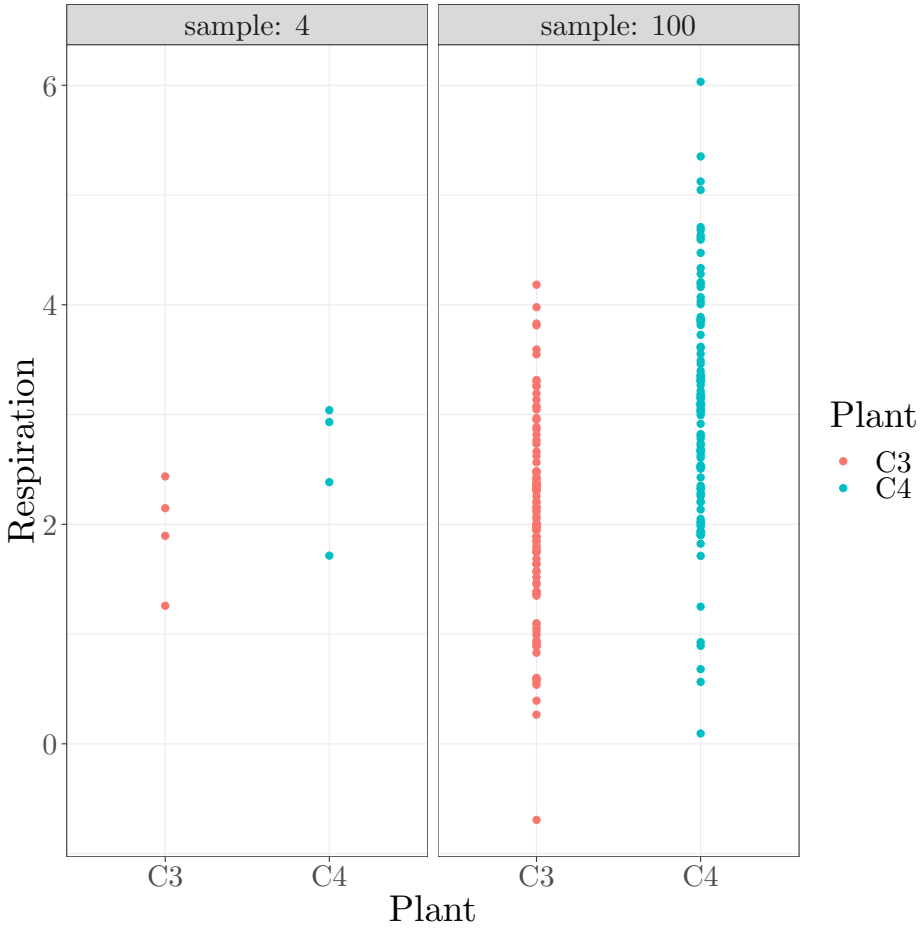

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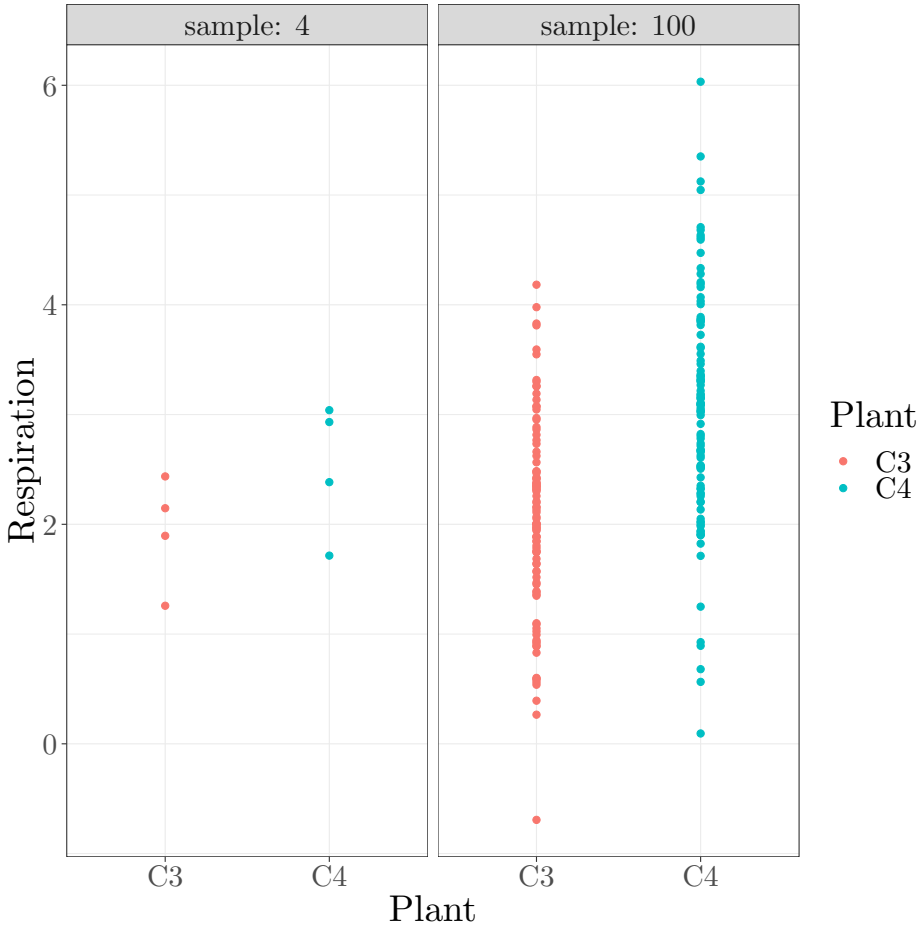

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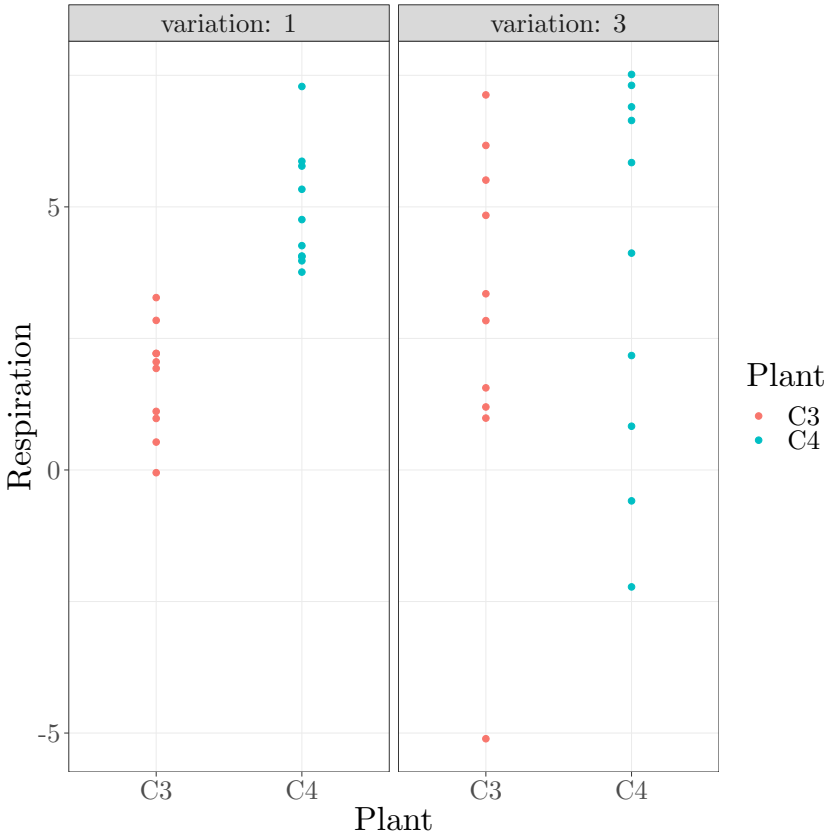

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Is it easier when unexplained variation is 1 or when it is 3?

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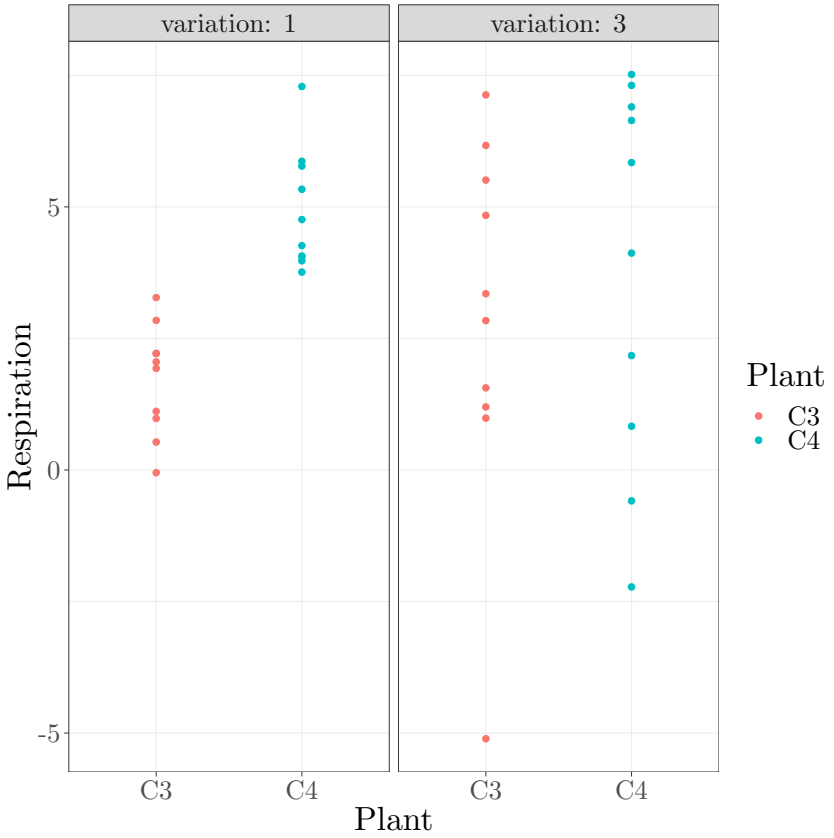

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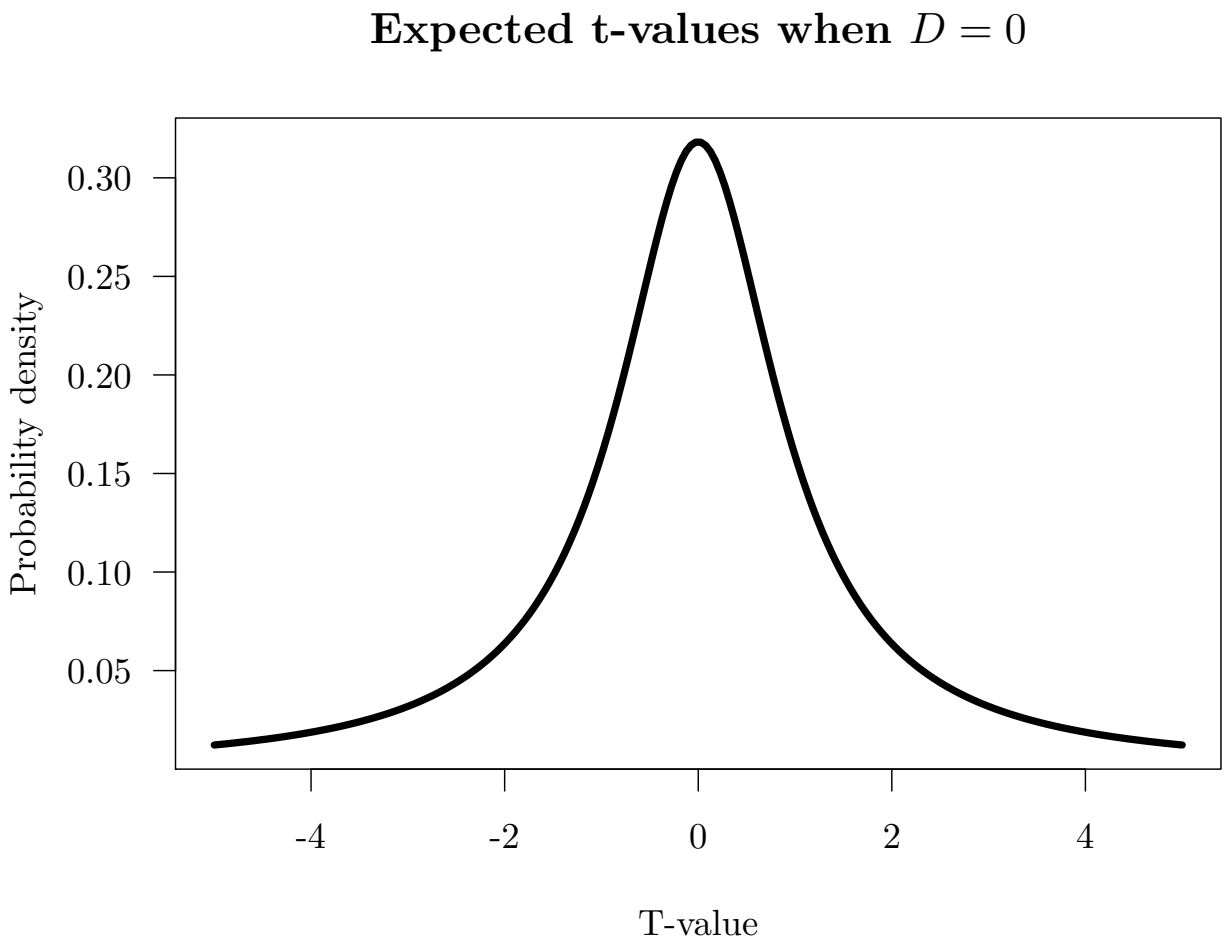
What makes t large:

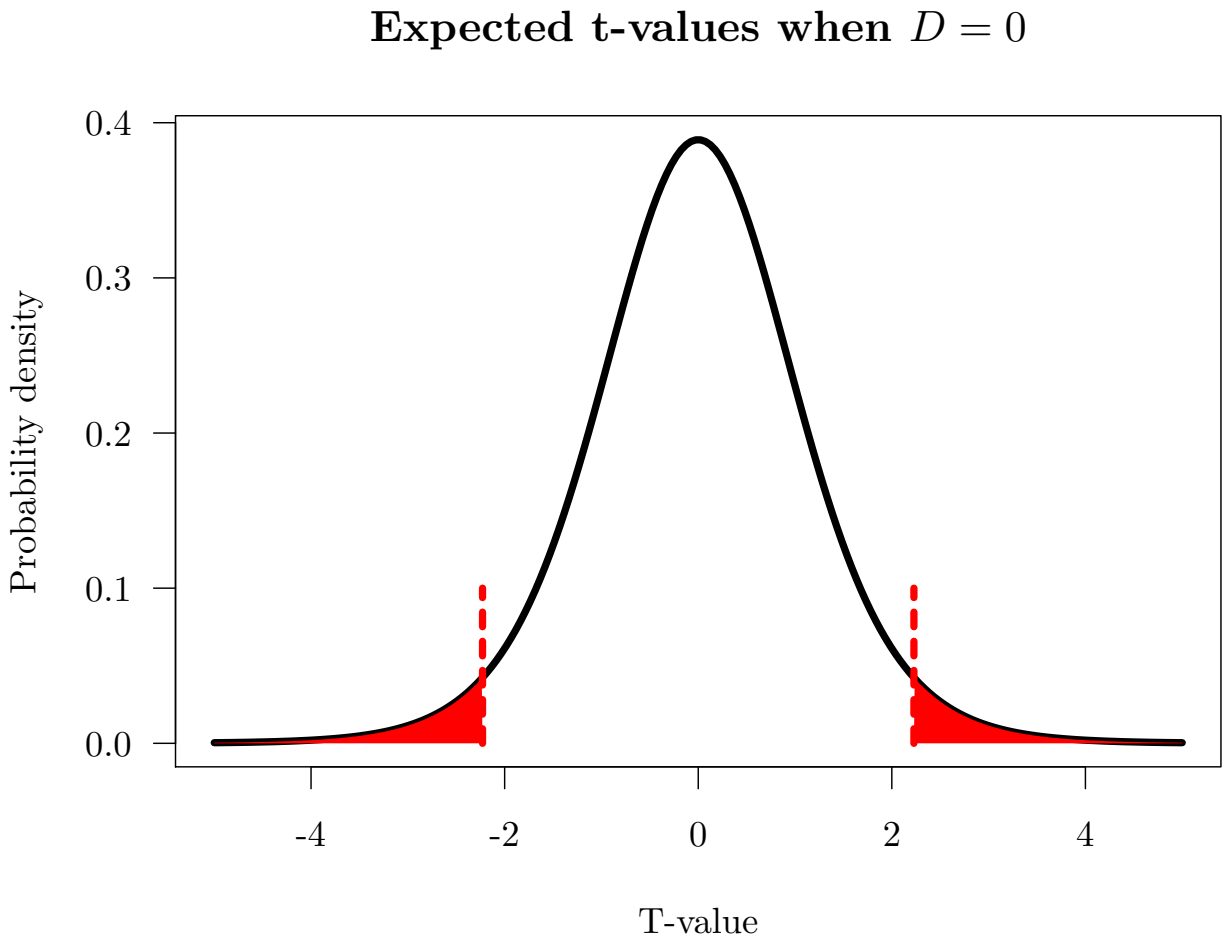
1. Large true difference between the means
2. Large sample size
3. Small unexplained variation

When can we know whether $D \neq 0$?



When can we know whether $D \neq 0$?

p-value: probability (area under curve) of getting a value as extreme as what you observed, when D=0



But really, what is a p-value?

Candy practical

You got 5 Halloween candies out of the bag. Does the bag contain more Halloween than normal candies?

Back to C3/C4 plants. Analyse real data in R

1. Set working directory (`setwd(‘‘ / ’’)`) or create a R-project
2. Load and check data

```
resp <- read.csv("d_respiration.csv")
str(resp)
View(resp)
```

3. Visualize data

```
library(ggplot2)
ggplot(resp,aes(Plant_type,rrarea,colour=Plant_type))+
    geom_point()+facet_wrap(~Variation)
```

Fit a t-test in R: `t.test()`

Subset data by Variation (High and Low)

```
resp_H <- subset(resp,Variation == "High")
resp_L <- subset(resp,Variation == "Low")
```

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Compare C3 and C4 plants in "High Variation" subset

```
t.test(rrarea~Plant_type, data=resp_H, var.equal=TRUE)
```

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```
Two Sample t-test
data:  rrarea by Plant_type
t = -0.93776, df = 10, p-value = 0.3705
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -1.7619349  0.7181446
sample estimates:
mean in group C3 mean in group C4
        2.720021         3.241916
```

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Compare C3 and C4 plants in "Low Variation" subset

```
t.test(rrarea~Plant_type, data=resp_L, var.equal=TRUE)
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Fit an anova in R: aov()

```
aov1 <- aov(rrarea~Plant_type, data=resp_H)
summary(aov1)
```

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```
             Df Sum Sq Mean Sq F value Pr(>F)
Plant_type    1  0.817  0.8171   0.879   0.37
Residuals    10  9.292  0.9292
```

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aov1 <- aov(rrarea~Plant_type, data=resp_H)
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Fit a linear model in R: `lm()`

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lm1<-lm(rrarea ~ Plant_type, data = resp_L)
summary(lm1)
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```
lm1<-lm(rrarea ~ Plant_type, data = resp_L)
summary(lm1)
```

```
lm(formula = rrarea ~ Plant_type, data = resp_H)

Residuals:
    Min      1Q  Median      3Q     Max
-1.7380 -0.4201 -0.1437  0.6706  1.6754

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)    2.7200     0.3935   6.912 4.13e-05 ***
Plant_typeC4   0.5219     0.5565   0.938     0.37
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.9639 on 10 degrees of freedom
Multiple R-squared:  0.08083,Adjusted R-squared:  -0.01109
F-statistic: 0.8794 on 1 and 10 DF,  p-value: 0.3705
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Fit a linear model in R: `lm()`

```
library(emmeans)
emmeans(lm1, ~Plant_type)
```

```
 Plant_type   emmean        SE df lower.CL upper.CL
 C3          2.720021 0.3935305 10 1.843180 3.596861
 C4          3.241916 0.3935305 10 2.365076 4.118757

Confidence level used: 0.95
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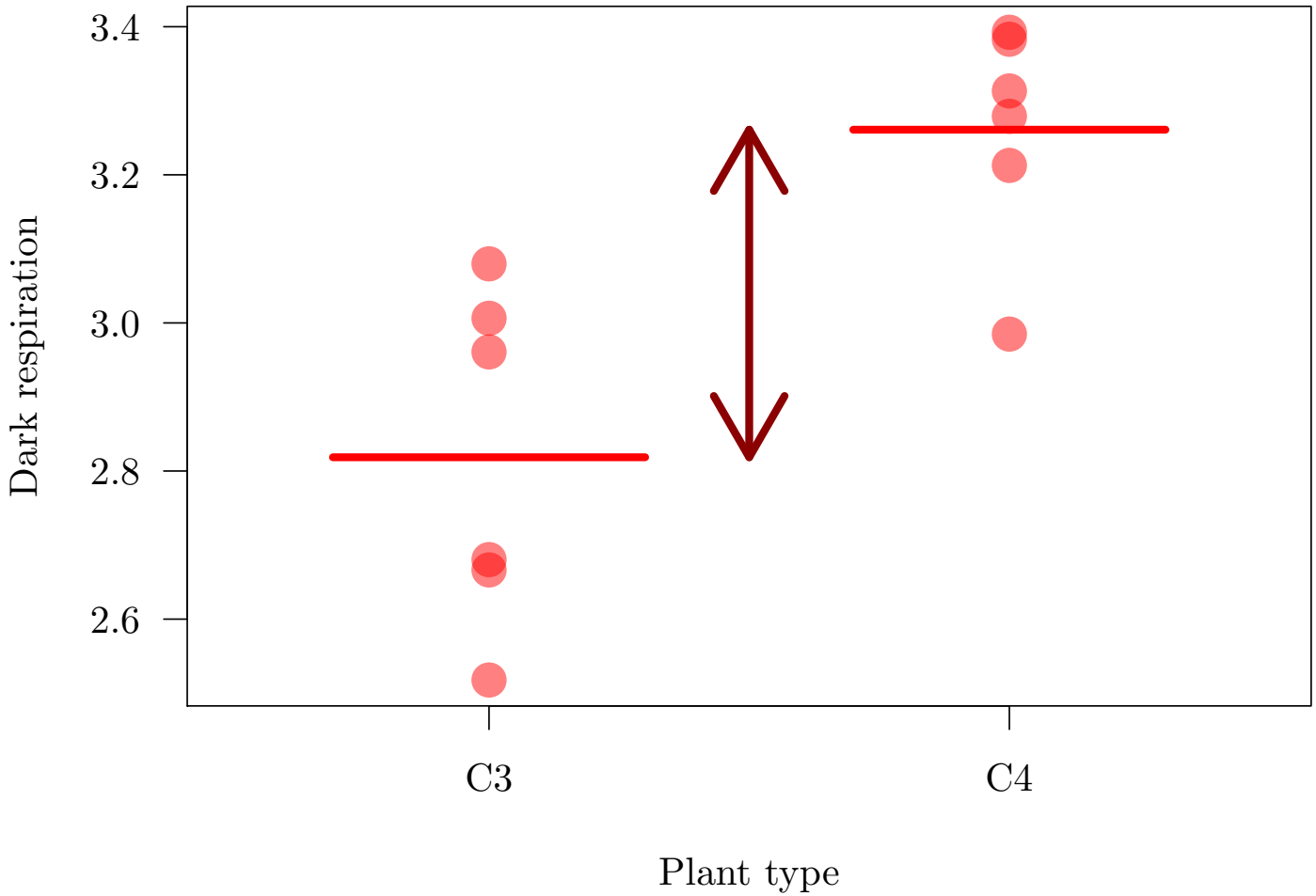
Compare the output from t.test, aov and lm

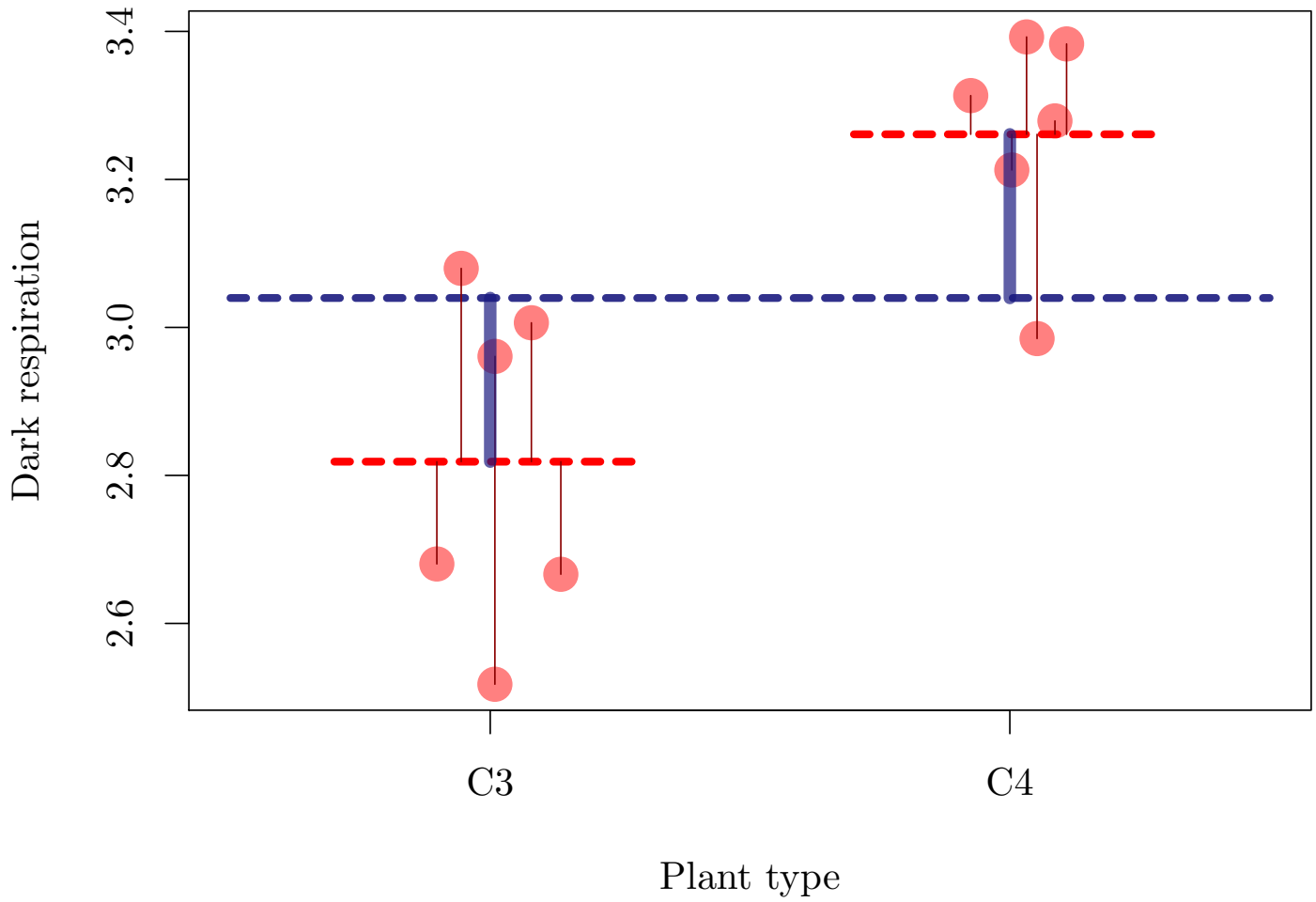
Three equivalent ways to look at data

T-test, focus on difference between two means



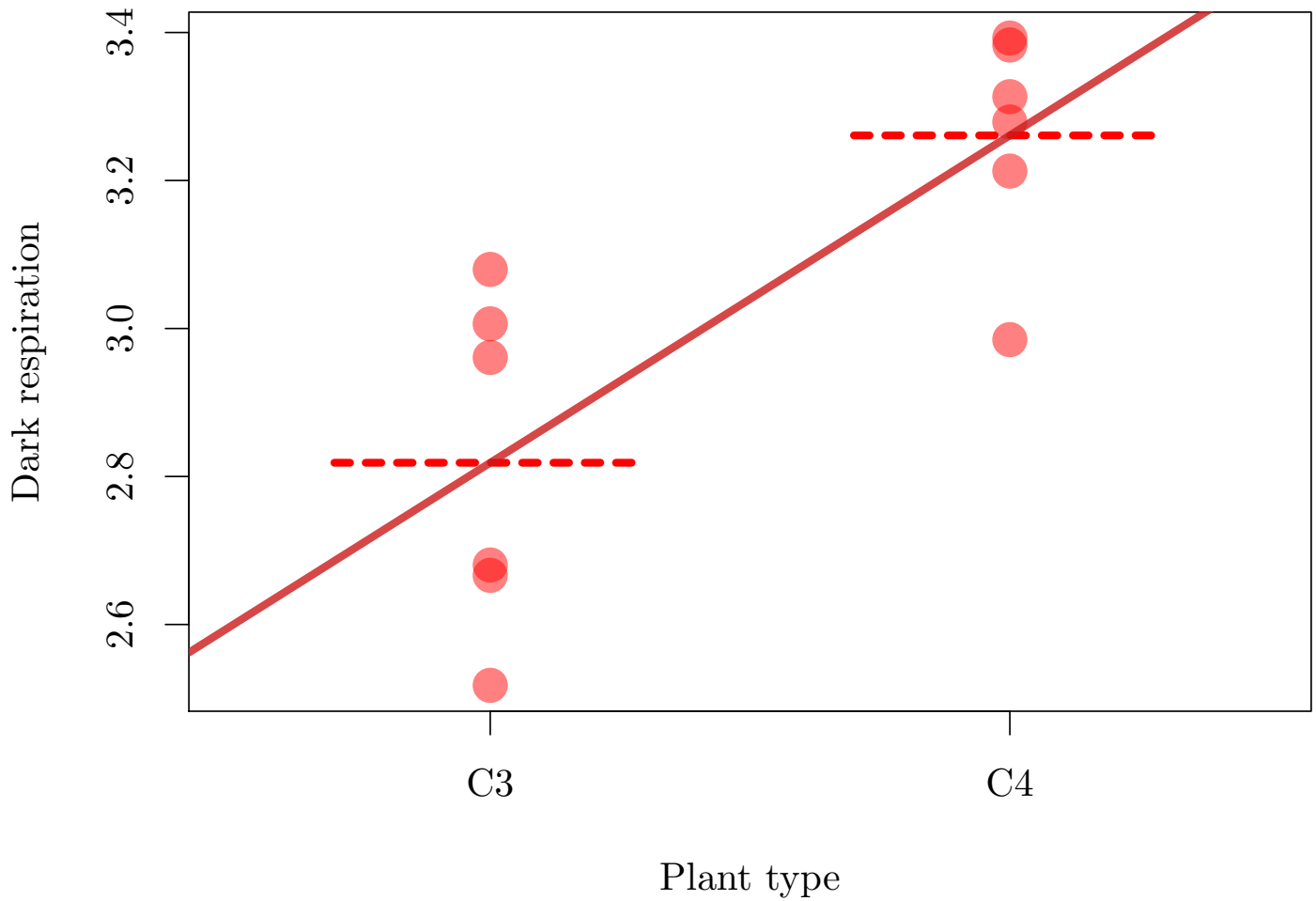
Three equivalent ways to look at data

ANOVA, focus on variation within VS. between



Three equivalent ways to look at data

Linear regression, focus on rate of change



All is one. . .

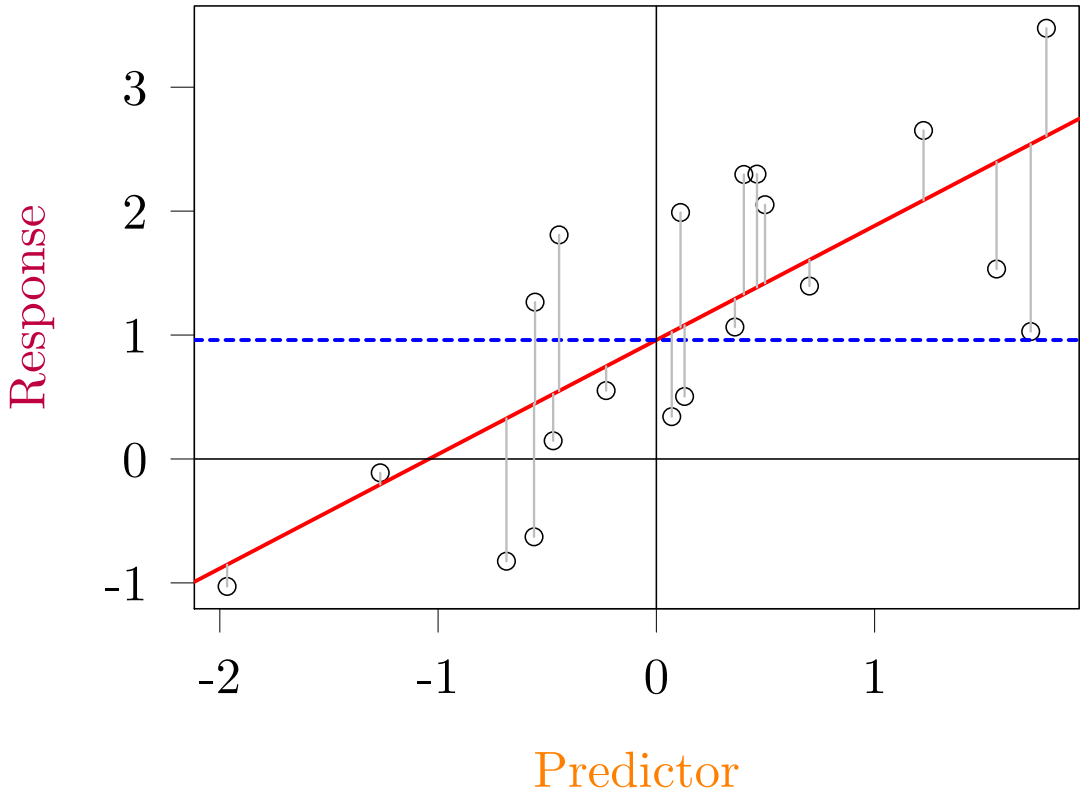
All is one...

...but `lm()` rules (IMH)

- t-test, ANOVA, regression and others can be mathematically equivalent
- In R, `lm()` and related functions can do them all...
- ...and much more!

Focus on linear models

Response = **Intercept** + **Slope** × **Predictor** + **Error**



A simple linear model

Response = **Intercept** + **Slope** × **Predictor** + **Error**

```
lm(response ~ 1 + predictor1 + predictor2, data=data)
```

equivalent to

```
lm(response ~ predictor1 + predictor2, data=data)
```

equivalent to

```
lm(response ~ predictor2 + predictor1, data=data)
```

- Intercept can be explicit or implicit
- Can remove intercept with `...~ 0 + ...`
- Error is implicit
- Feed the option `data=` to keep code short, reliable and flexible
- Order of predictors do not matter

1. Cautionary tales from the front
2. Introduction to Statistical Modelling
3. Another look at essential steps

General approach

1. Scientific question

General approach

1. Scientific question
↓
2. Model and Statistical question

General approach

1. Scientific question

$\downarrow$

2. Model and Statistical question $\longleftrightarrow$ 3. Data collection

General approach

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↓

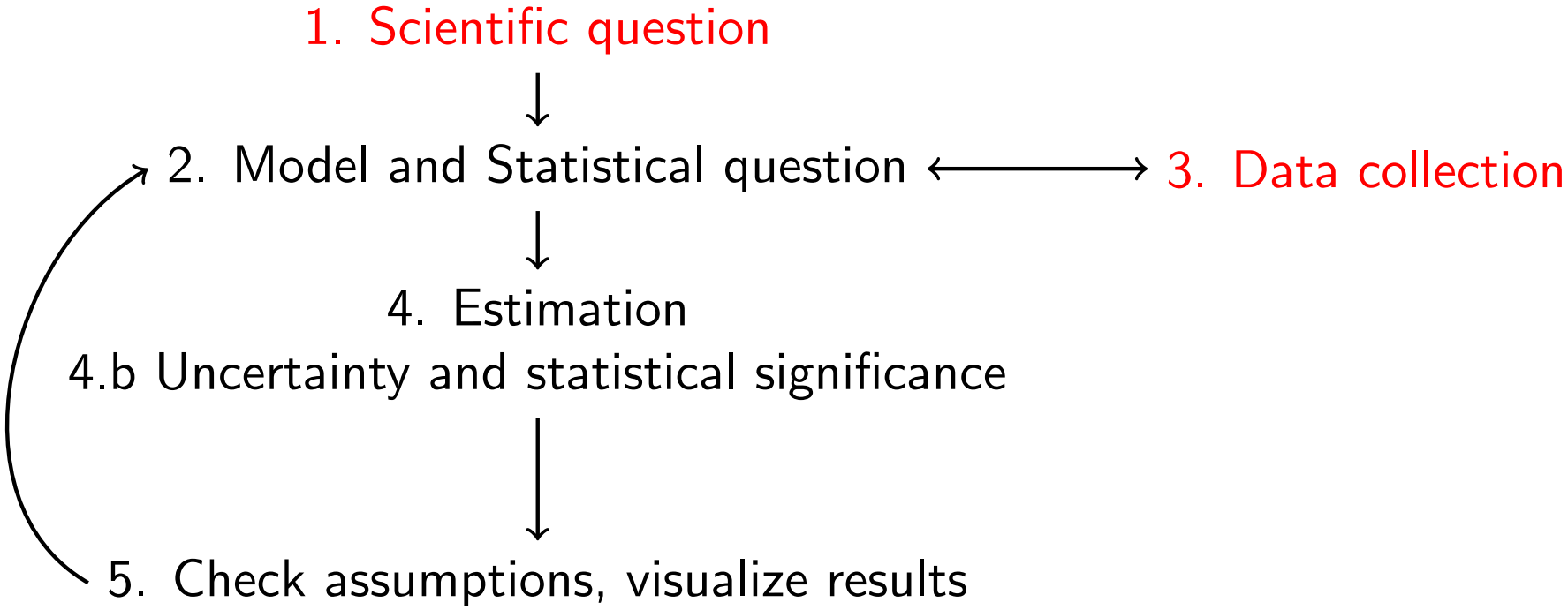
2. Model and Statistical question ⟷ 3. Data collection

↓

4. Estimation

4.b Uncertainty and statistical significance

General approach



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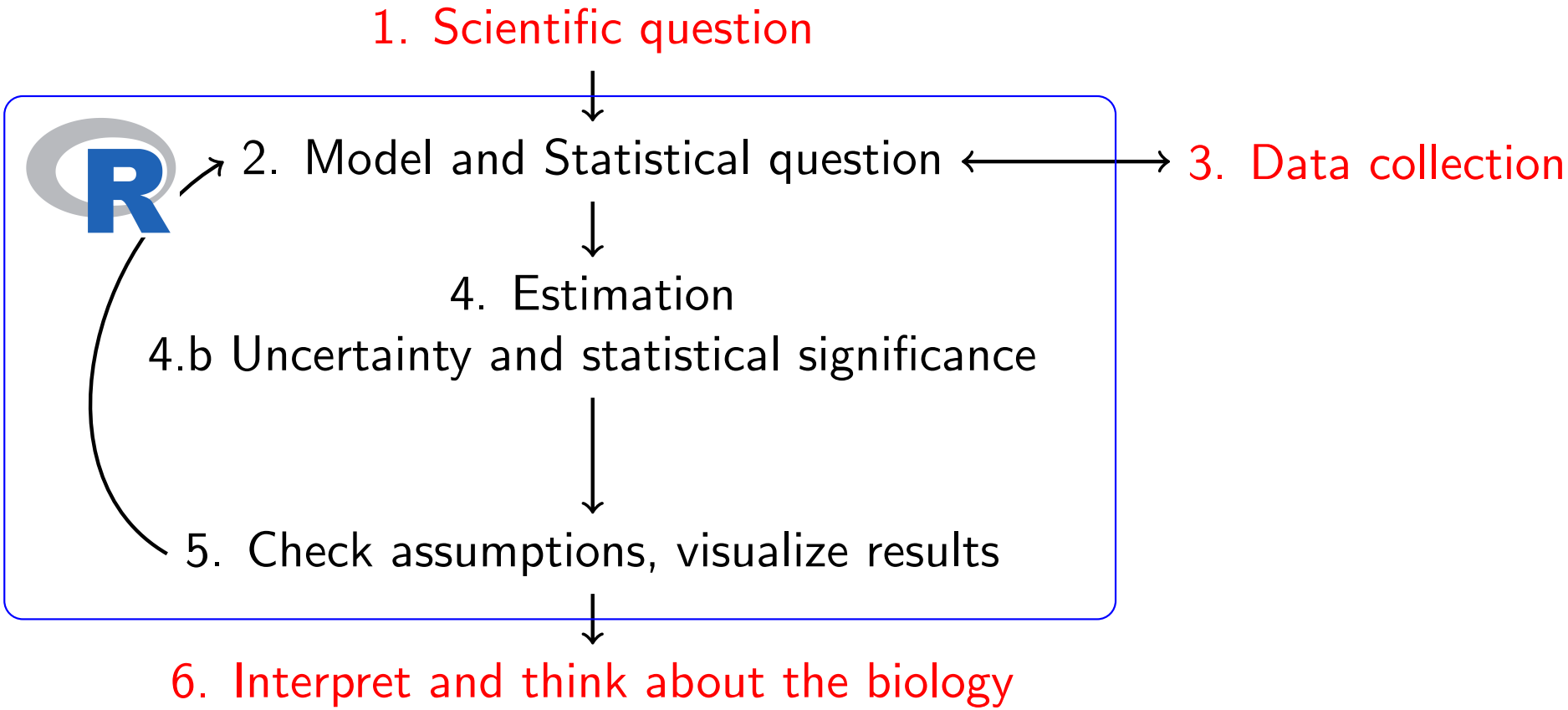

Back to C3/C4

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lmL<-lm(rrarea ~ Plant_type, data = resp_L)
summary(lmL)
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             Estimate Std. Error t value Pr(>|t|)
(Intercept)   2.81857    0.07856  35.878 6.72e-12 ***
Plant_typeC4  0.44235    0.11110   3.982  0.00259 ** ---
...
```

Estimation:

$response = A + D \times predictor + \epsilon$

A =?, D =?

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What do we do next?

Check assumptions, visualize results

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Linear model basic assumptions

- Predictor not perfectly correlated
  Risk: Model won't run, unstable convergence, or huge SE

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  Risk: Poor predictions
- Homoscedasticity (constant error variance)
  Risk: Over-optimistic uncertainty, unreliable predictions
- Independence of error
  Risk: Bias and over-optimistic uncertainty

Check assumptions, visualize results

Assessing model assumptions in R:

```
lmL<-lm(rrarea ~ Plant_type, data = resp_L)
plot(lmL)
summary(lmL)
```

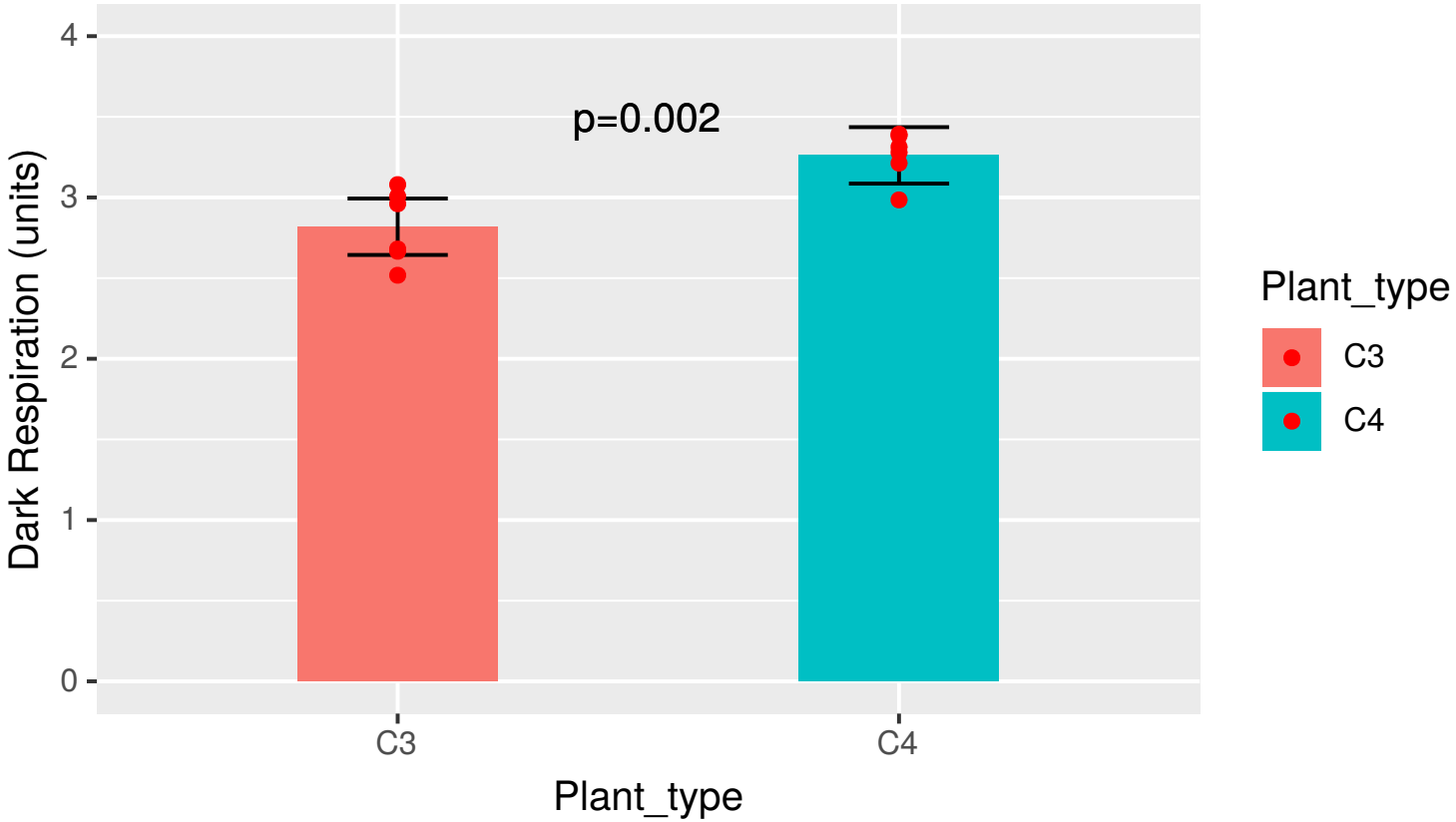
Check assumptions, visualize results

Visualize and report results

```
lm1.results<-summary(emmeans(lm1,~Plant_type))

ggplot(lm1.results,aes(Plant_type,emmean, fill=Plant_type))+
  geom_bar(stat="identity", width=.4)+
  geom_errorbar(aes(ymin =lm1.results$lower.CL,
  ymax = lm1.results$upper.CL), width=.2)+
  ylim(0,4)+
  geom_point(data=resp_L, aes(x=Plant_type, y=rrarea), color="red")+
  labs(y = "Dark Respiration (units)")+
  geom_text(aes(x=1.5, y=3.5, label="p=0.002"))
```

Check assumptions, visualize results



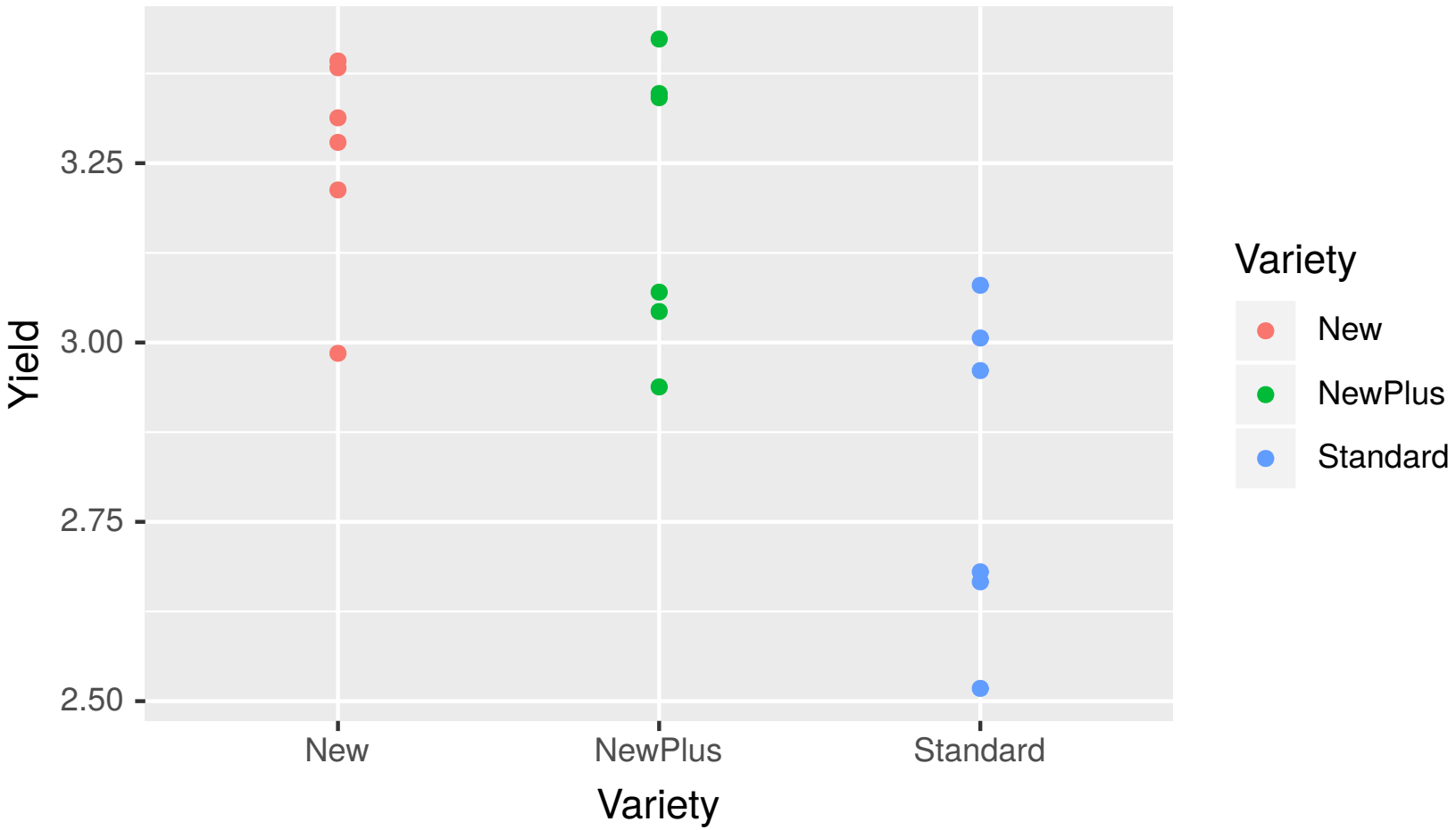
Another example

Compare wheat yields between 3 varieties

- Outcome measure: Tonnes/hectare
- Experimental factor: Variety (new/newPLUS/standard)
- Data: 6 plots/ variety

How many parameters in this model?

Results from Wheat Yield Experiment with 3 Varieties



Analyse these data in R

```
 wheat2<-read.csv("wheat yield PLUS.csv")
str(wheat2) #check data types for each variable
View(wheat2) #View data
ggplot(wheat2, aes(Variety, Yield, colour=Variety)) +
            geom_point()
```

Sample analysis in R: 1-way ANOVA

```
aov1=aov(Yield~Variety, data = wheat2)
summary(aov1)
```

```
            Df  Sum Sq   Mean Sq  F value   Pr(>F)
Variety      2  0.6820    0.3410    8.951  0.00276 **
Residuals   15  0.5714    0.0381
```

ANOVA

- Compares means between TWO or MORE GROUPS
- Relies on F-statistic $= \frac{\text{Between-groups variance}}{\text{Within groups variance}} = \frac{\text{Explained}}{\text{Unexplained}}$
- One test for significance of all groups

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- One test for significance of all groups

But which groups are different???

Using emmeans to extract group estimates

```
emmeans(aov1, ~Variety)
```

```
Variety     emmean    SE  df    lower.CL    upper.CL
 Standard  2.81   0.079  15  2.64   2.98
 New       3.26   0.079  15  3.09   3.43
 NewPlus   3.19   0.079  15  3.02   3.36

Confidence level used: 0.95
```

Using emmeans to compare groups

```
emmeans(aov1, pairwise~Variety)
```

```
$contrasts
 contrast                estimate        SE  df    t.ratio  p.value
 Standard - New      -0.44     0.112  15   -3.925  0.0036
 Standard { NewPlus  -0.37     0.112  15   -3.330  0.0120
 New - NewPlus        0.06     0.112  15    0.595  0.8248

P value adjustment: tukey method for comparing a family of 3 estimates
```

Equivalent with lm()

```
lm2<-lm(Yield ~ Variety, data = wheat2)
anova(lm2)
summary(lm2)
```

```
Analysis of Variance Table
 Response: Yield
           Df   Sum Sq  Mean Sq     F value   Pr(>F)
Variety       2  0.68203    0.34101        8.9513    0.002764 **
Residuals 15  0.57145   0.03810
```

```
Coefficients:
                 Estimate  Std. Error    t value     Pr(>|t|)
(Intercept)       2.81857    0.07968     35.372     7.26e-16 ***
VarietyNew        0.44235    0.11269       3.925     0.00135 **
VarietyNewPlus  0.37529    0.11269        3.330     0.00457 **
```

Equivalent with `lm()`

```
emmeans(lm2, pairwise~Variety)
```

```
$emmeans
 Variety    emmean         SE      df    lower.CL upper.CL
 Standard     2.81       0.0796    15       2.64       2.98
 New            3.26        0.0796   15        3.09      3.43
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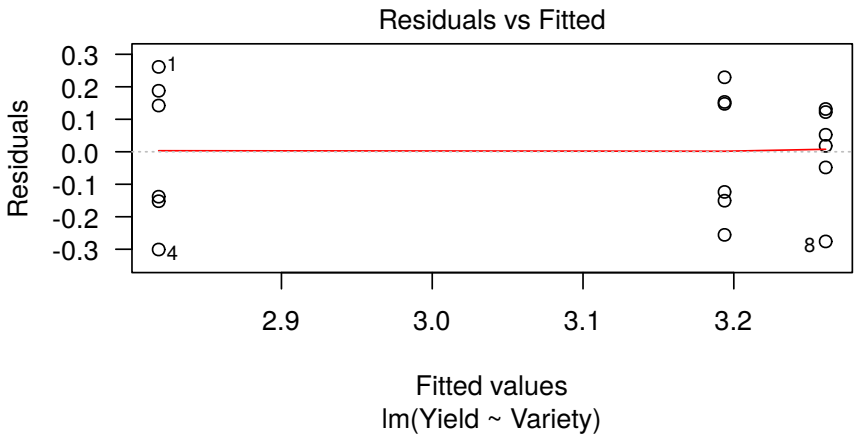
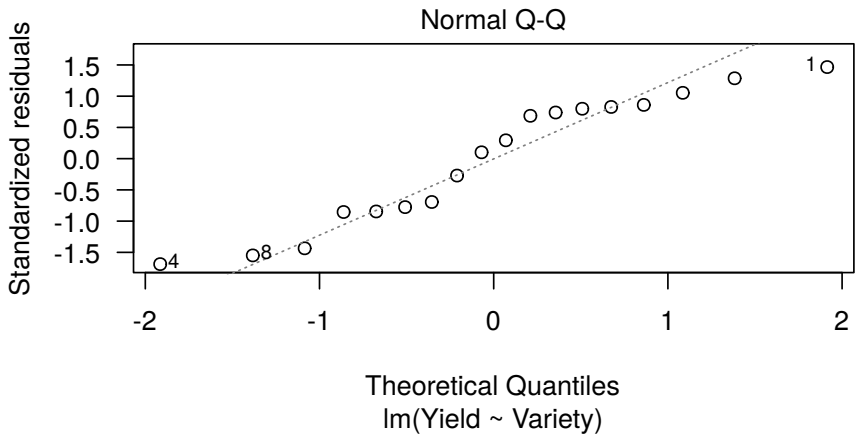
Assessing model assumptions for `lm()`

```
plot(lm2)
```





Summary of results with post hoc comparisons: