Statisitcal Thinking in Biology Research

Chapter 1

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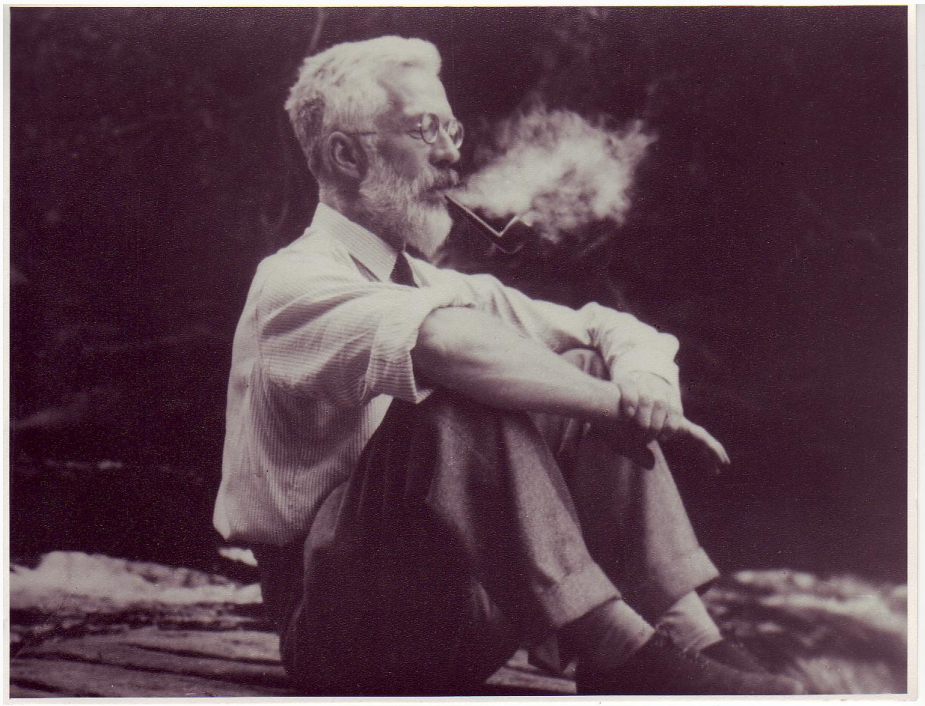
Research School Biology and Biological Data Science Institute

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)

The big picture

To call in the statistician after the experiment is done may be no more than asking him to perform a post-mortem examination: he may be able to say what the experiment died of.

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Statistics is...

1. interesting
2. a unifying language of sciences
3. empowering

Approximate plan

Monday morning	Cautionary tales, General approach to modelling
Monday afternoon	Experimental design
Tuesday morning	Mean structure, *additive effects and interactions*
Tuesday afternoon	Variance structure, *mixed models*
Wednesday	Data generating process, *GLMs*; + practice with your data?

If you want, send me your data with a brief explanation of they are and what you want to do

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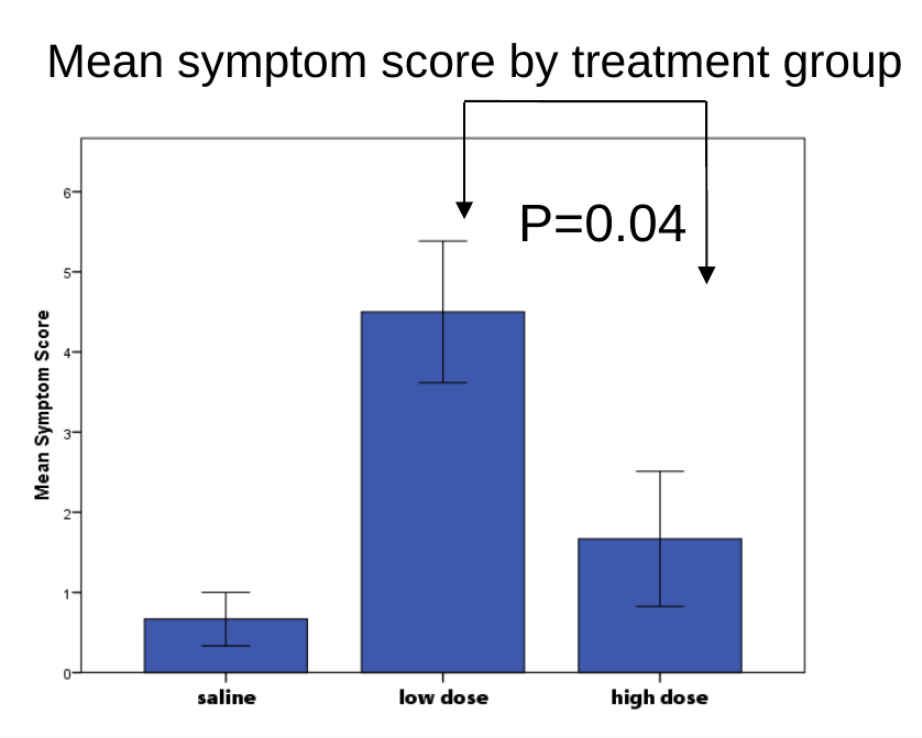
- Statistics in biology = study biological variation
- Undestanding statistical ideas about biological variation:
    - Informs the design of experiments
    - Informs the analysis of experiments
- Statistical thinking is an essential component of scientific thinking

Cautionary tales from the front

Introduction to Statistical Modelling

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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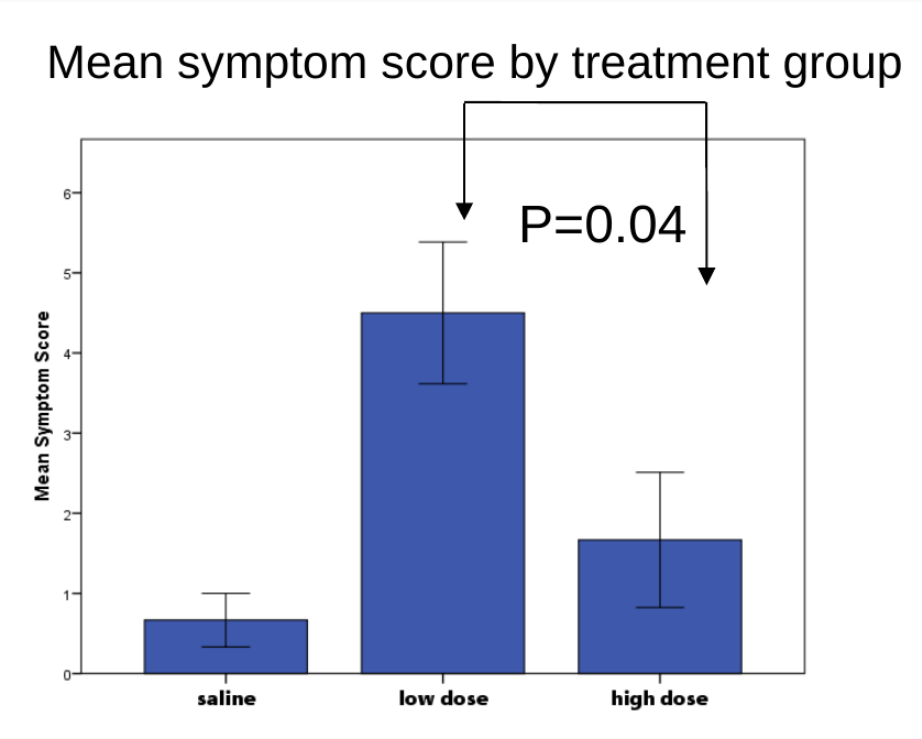

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Do you think the vaccine works? What is strange?

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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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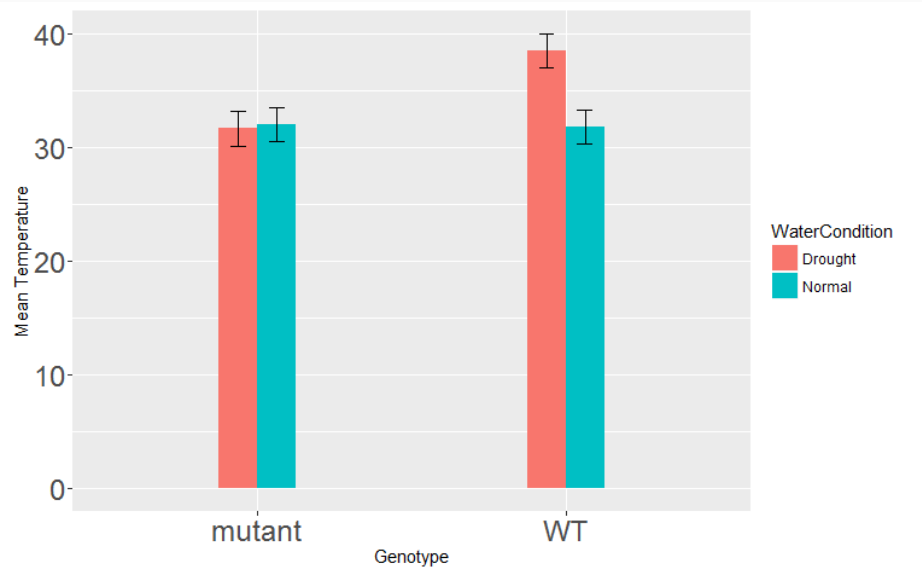

Are temperature mechanisms modified in a genetically modified tomato plant?

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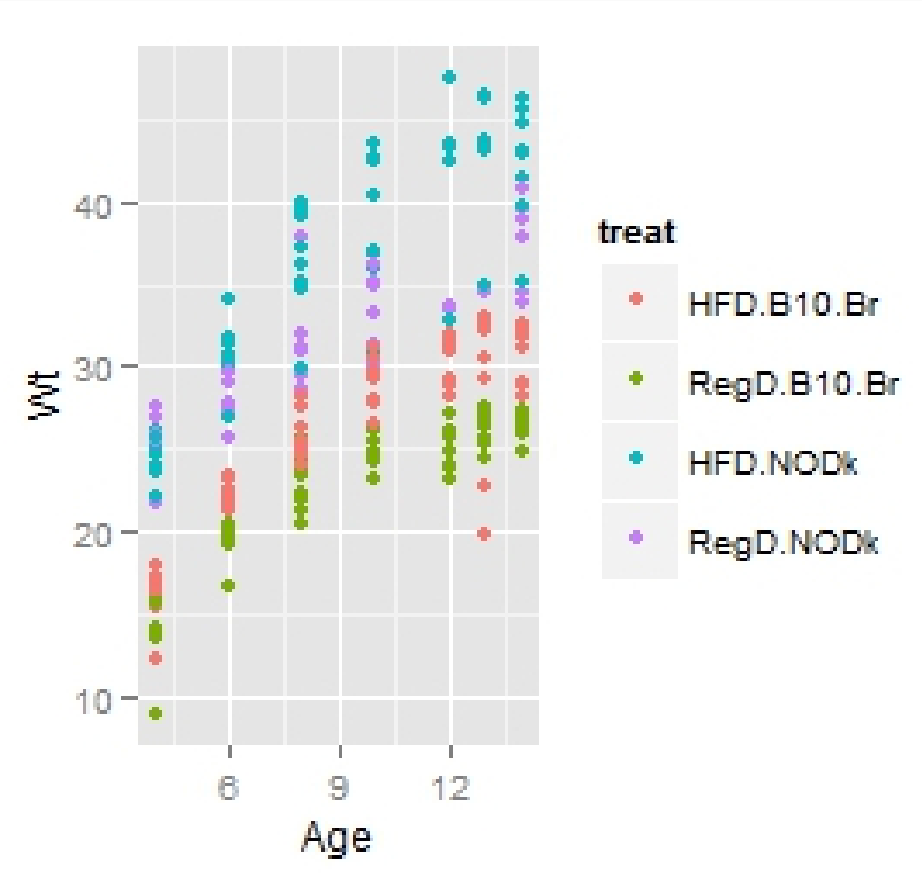
Research question: Are mice susceptible to obesity when exposed to a high fat diet?



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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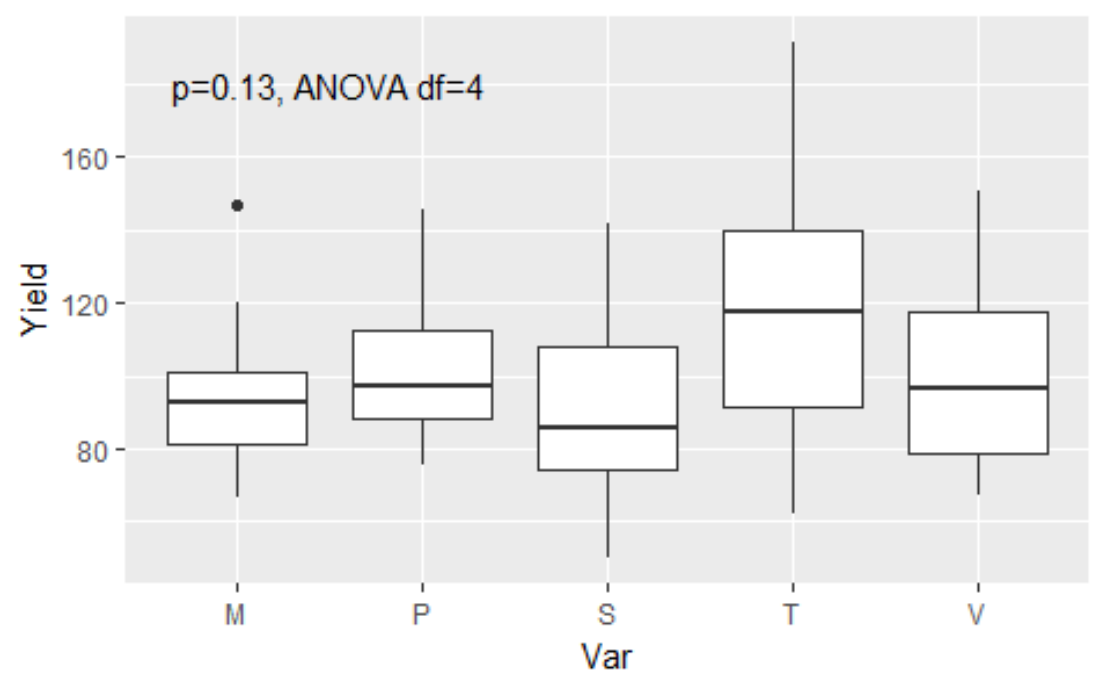
Comparing yield in five barley varieties (1930s)

Experimental factors: 5 varieties of barley, 6 locations, 2 time points. Outcome measure: yield



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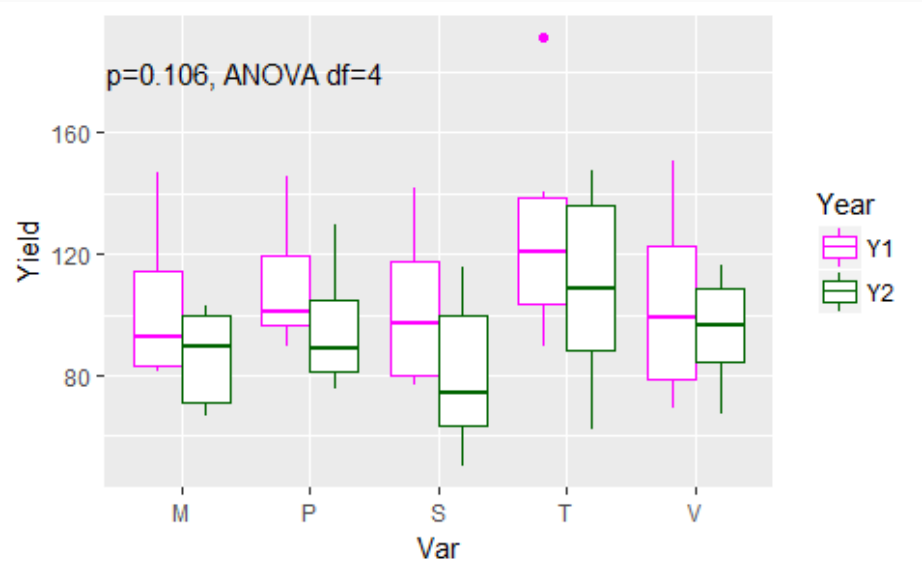

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- Controlling for year = comparing yield WITHIN years and combining these

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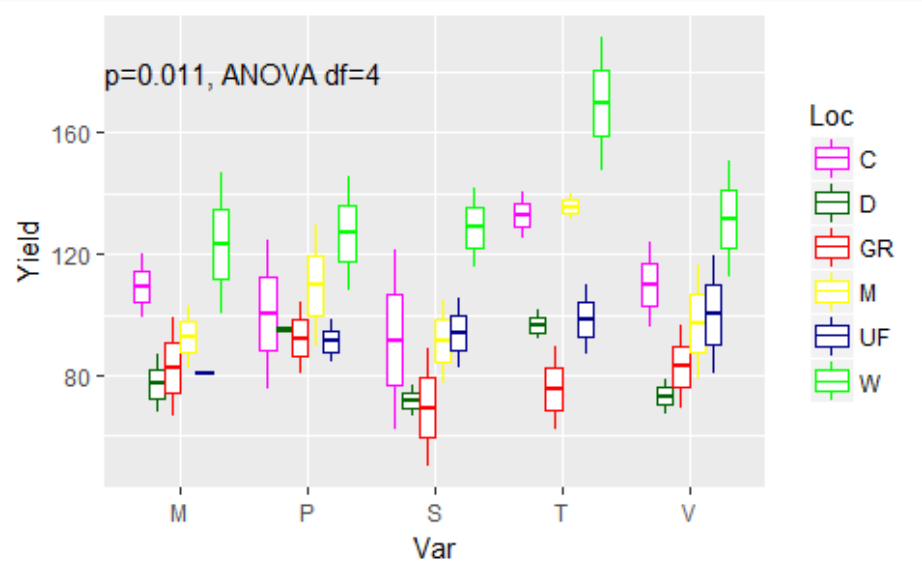

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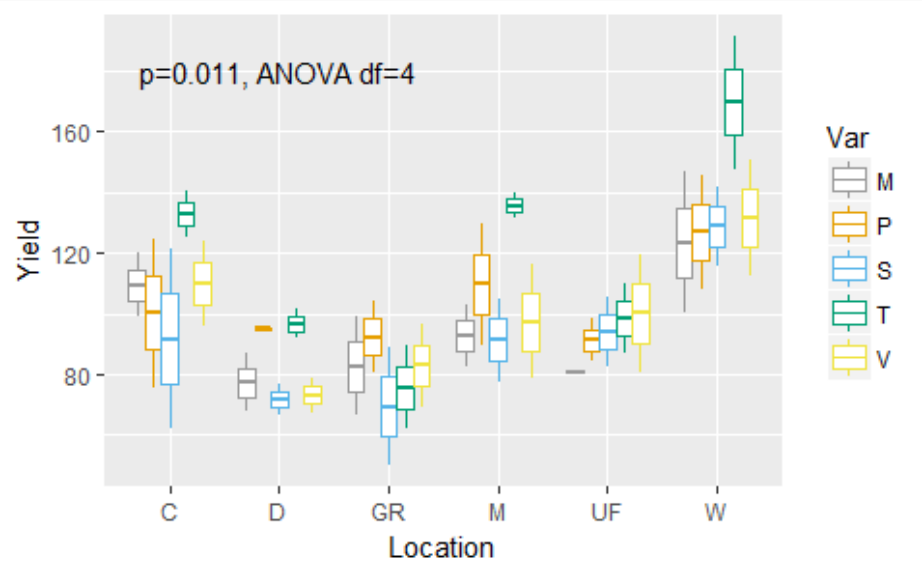

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Research question: How does cold duration impact upon germination in alpine plant *A. glacialis*?

Experimental set-up:

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

What factors other than temperature to consider?

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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.**

Cautionary tales from the front

Introduction to Statistical Modelling

Another look at essential steps

Introduction to Statistical Modelling

- What is a statistical model?
- What models can do:
    - summary of data
    - predictions
    - correlation/causal inference
- 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
  - 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

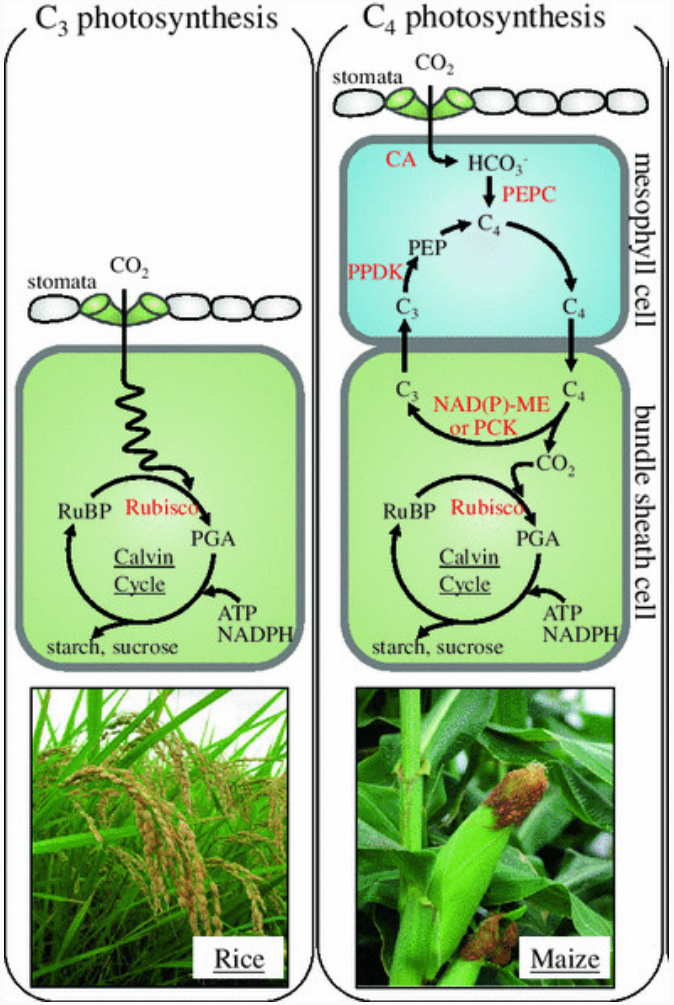
- To compare the mean response of an organism/system to a set of different experimental conditions.
    - Obtain estimate of "Treatment effect"
    - Is this "effect" different in subgroups of interest?
- What are the most important factors influencing the mean response?
- Subsidiary question: how can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

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

A and D are the model PARAMETERS.

We want to infer whether D is different from 0

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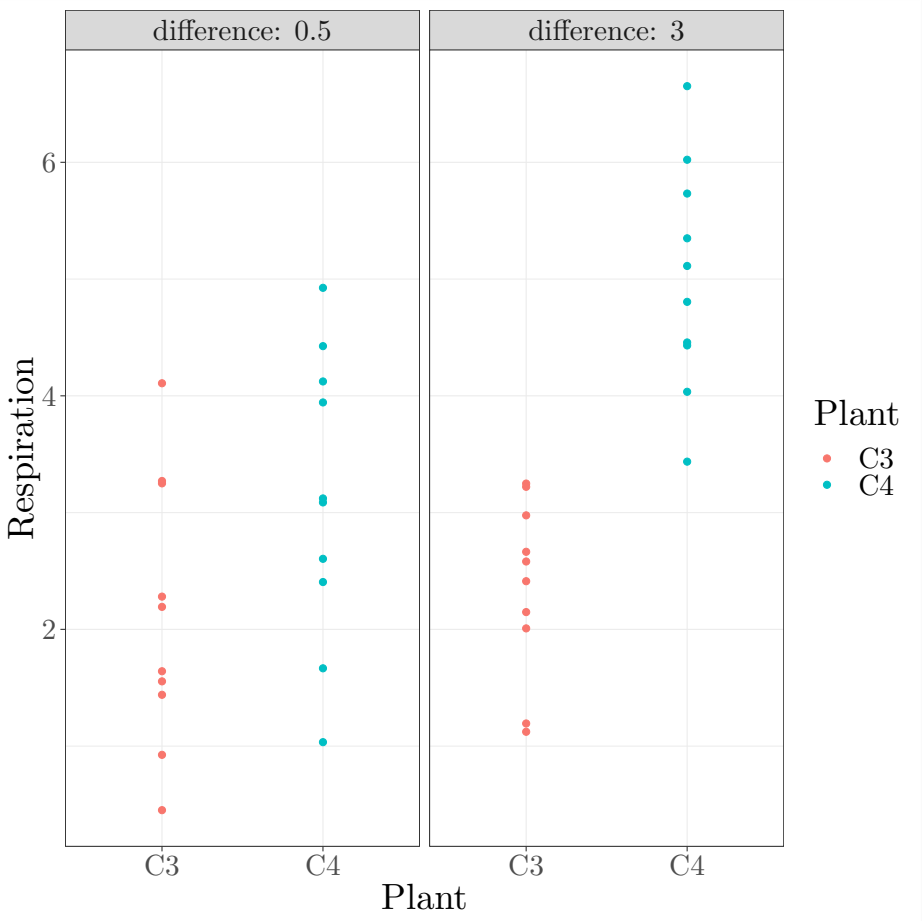
When can we know whether $D \neq 0$?



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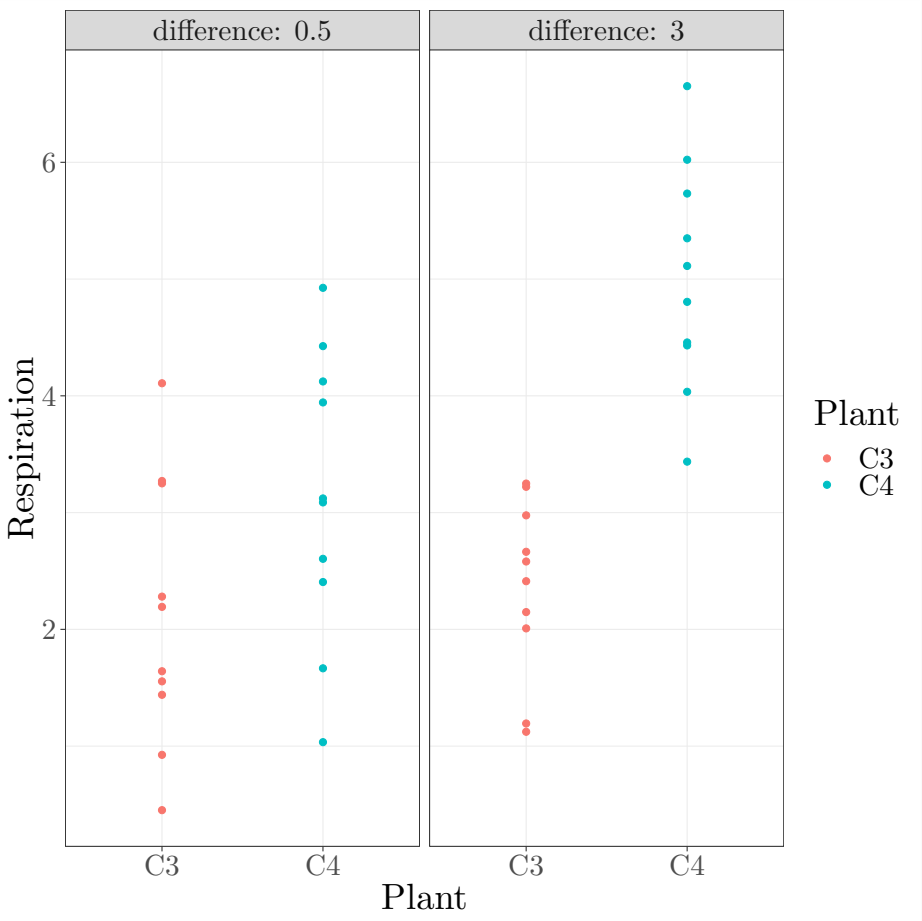

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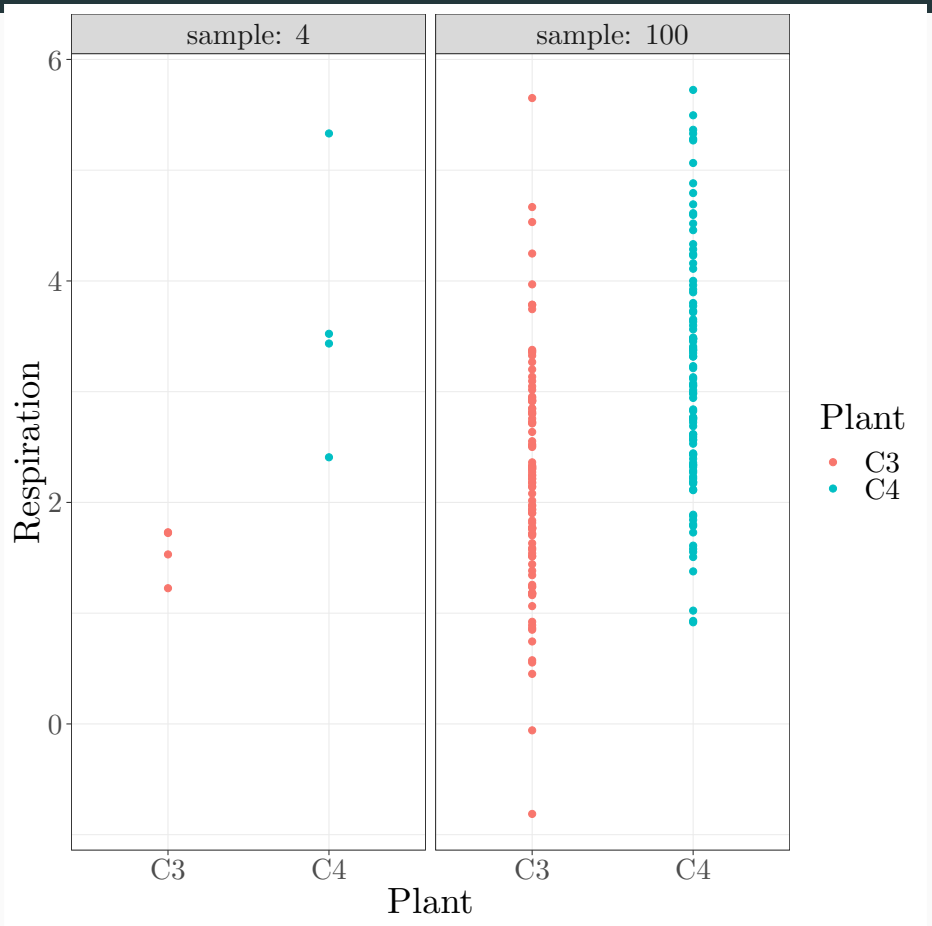

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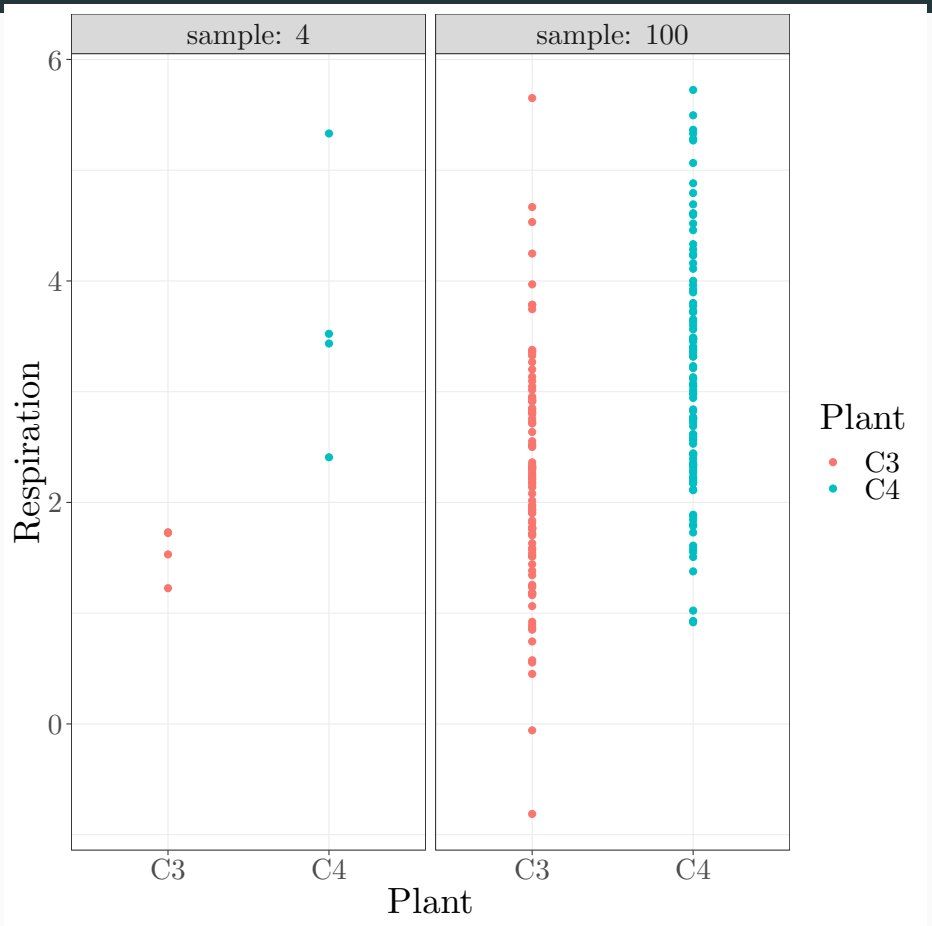

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2. Large sample size

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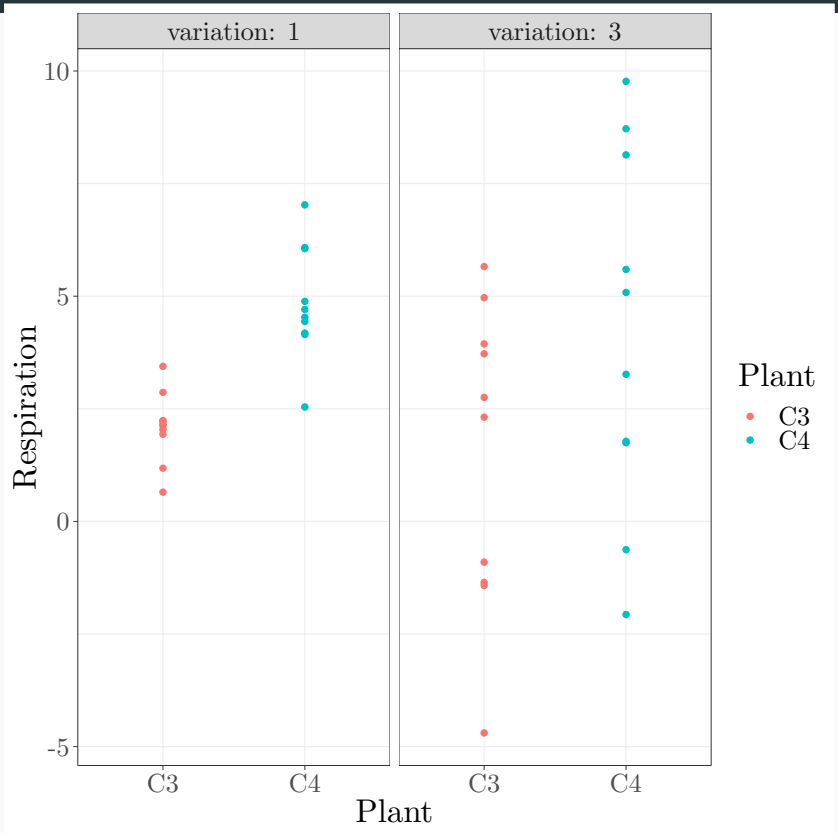

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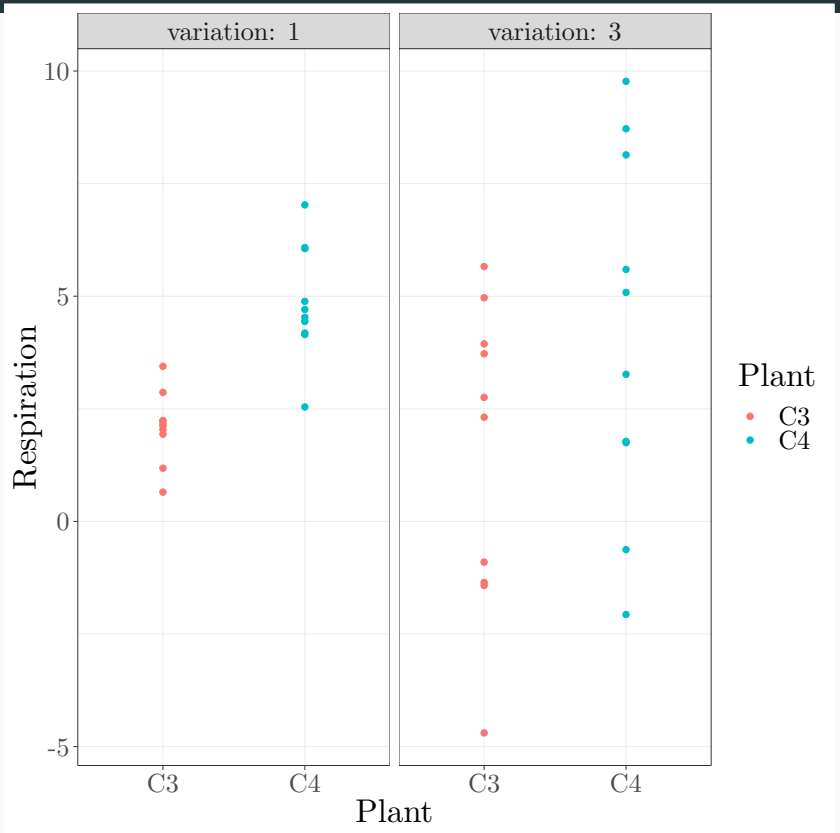

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What makes t large:

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

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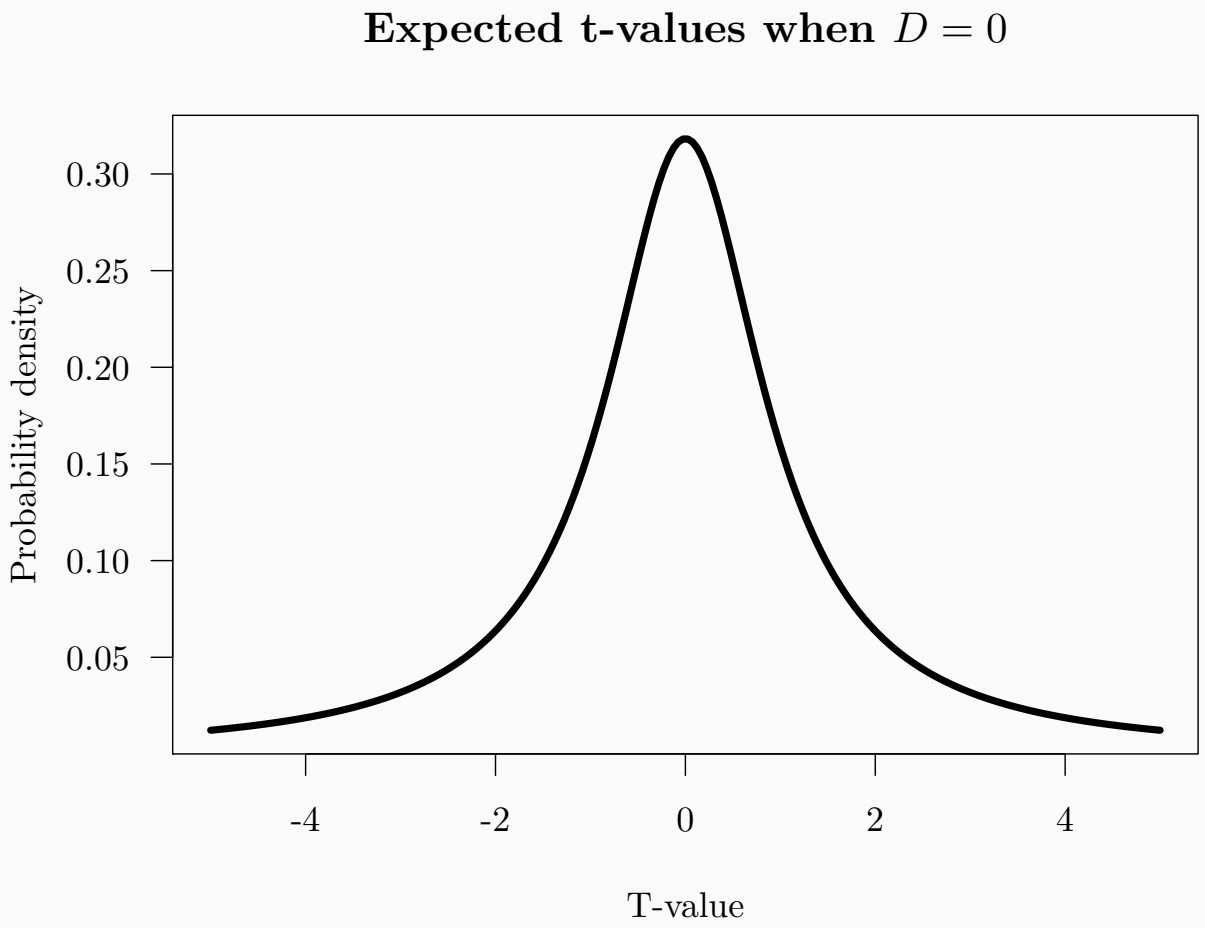
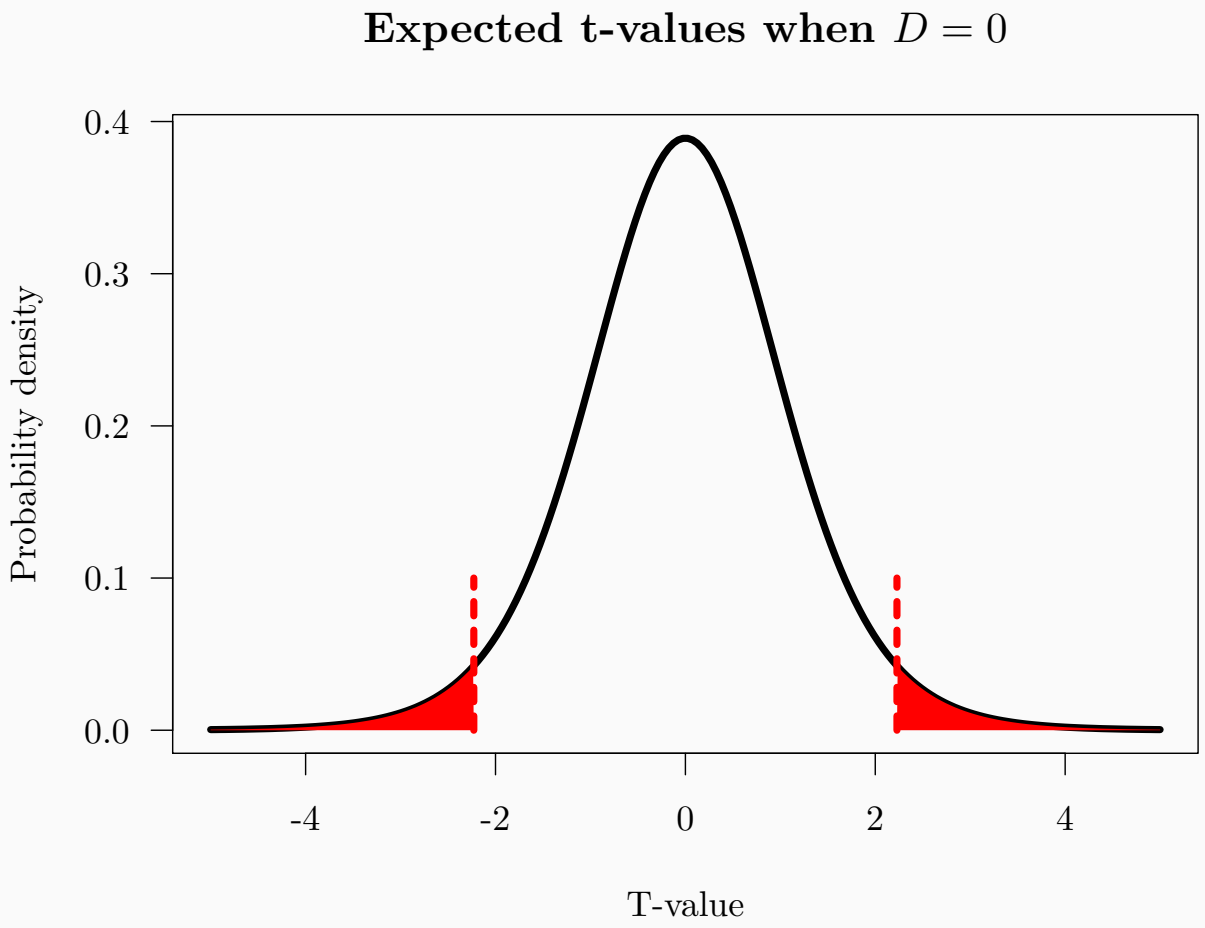


When can we know whether $D \neq 0$?

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



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Candy practical

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- And so on, until 5 candies. You wonder if you have been cheated.

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- Are there more Halloween than Fruit candies in that pack?

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- You pick a second one. Again a disgusting Halloween candy! You put it back
- And so on, until 5 candies. You wonder if you have been cheated.
- Are there more Halloween than Fruit candies in that pack?
- You decide to use statistics to find out

How to?

- Draw 5 candies out of the pack
- Write down how many Halloween candies
- How often is it 5?

But really, what is a p-value?

https://docs.google.com/spreadsheets/d/1Y9512z1xxkphjAZ_dYT9SqfQH02UqTDKlW2X0mDEwZY/edit?usp=sharing

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- Estimate the p-value for the test "candies have same frequency"

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- Redo the experiment in R, using random sampling (rbinom)

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- Redo the experiment in R, using random sampling (rbinom)
- What is the correct null-distribution?

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

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

Compare C3 and C4 plants in "Low Variation" subset

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

Fit an anova in R: aov()

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

Fit an anova in R: aov()

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

```
            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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```

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

Fit a linear model in R: `lm()`

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

Fit a linear model in R: `lm()`

```
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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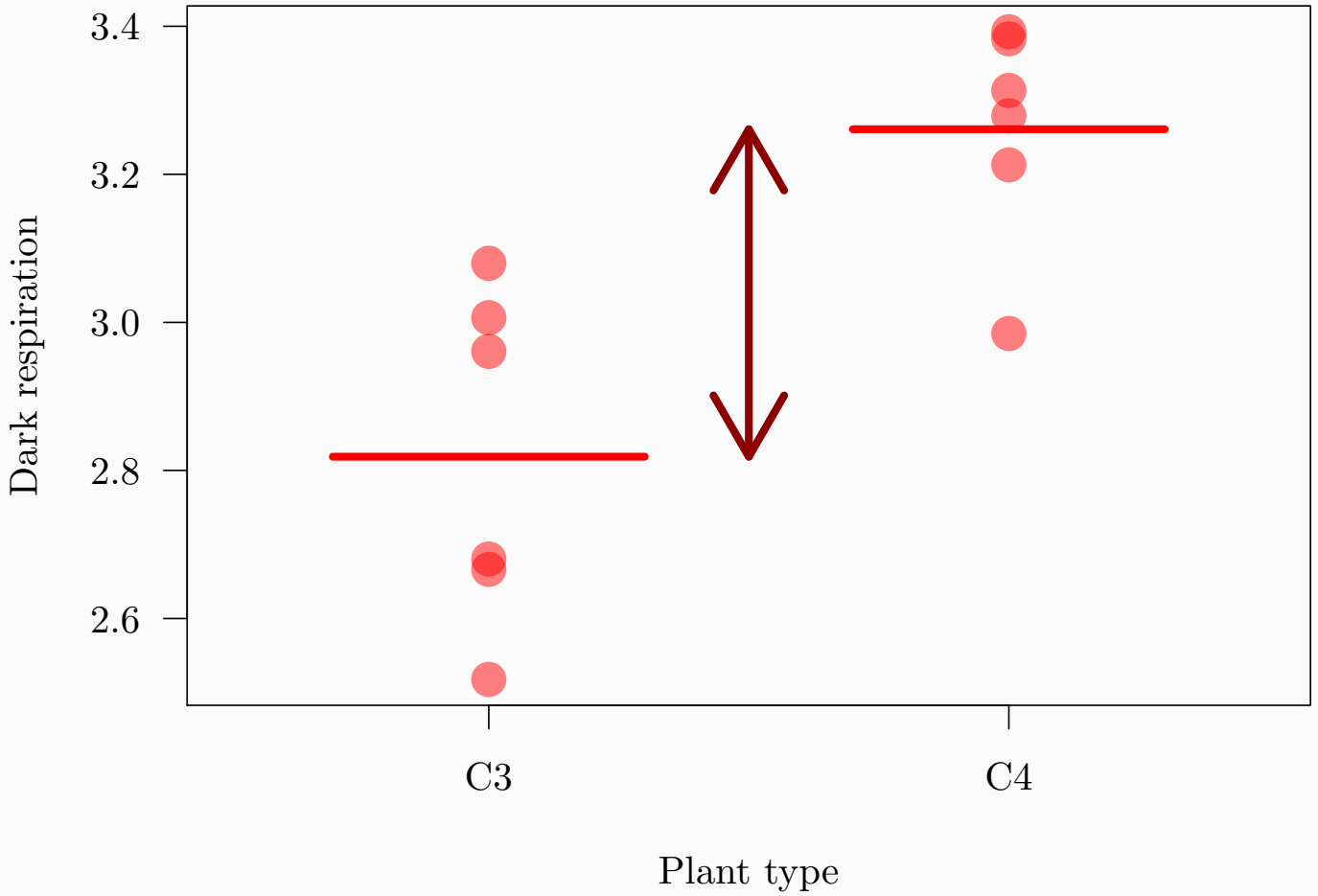
Confidence level used: 0.95
```

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

Compare the output from t.test, aov and lm

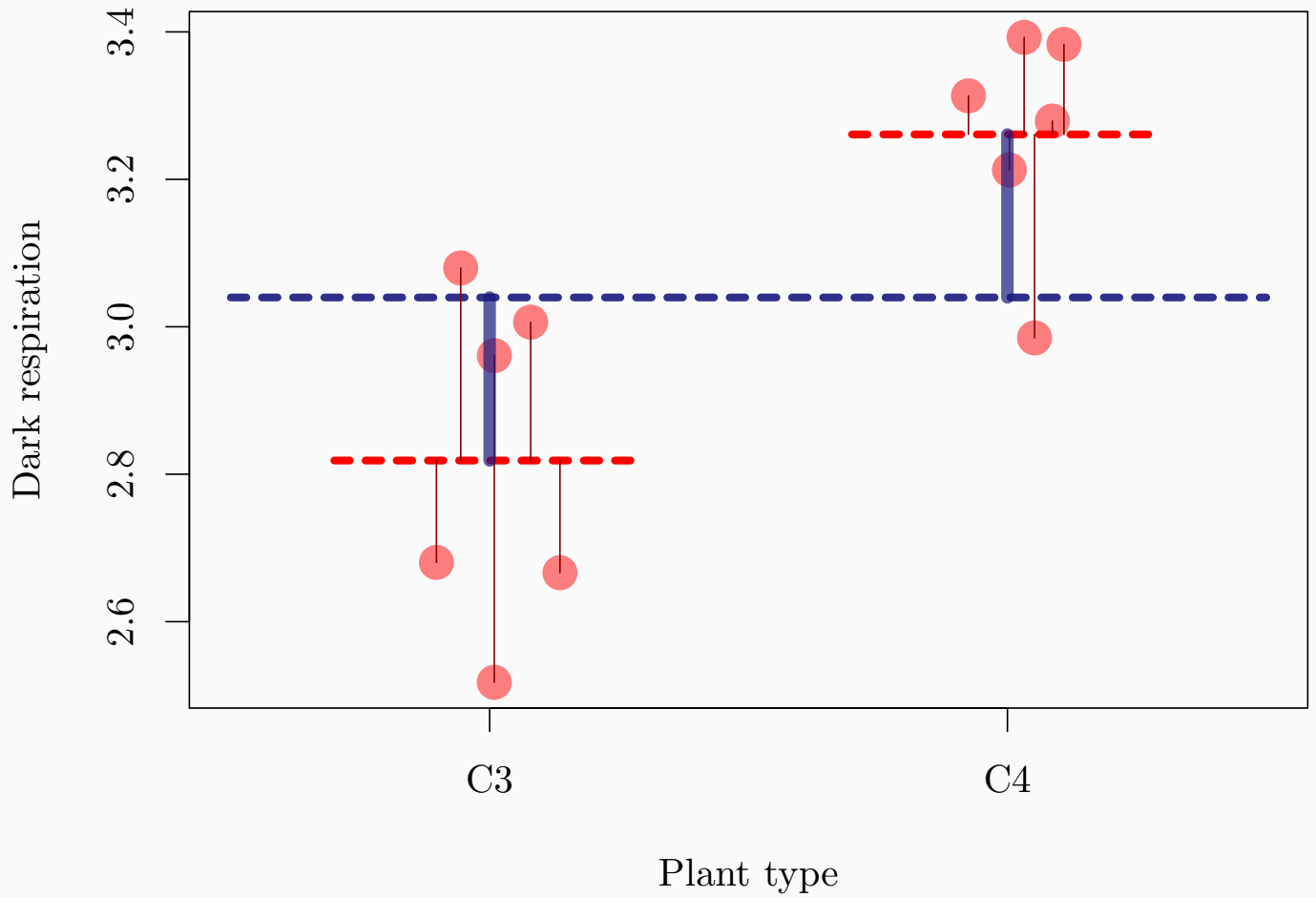
Three equivalent ways to look at data

T-test, focus on difference between two means



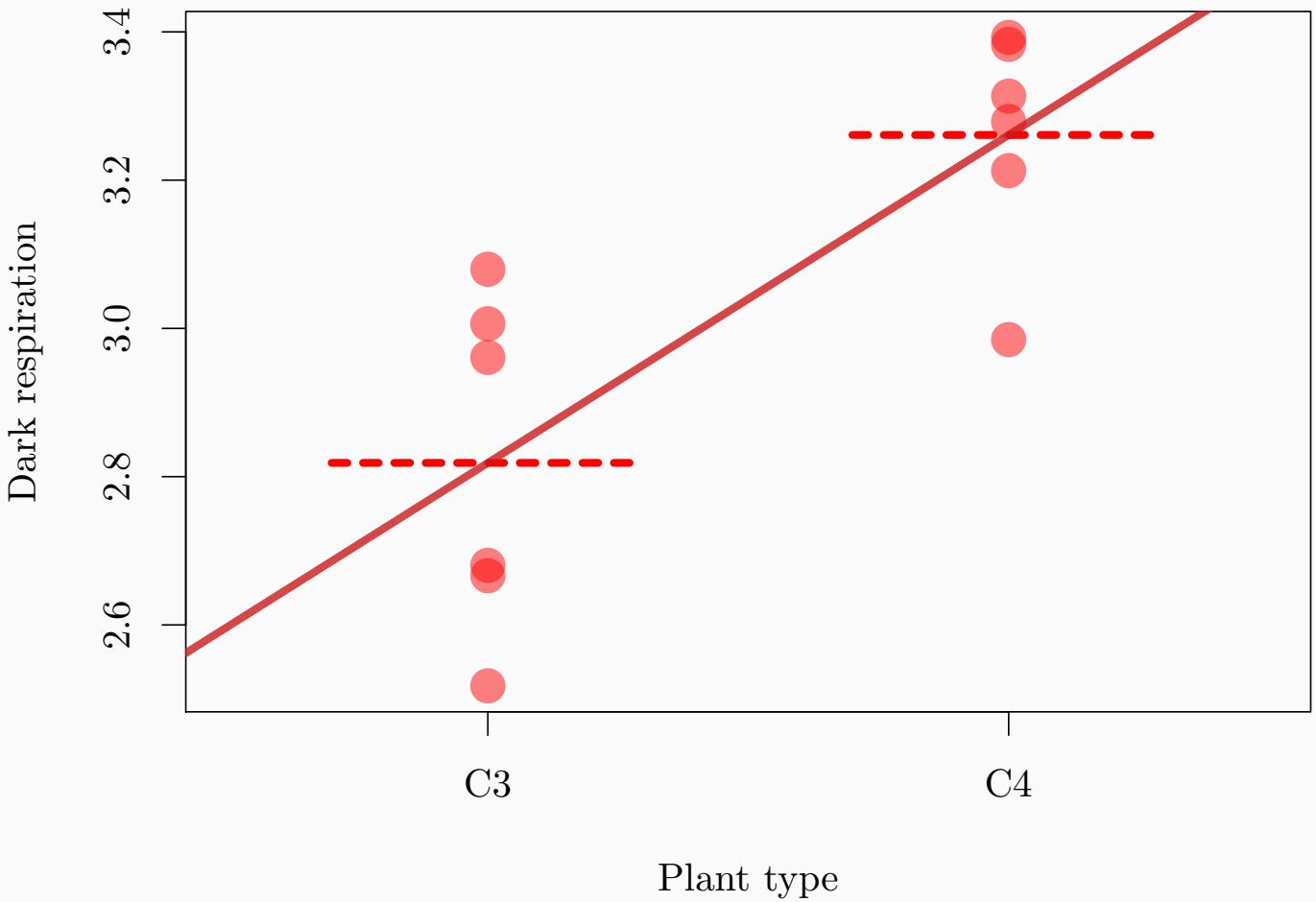
Three equivalent ways to look at data

ANOVA, focus on variation within VS. between



Three equivalent ways to look at data

Linear regression, focus on rate of change



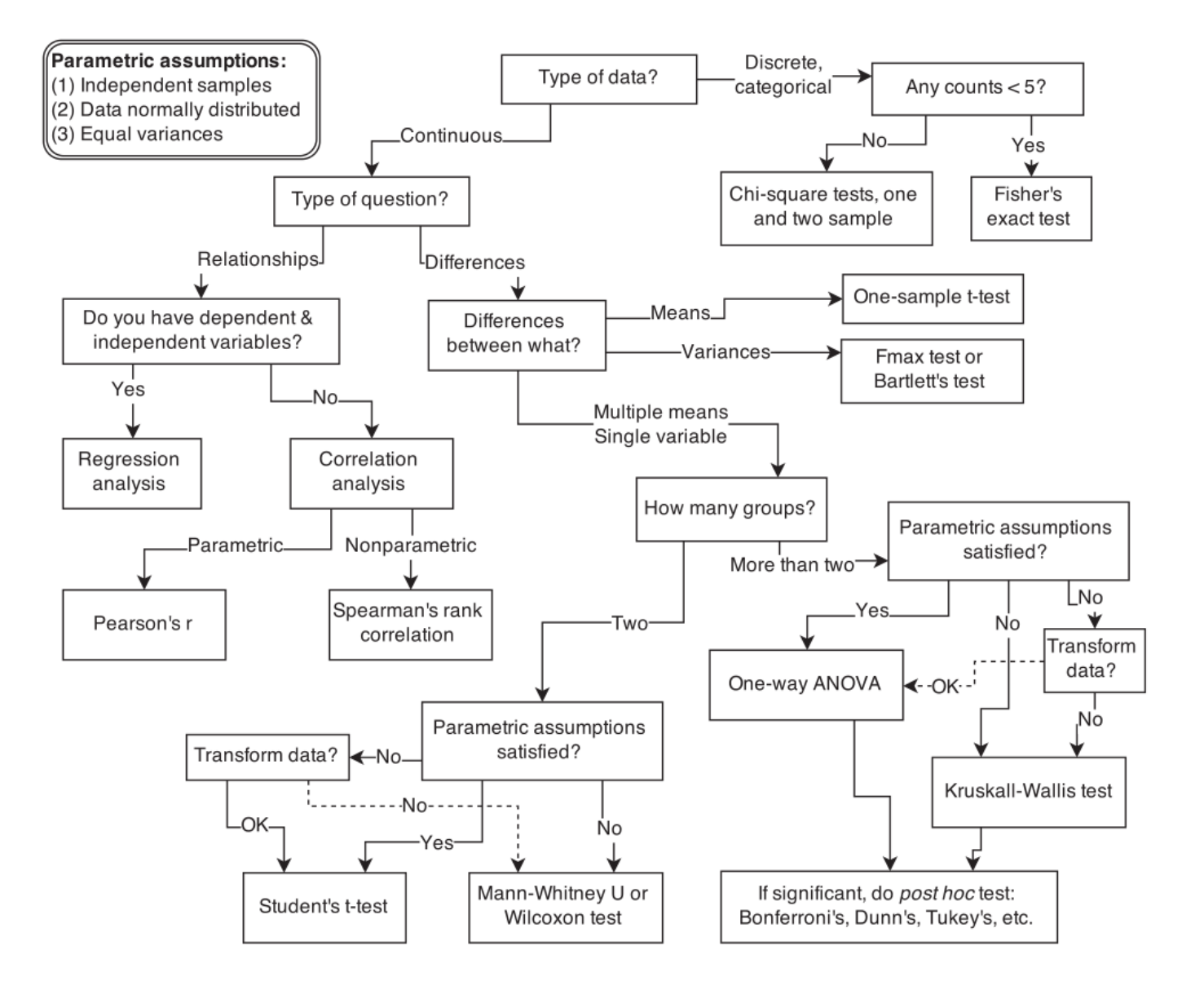
All is one. . .

All is one...

...but `lm()` rules (IMHO)

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

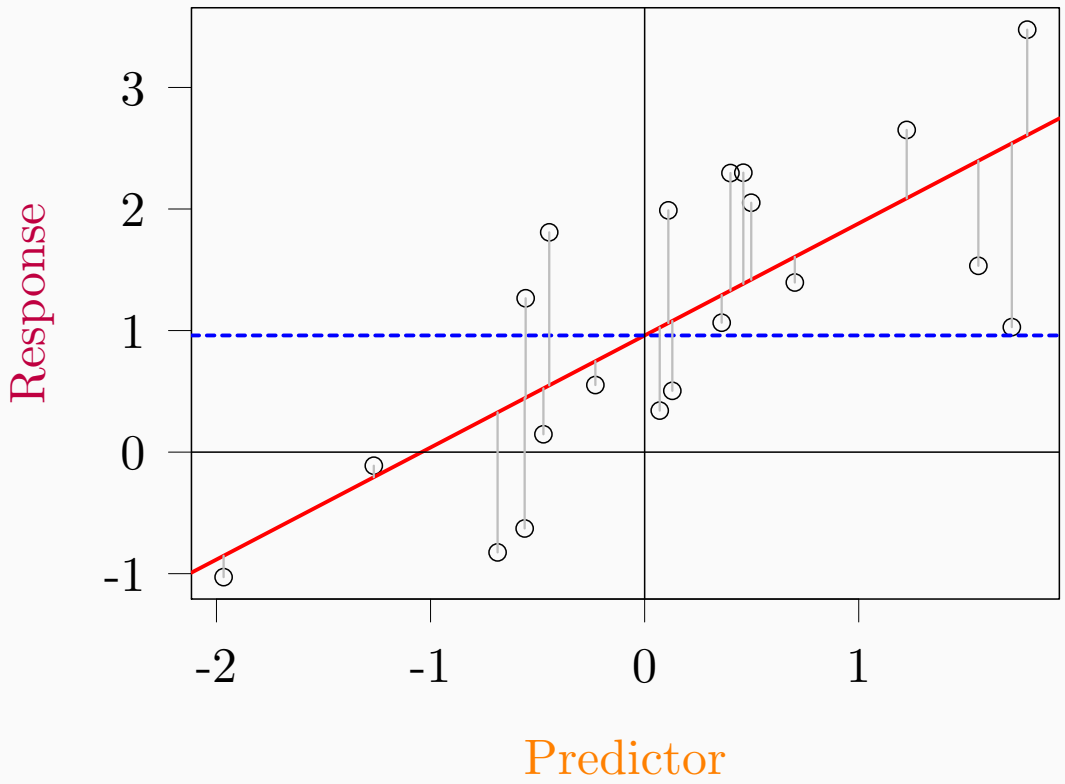
All is one. . .



ALL can be done as linear models

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

Cautionary tales from the front

Introduction to Statistical Modelling

Another look at essential steps

General approach

1. Scientific question

General approach

1. Scientific question

↓

2. Model and Statistical question

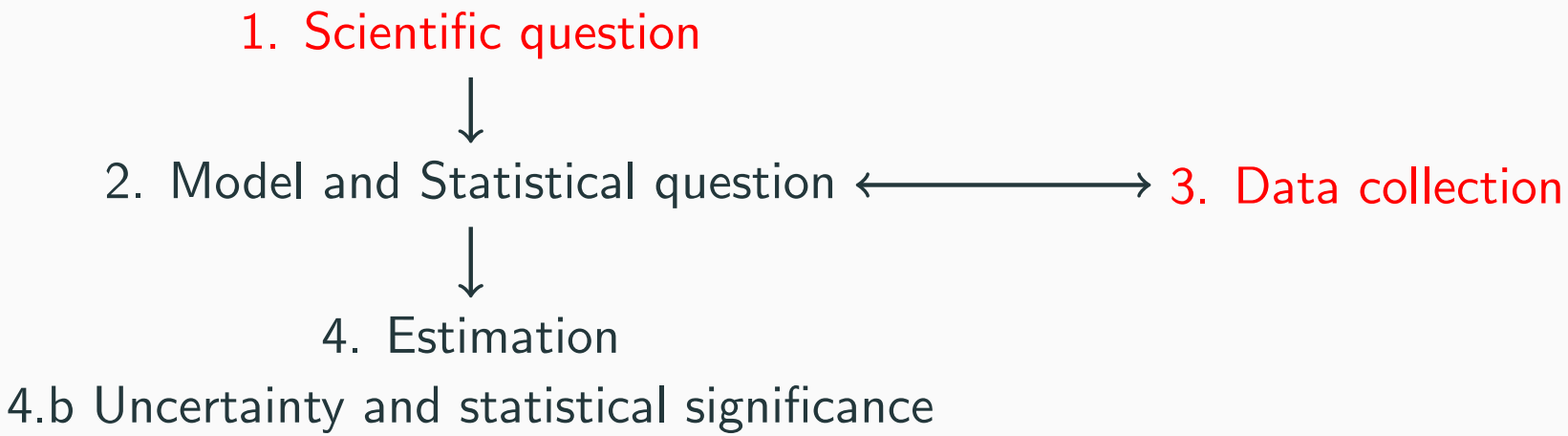
General approach

1. Scientific question

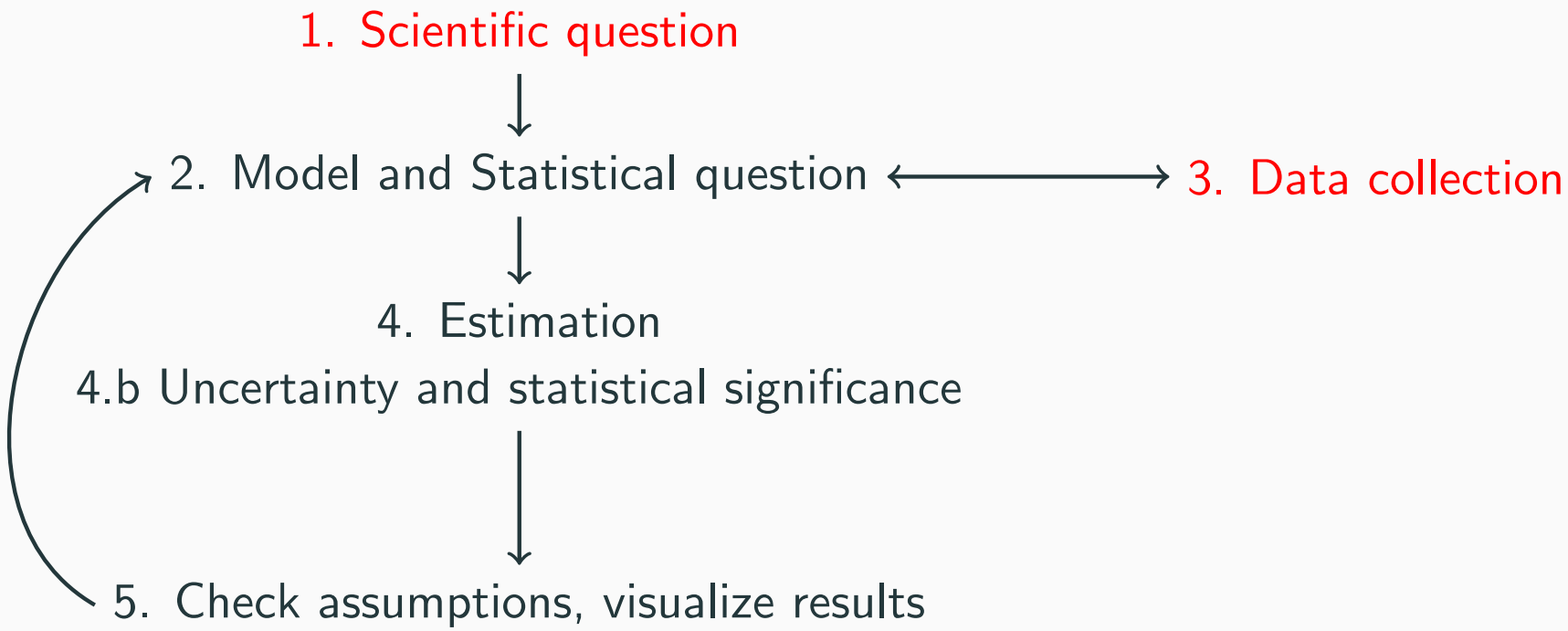
↓

2. Model and Statistical question ⟷ 3. Data collection

General approach



General approach



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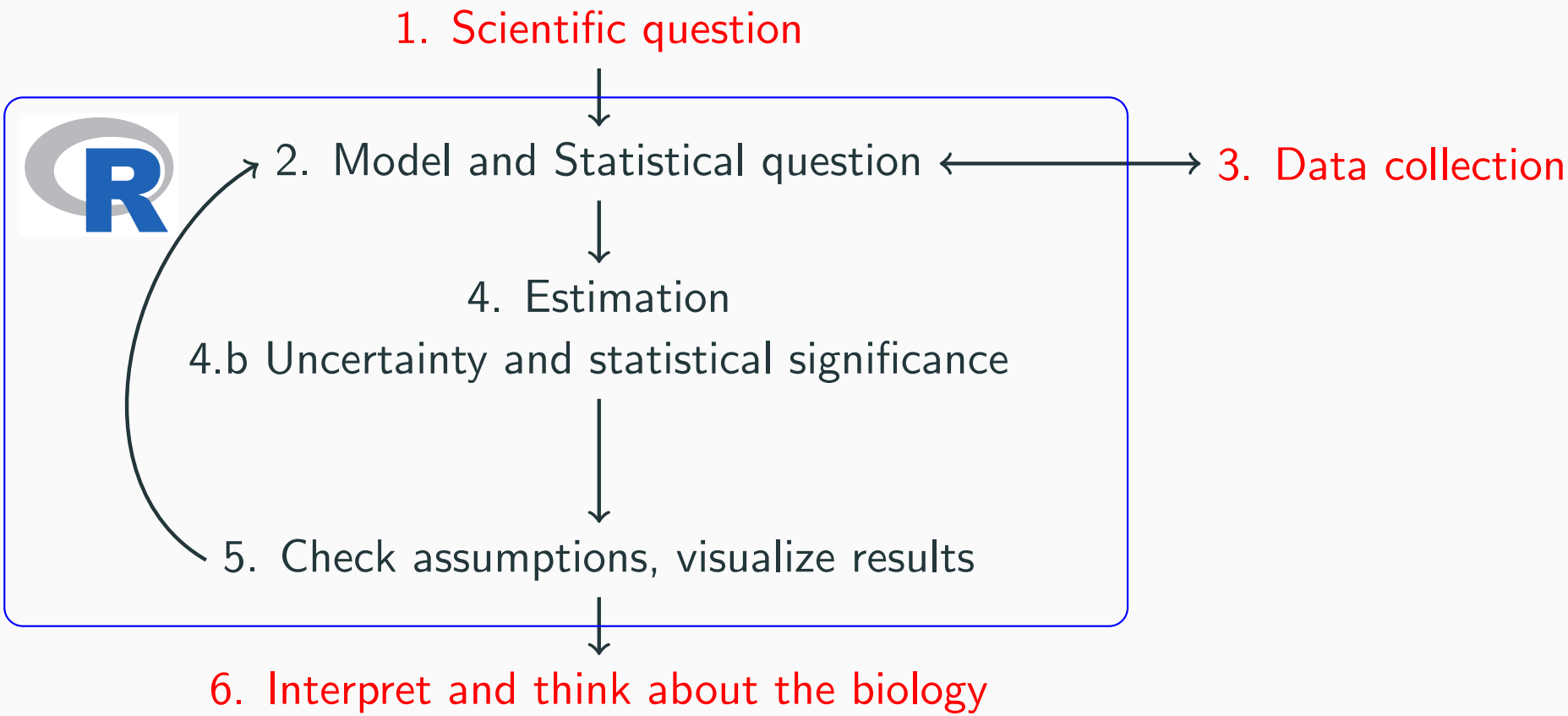

Back to C3/C4

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summary(lmL)
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             Estimate Std. Error t value Pr(>|t|)
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...
```

Estimation:

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

$A =?$, $D =?$

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$A = 2.81857$, $D = 0.44235$

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For D SE= 0.11110 ; p-value=0.00259

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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: Over-optimistic uncertainty, unreliable predictions

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- Gaussian error distribution
  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)
```

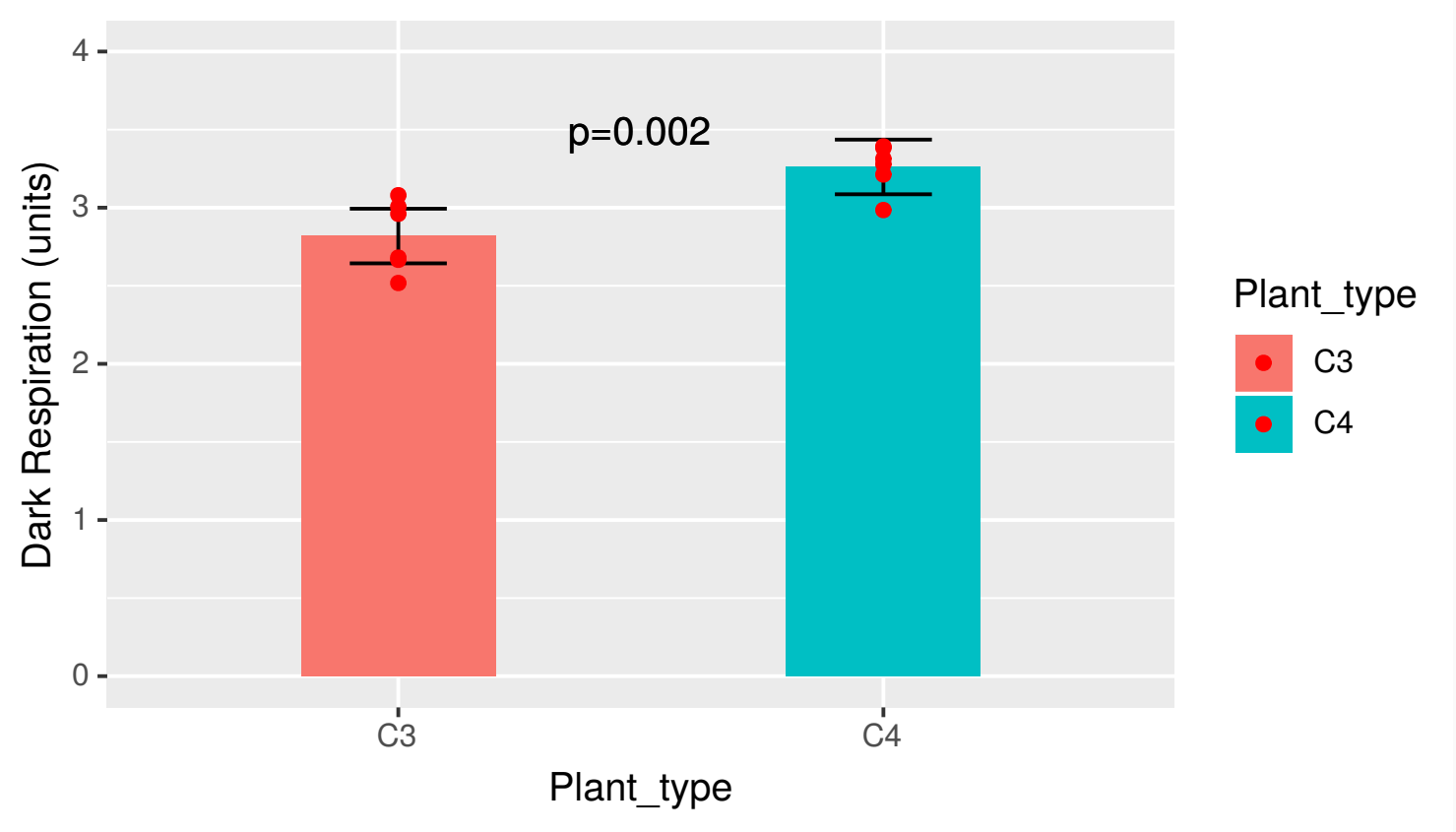
Check assumptions, visualize results

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

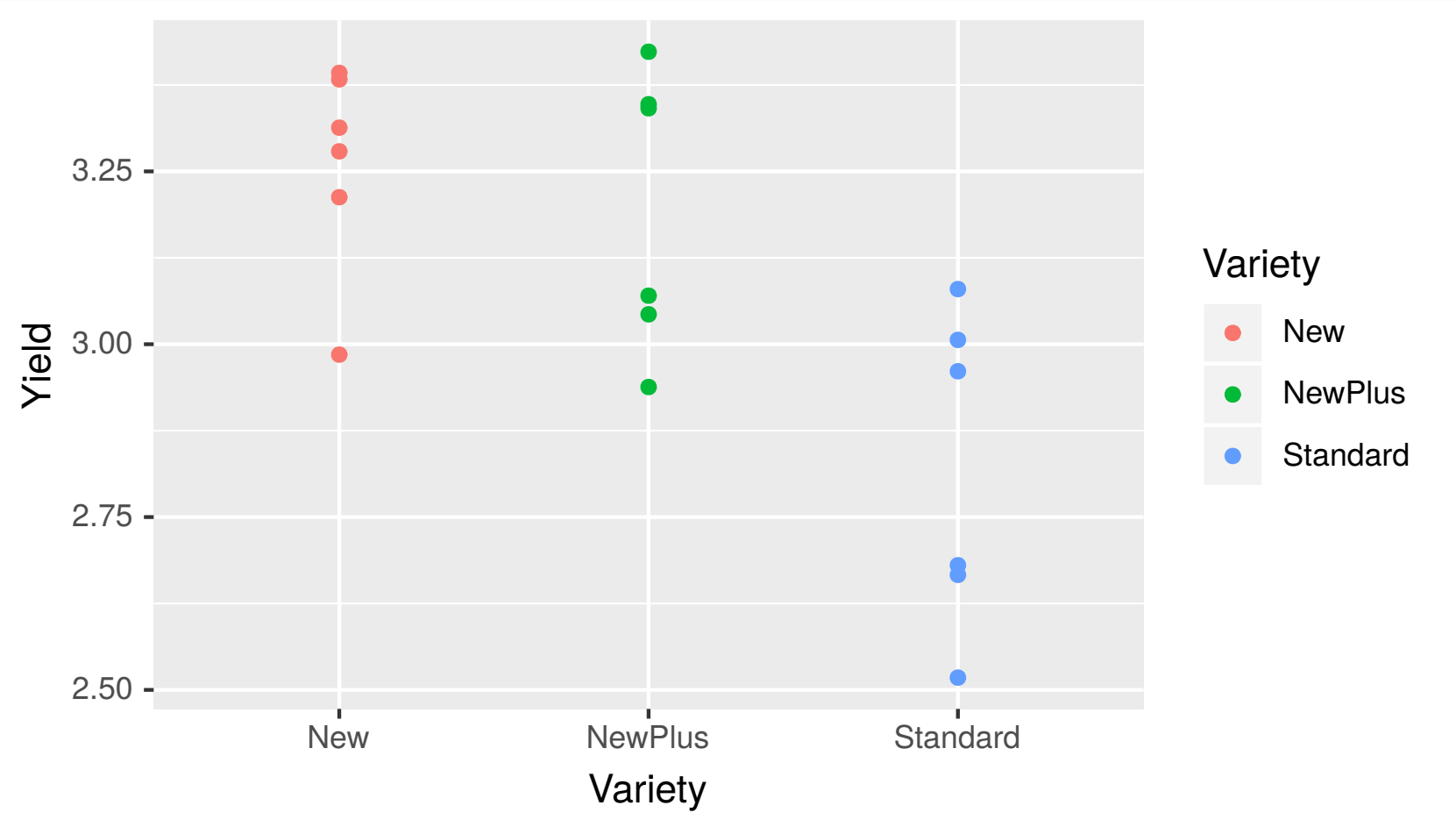
Compare wheat yields between 3 varieties

- 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
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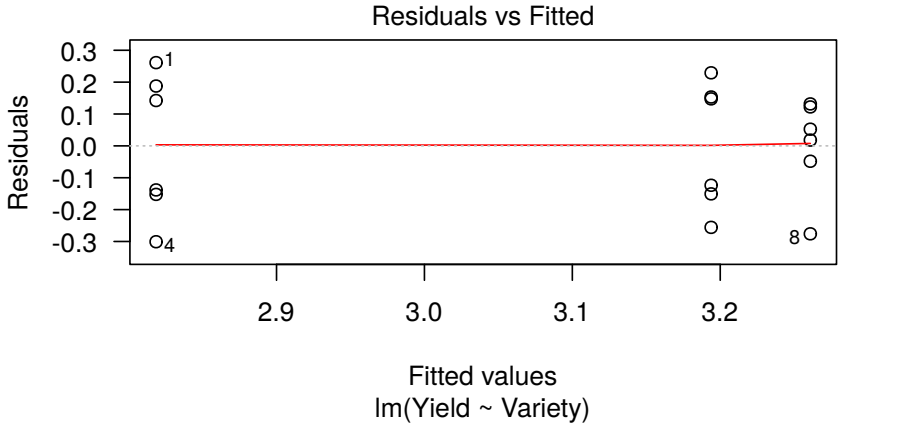
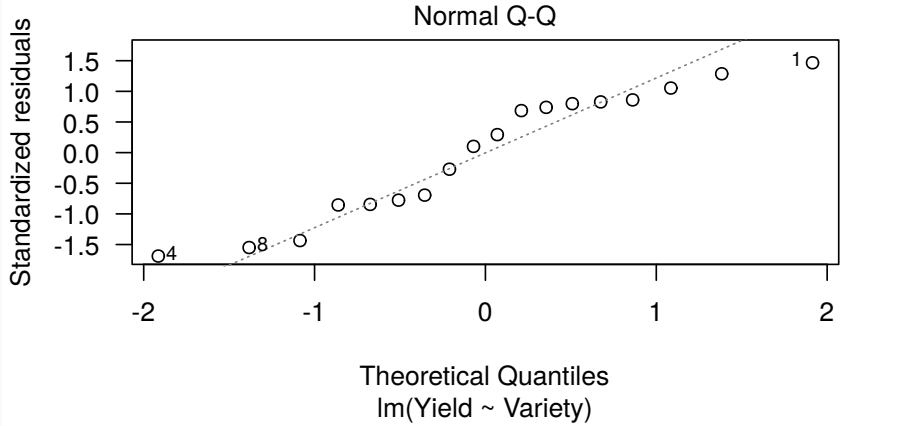
Assessing model assumptions for `lm()`

```
plot(lm2)
```





Summary of results with post hoc comparisons: