@CSoneson

Principles of differential expression analysis

Charlotte Soneson

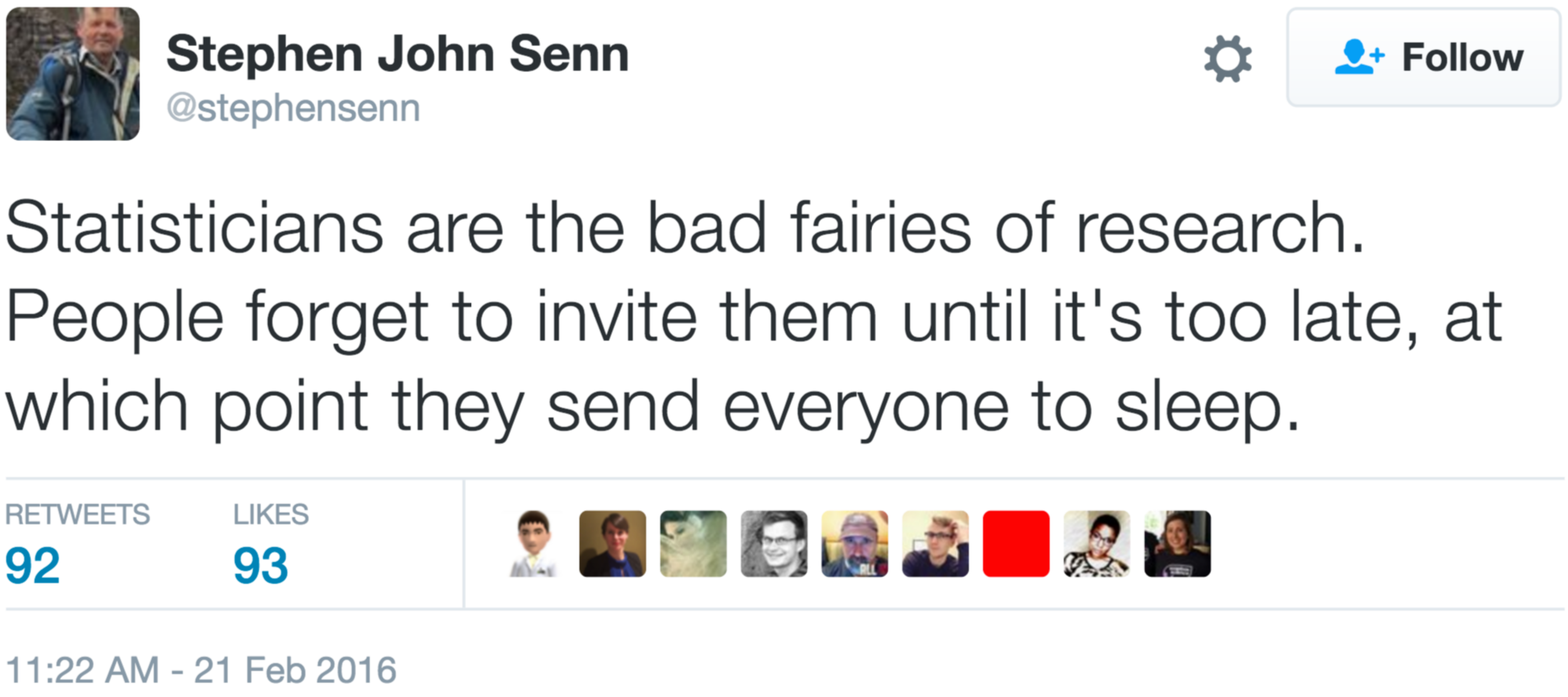
Friedrich Miescher Institute for Biomedical Research &
SIB Swiss Institute of Bioinformatics

SIB Swiss Institute of Bioinformatics

FMI Friedrich Miescher Institute for Biomedical Research

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

Sir Ronald Fisher, Indian Statistical Congress, Sankhya, around 1938



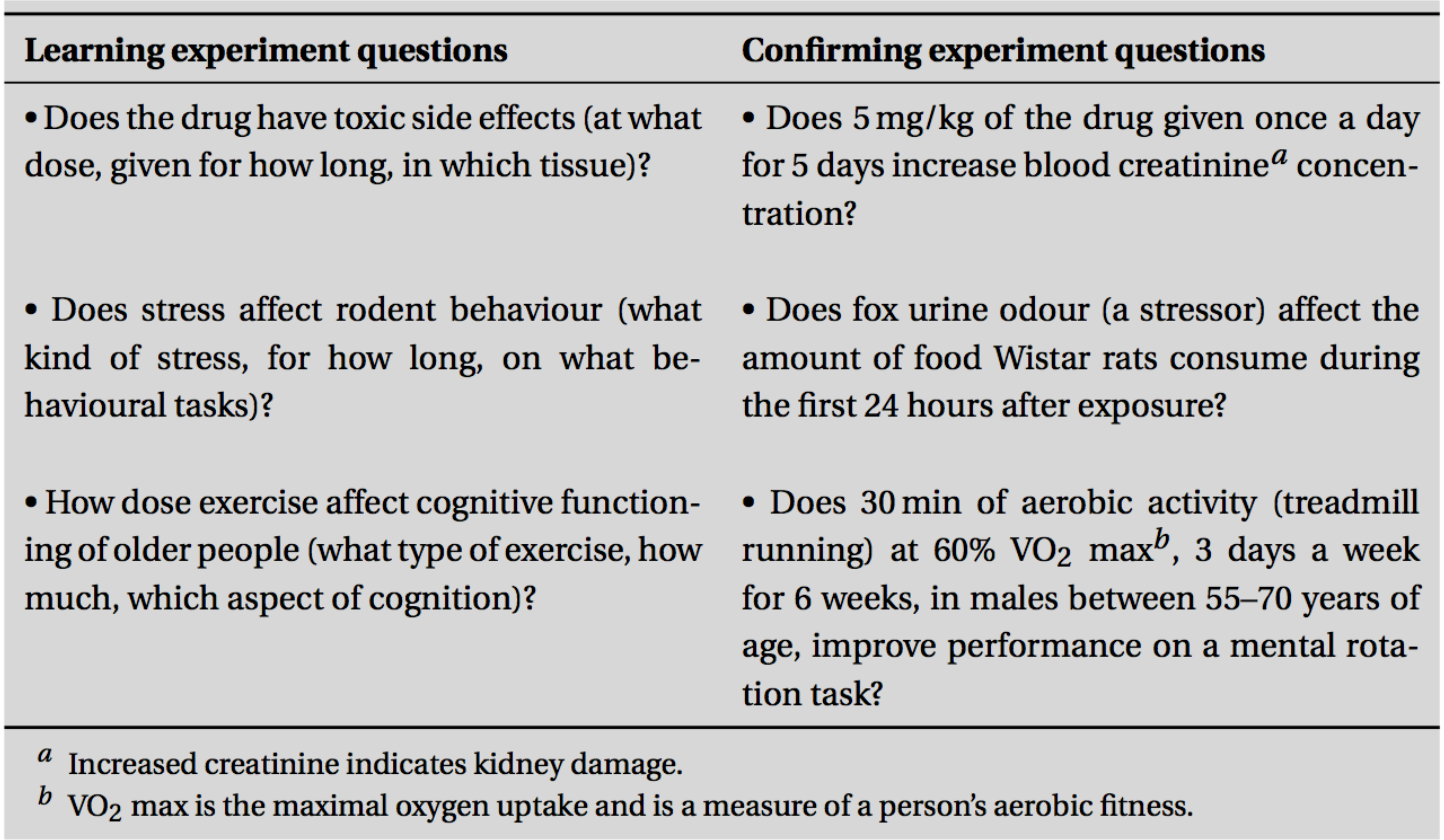
Stephen John Senn
@stephensenn

Statisticians are the bad fairies of research. People forget to invite them until it's too late, at which point they send everyone to sleep.

RETWEETS 92
LIKES 93

11:22 AM - 21 Feb 2016

Different types of experiments



Learning experiment questions	Confirming experiment questions
• Does the drug have toxic side effects (at what dose, given for how long, in which tissue)?	• Does 5 mg/kg of the drug given once a day for 5 days increase blood creatinine[a] concentration?
• Does stress affect rodent behaviour (what kind of stress, for how long, on what behavioural tasks)?	• Does fox urine odour (a stressor) affect the amount of food Wistar rats consume during the first 24 hours after exposure?
• How dose exercise affect cognitive functioning of older people (what type of exercise, how much, which aspect of cognition)?	• Does 30 min of aerobic activity (treadmill running) at 60% VO_2 max[b], 3 days a week for 6 weeks, in males between 55–70 years of age, improve performance on a mental rotation task?

[a] Increased creatinine indicates kidney damage.

[b] VO_2 max is the maximal oxygen uptake and is a measure of a person's aerobic fitness.

[Lazic, 2016]

What is experimental design?

The organization of an experiment, to ensure that the **right type** of data, and **enough** of it, is available to answer the **questions of interest** as clearly and efficiently as possible.

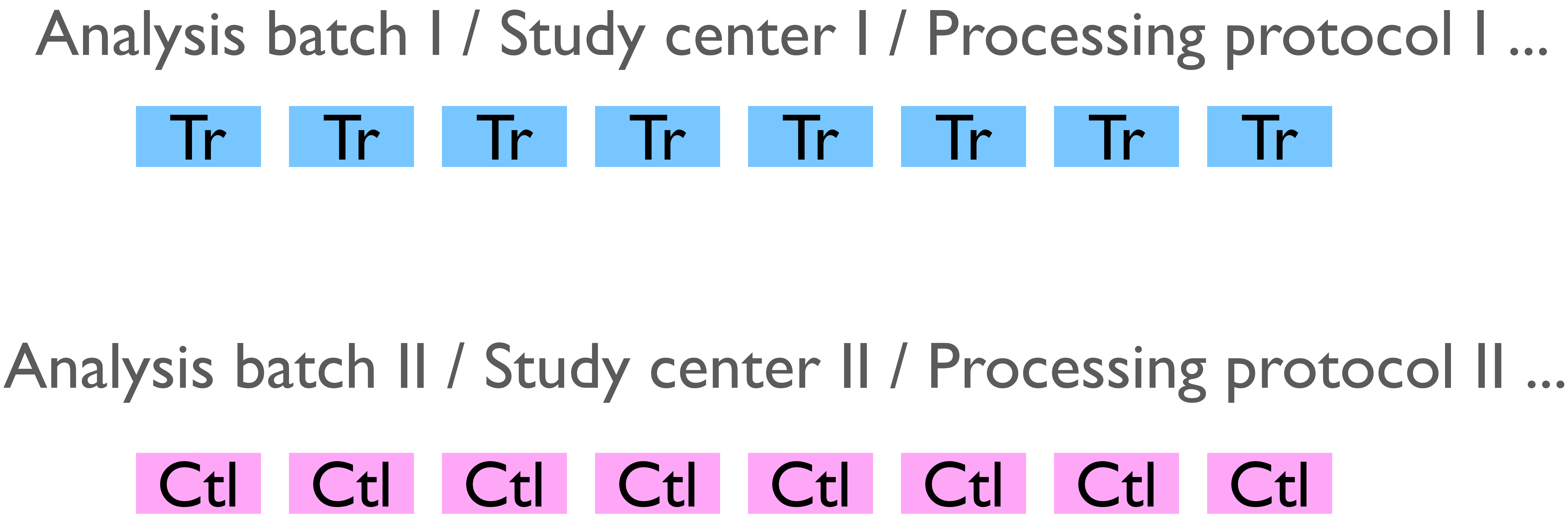
http://www.stats.gla.ac.uk/steps/glossary/anova.html#expdes

What affects the outcome of an experiment?

$$\text{Outcome} = \underbrace{\text{Treatment effects}}_{\substack{\text{Environment}\\ \text{Compound}\\ \text{Inhibitor}\\ \text{siRNA}\\ \text{Dose}\\ \text{Time}}} + \underbrace{\text{Biological effects}}_{\substack{\text{Sex}\\ \text{Age}\\ \text{Weight}\\ \text{Litter}\\ \text{Genotype}\\ \text{Species}\\ \text{Cell line}}} + \underbrace{\text{Technical effects}}_{\substack{\text{Technician}\\ \text{Batch}\\ \text{Plate}\\ \text{Cage}\\ \text{Array}\\ \text{Day}\\ \text{Order}\\ \text{Source}}} + \underbrace{\text{Error}}_{\substack{\text{Experimental}\\ \text{Treatment}\\ \text{Sampling}\\ \text{Measurement}}}$$

[Lazic, 2016]

What is **bad** experimental design?



Analysis batch I / Study center I / Processing protocol I ...

Tr Tr Tr Tr Tr Tr Tr Tr

Analysis batch II / Study center II / Processing protocol II ...

Ctl Ctl Ctl Ctl Ctl Ctl Ctl Ctl

What is **bad** experimental design?

Analysis batch I / Study center I / P essing protocol I ...

Tr Tr Tr Tr T Tr Tr

Confounding!

Analysis batch center II / Processing protocol II ...

Ctl Ctl Ctl Ctl Ctl Ctl Ctl Ctl

What can happen with bad experimental design?

- Example: gene expression study comparing 60 CEU and 82 ASN HapMap individuals
- 26% of the genes were found to be significantly differentially expressed (78% with less restrictive multiple testing correction)
- **But**: all CEU samples were processed (sometimes years) before all the ASN samples!

Akey et al., Nature Genetics 2007; Spielman et al., Nature Genetics 2007

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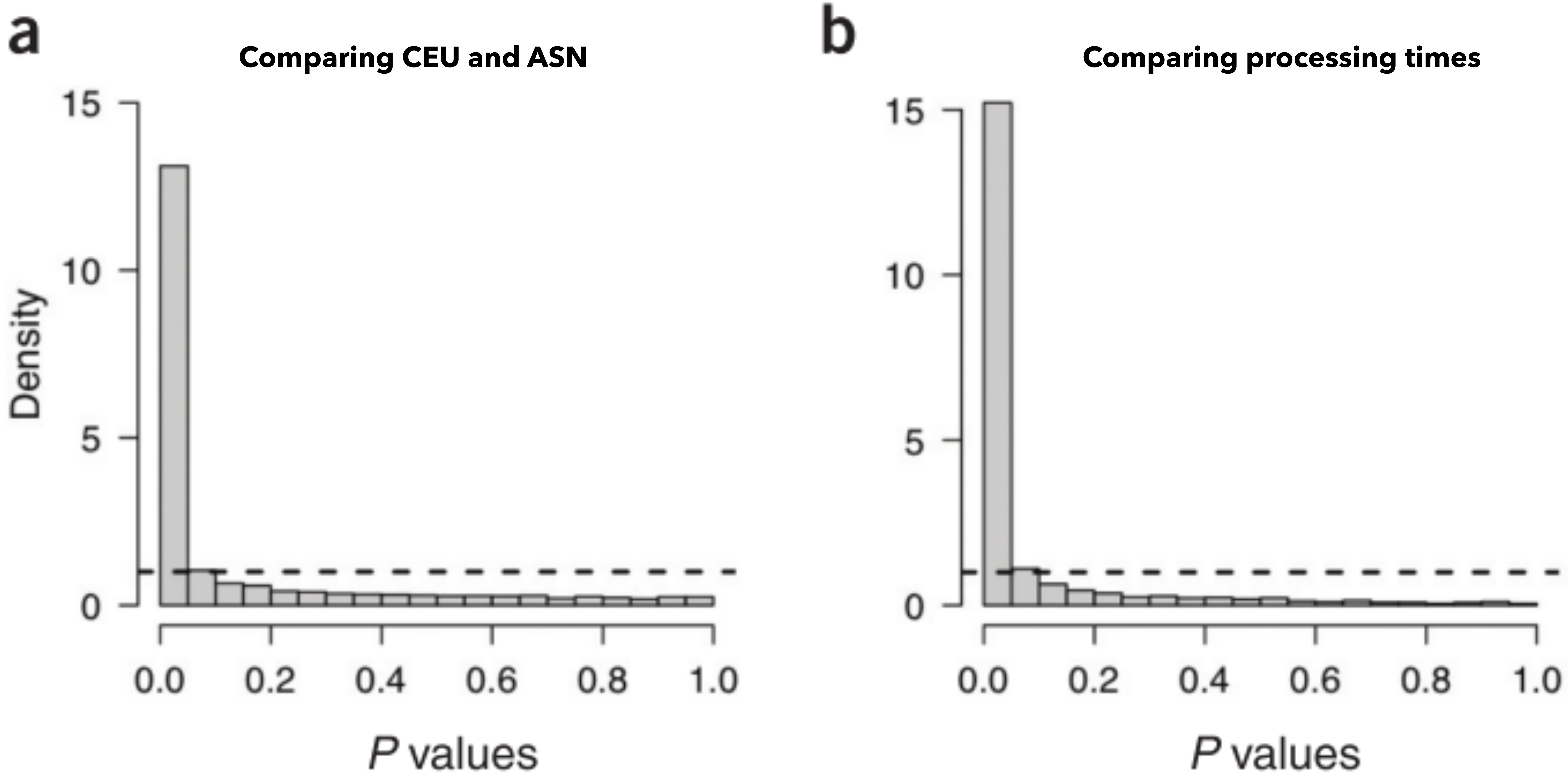


Comparing CEU and ASN

Comparing processing times

78% differentially expressed

96% differentially expressed

Akey et al., Nature Genetics 2007; Spielman et al., Nature Genetics 2007

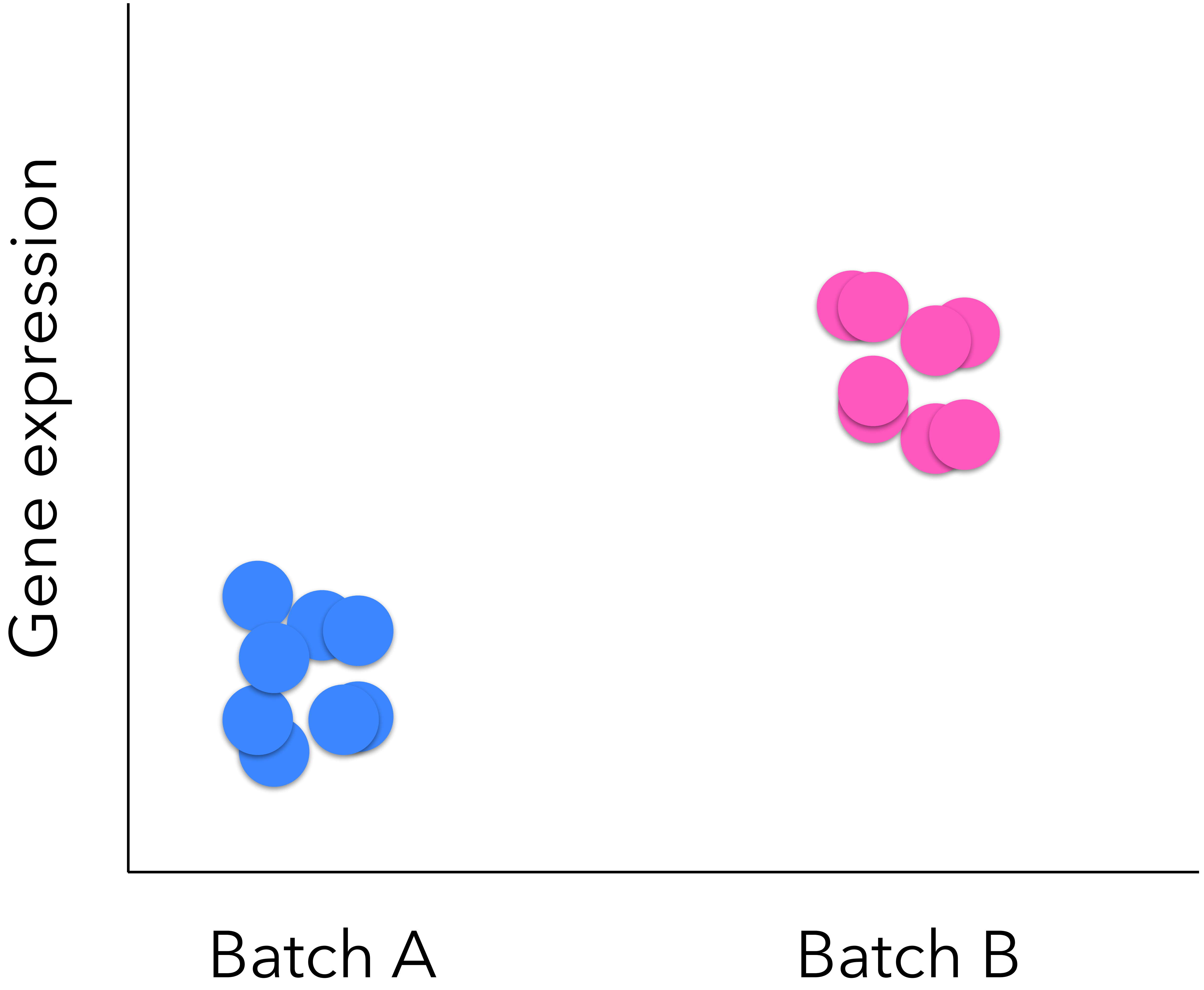
What would be a better experimental design?

- Process all samples at the same time/in one batch (not always feasible)
- Minimize confounding as much as possible through
  - blocking
  - randomization
- Batch effects may still be present, but with an appropriate design we can account for them

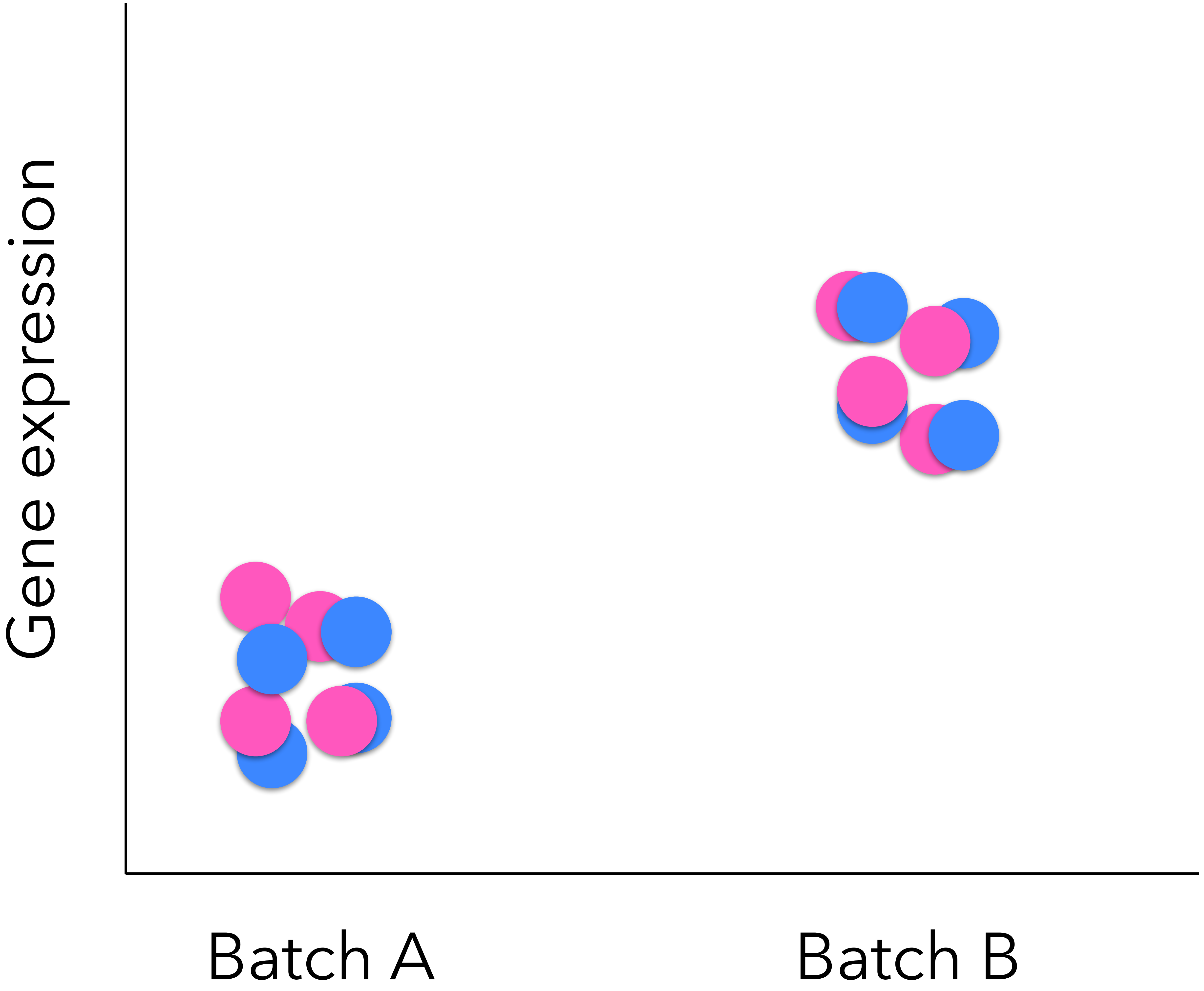
Nonzero batch effect
Zero treatment effect

Treated
Untreated

✗ Confounded design



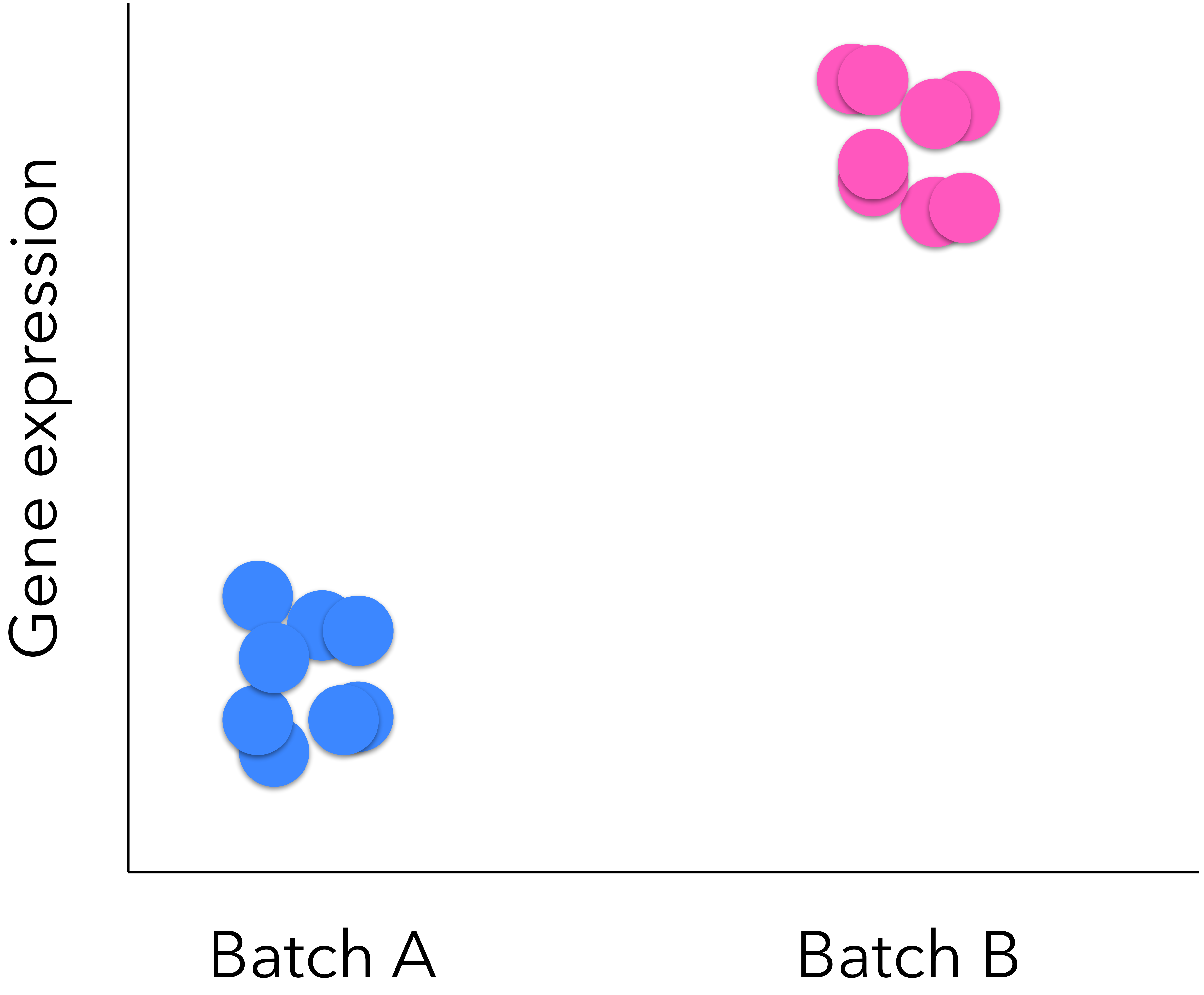
✓ Non-confounded design



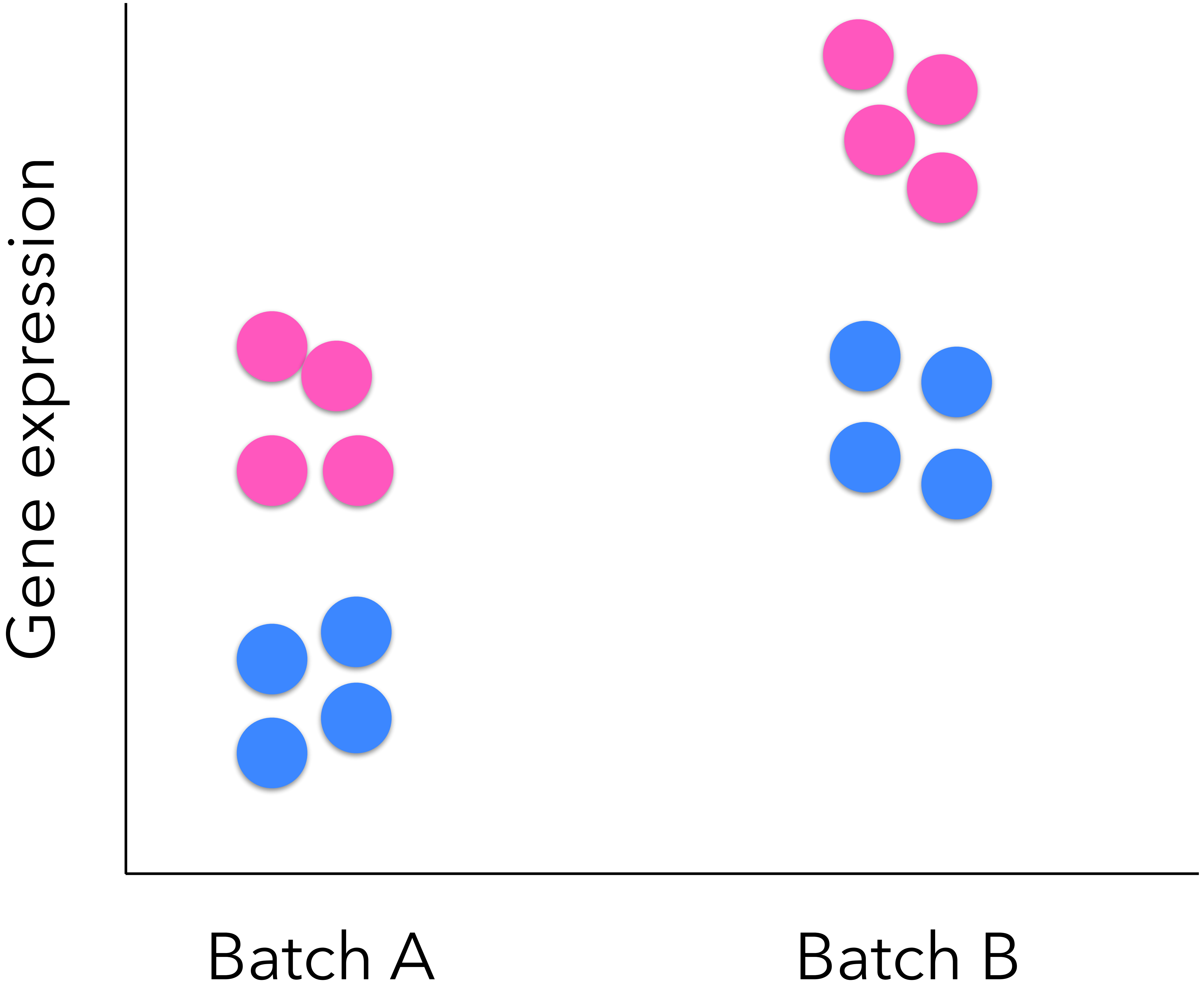
Nonzero batch effect
Nonzero treatment effect

Treated
Untreated

✗ Confounded design



✓ Non-confounded design



Dealing with batch effects

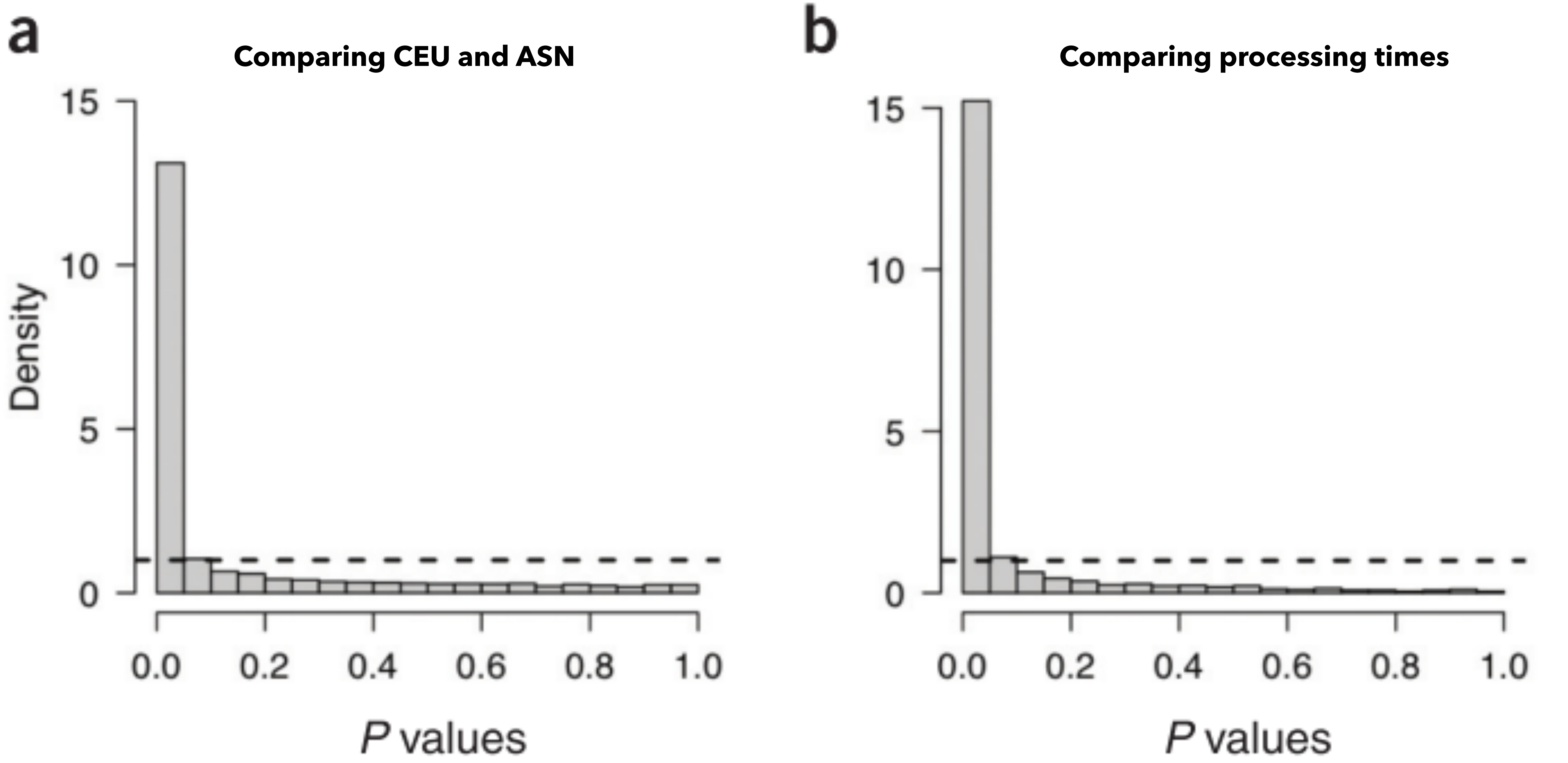
- In statistical modeling, batch effects can be included as **covariates** (additional predictors) in the model.

- For exploratory analysis, we often attempt to “eliminate” or “adjust for” such unwanted variation in advance, by subtracting the estimated effect from each variable (e.g. the expression of a gene).

- Even partial confounding between batch and signal of interest can lead to problems.

Nygaard et al., Biostatistics 2016

What can happen with bad experimental design?



Comparing CEU and ASN

Comparing processing times

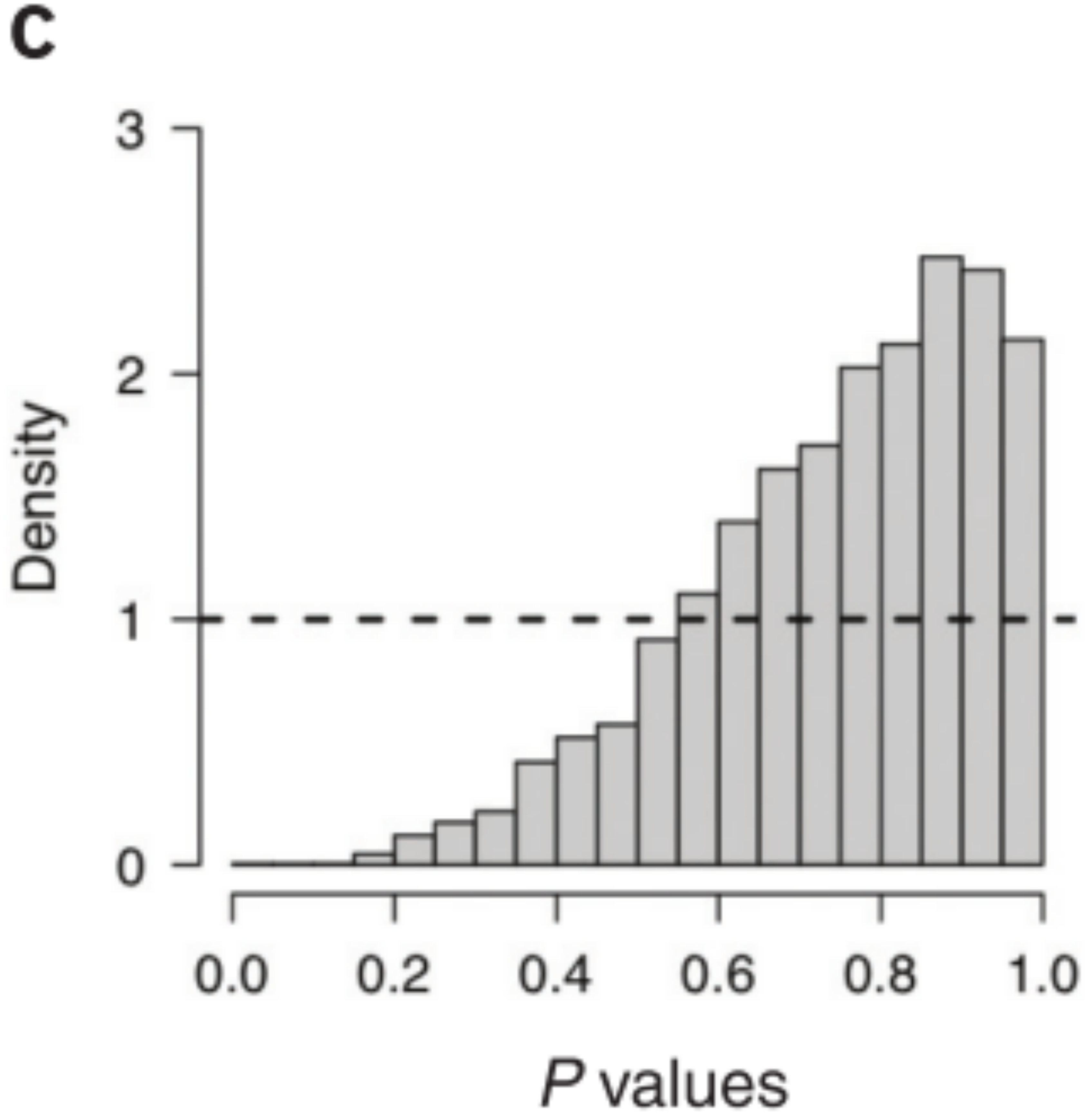
78% differentially expressed

96% differentially expressed

Akey et al., Nature Genetics 2007; Spielman et al., Nature Genetics 2007

“Batch effect correction” won’t work here

p-values from test comparing CEU and ASN, after controlling for the processing year

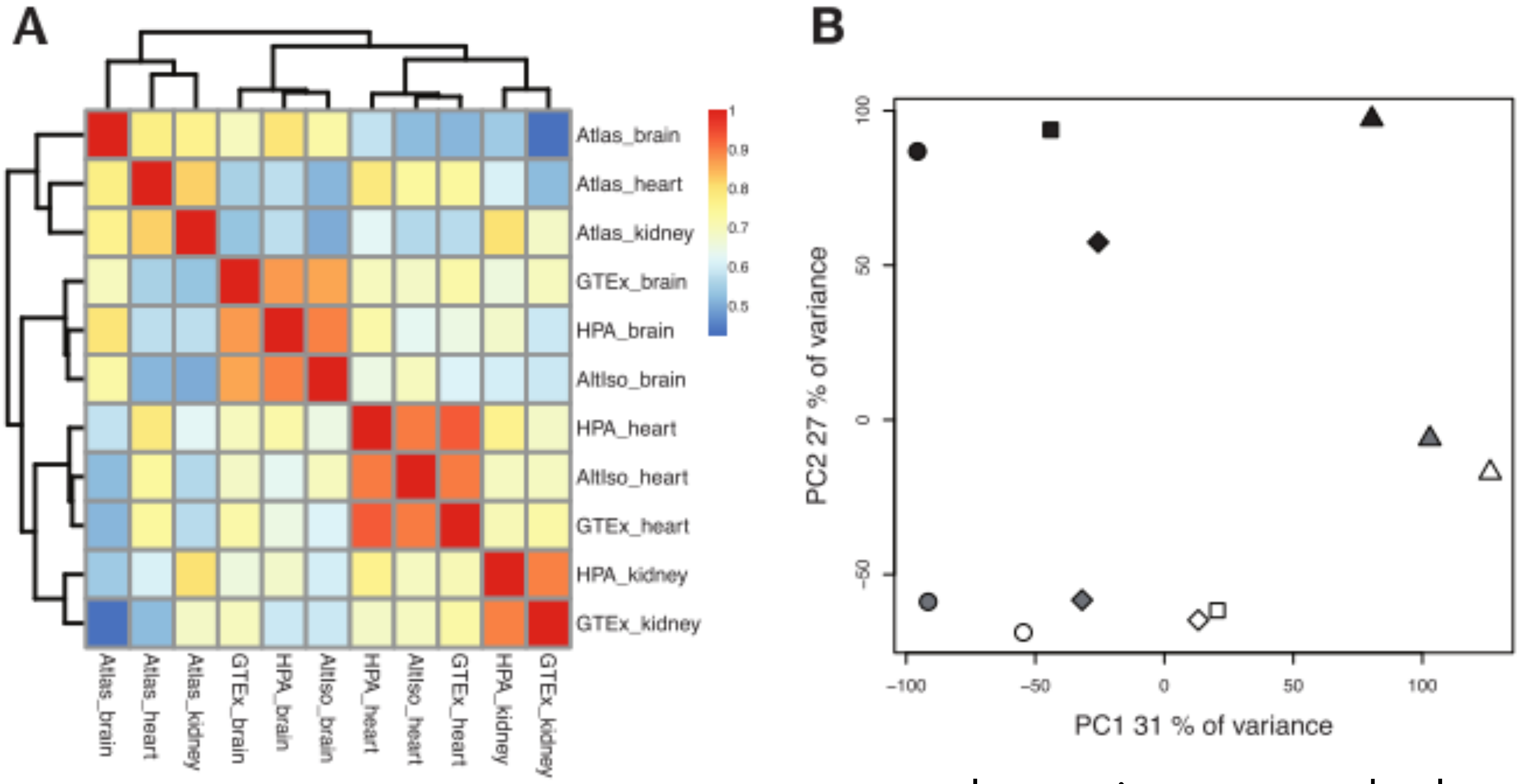


0% differentially expressed

Akey et al., Nature Genetics 2007

Accounting for batch effects in practice

Public, processed RNA-seq data from 3 tissues, 4 studies show strong "study" (=batch) signal

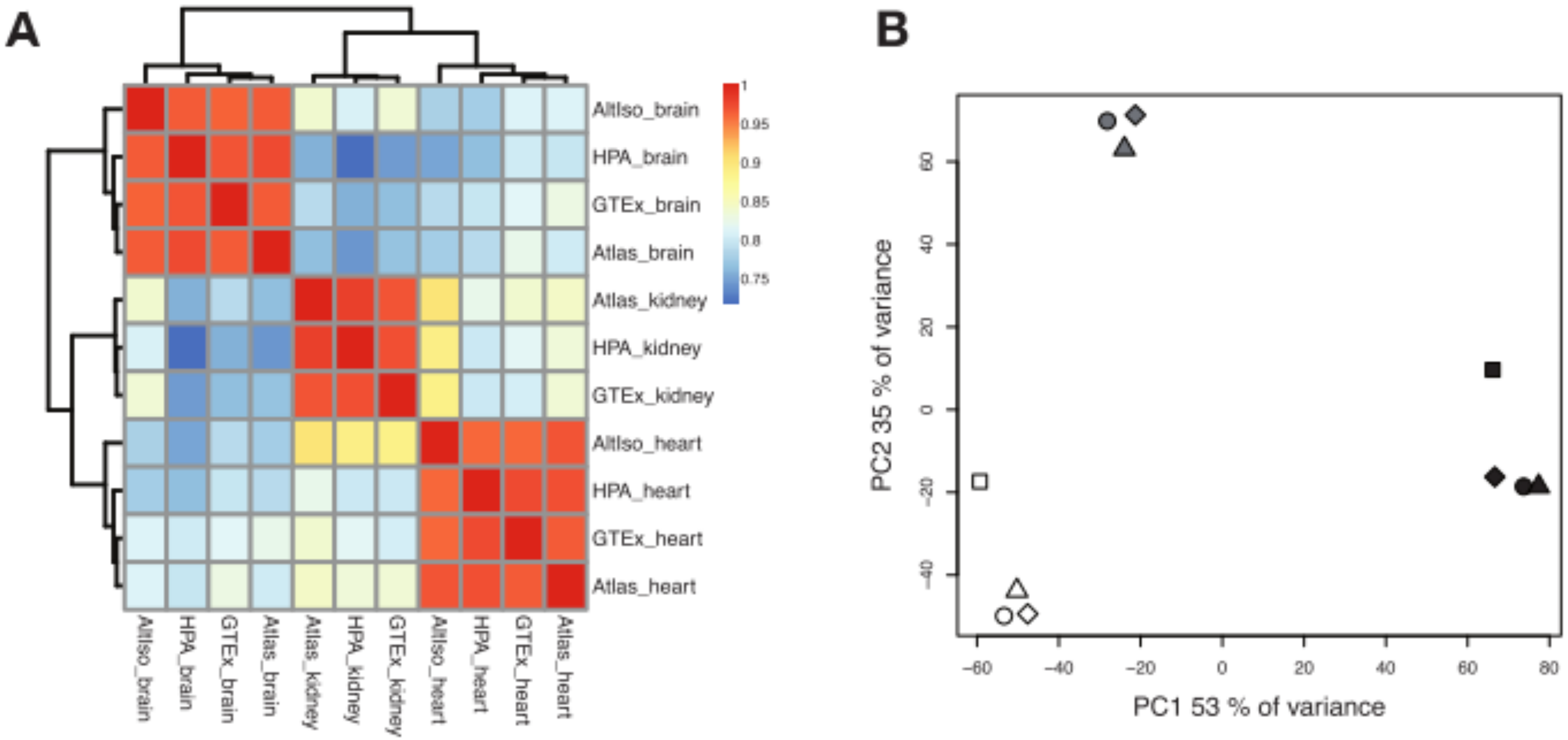


color = tissue; symbol = study (batch)

Danielsson et al., Briefings in Bioinformatics 2015

Accounting for batch effects in practice

Accounting for the batch effect brings out the signal of interest



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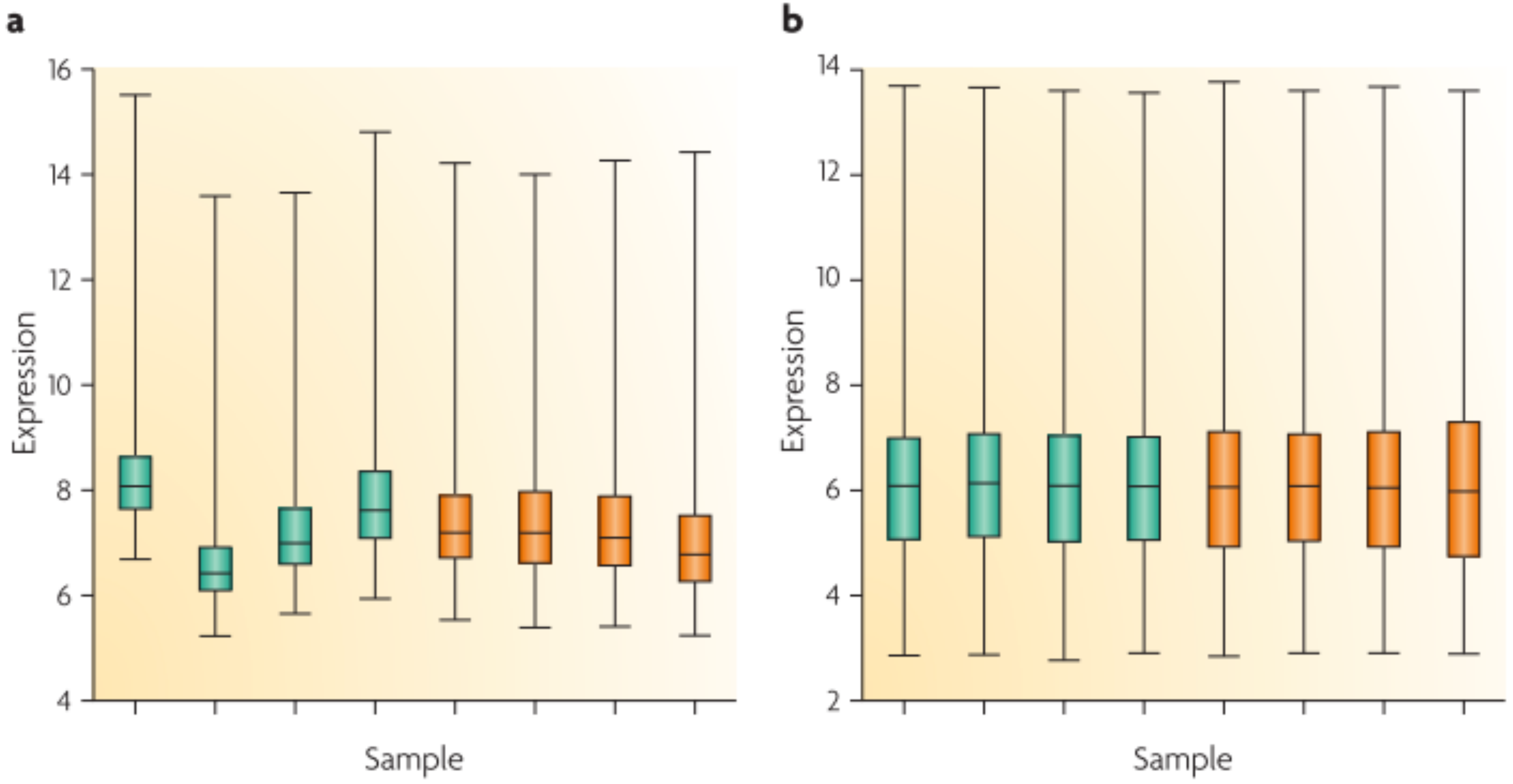
Batch effect adjustment vs normalization

Batch effect adjustment goes *beyond* the “global” between-sample normalization methods



Leek et al., Nature Reviews Genetics 2010

Batch effect adjustment vs normalization

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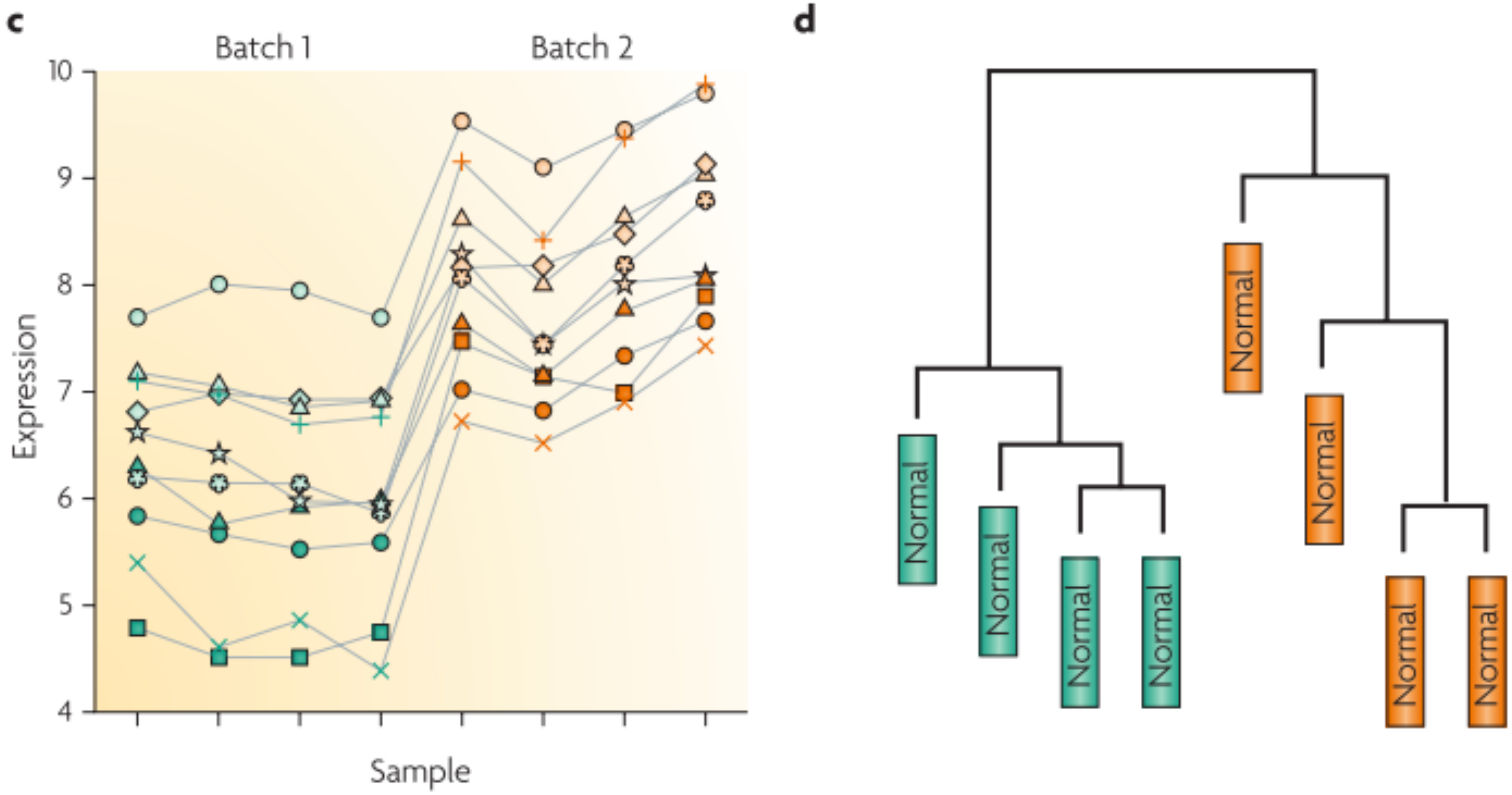
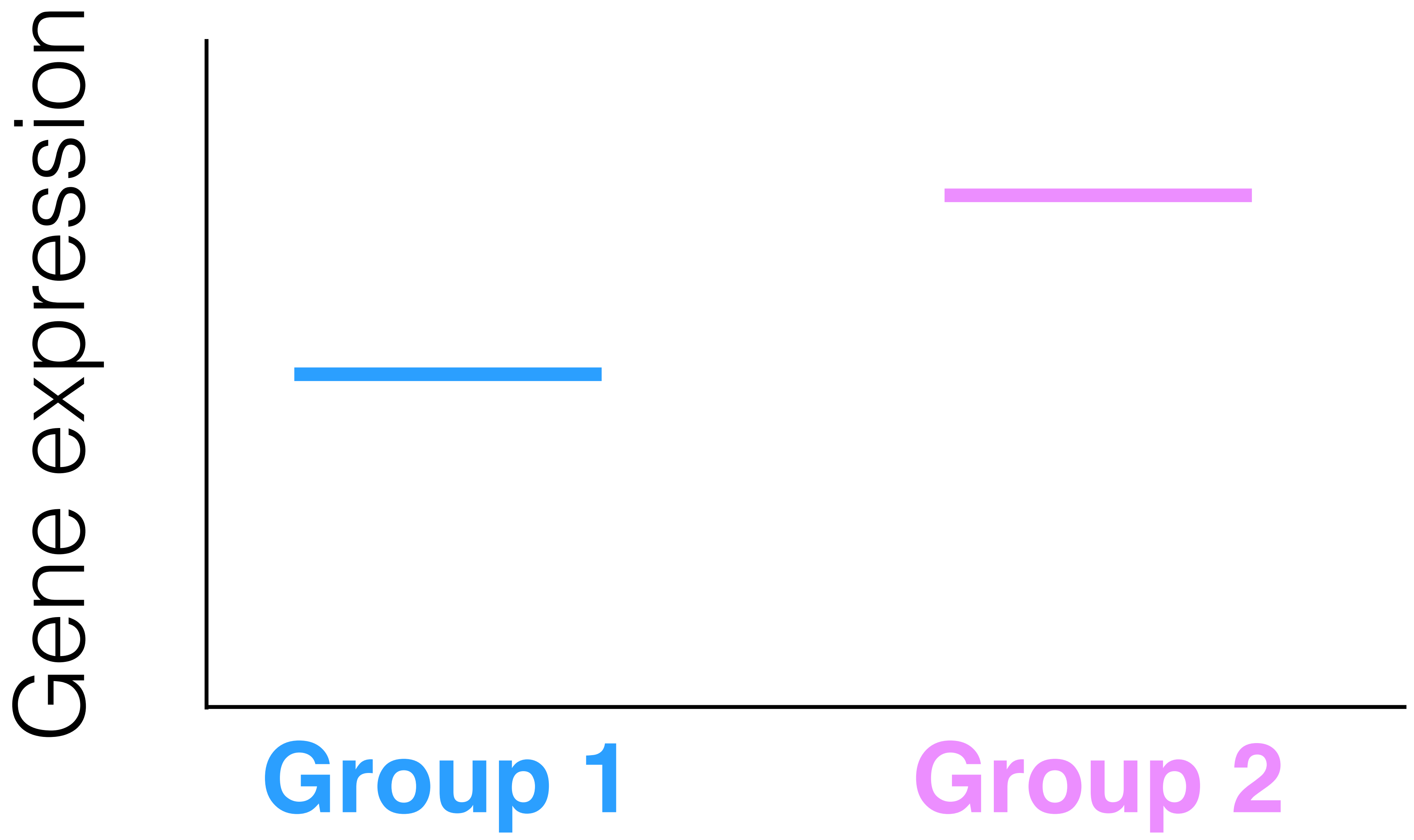


Leek et al., Nature Reviews Genetics 2010

Other design issues: replication

- Replicates are **necessary** to estimate within-condition variability.

- Variability estimates are, in turn, **vital** for statistical testing.



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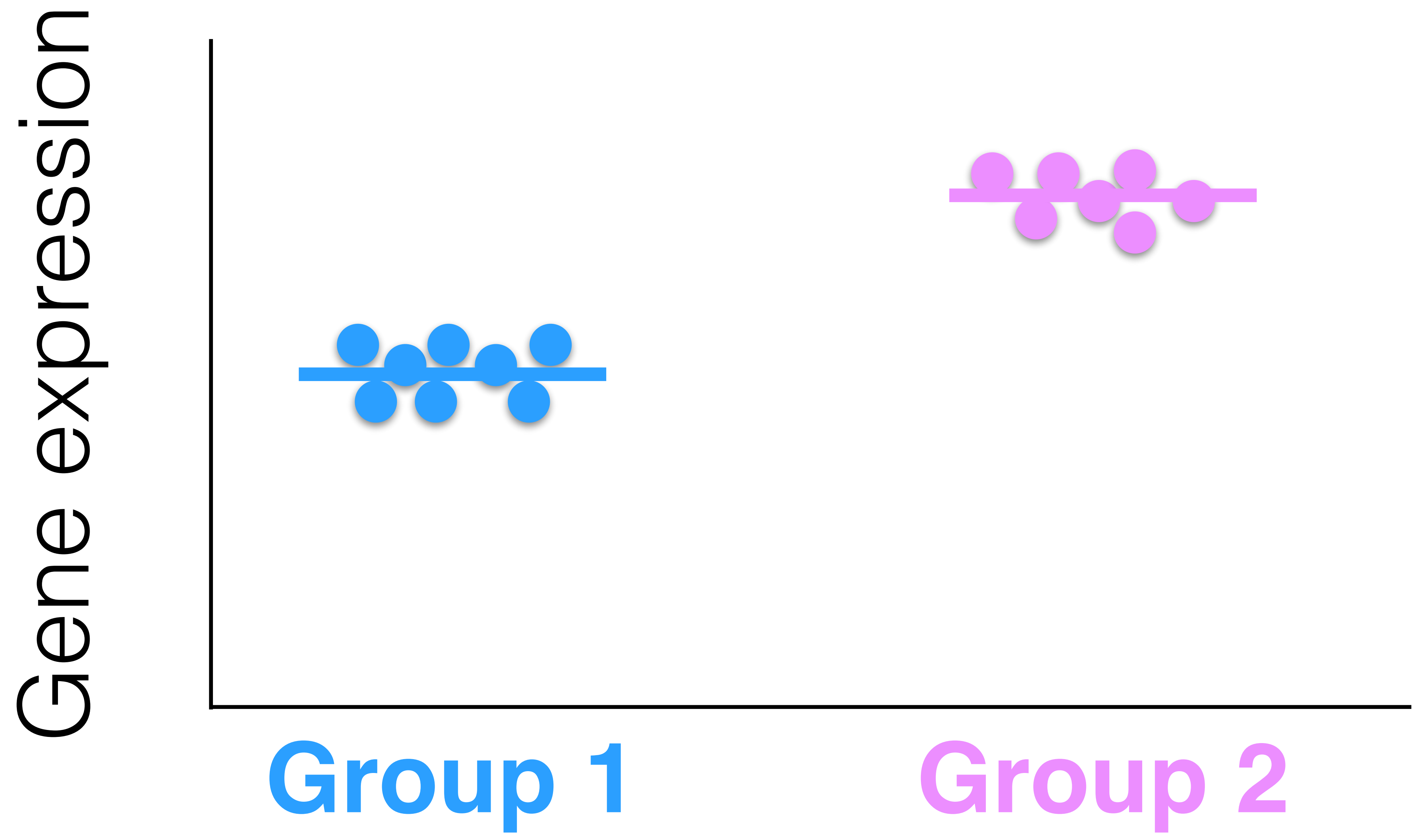

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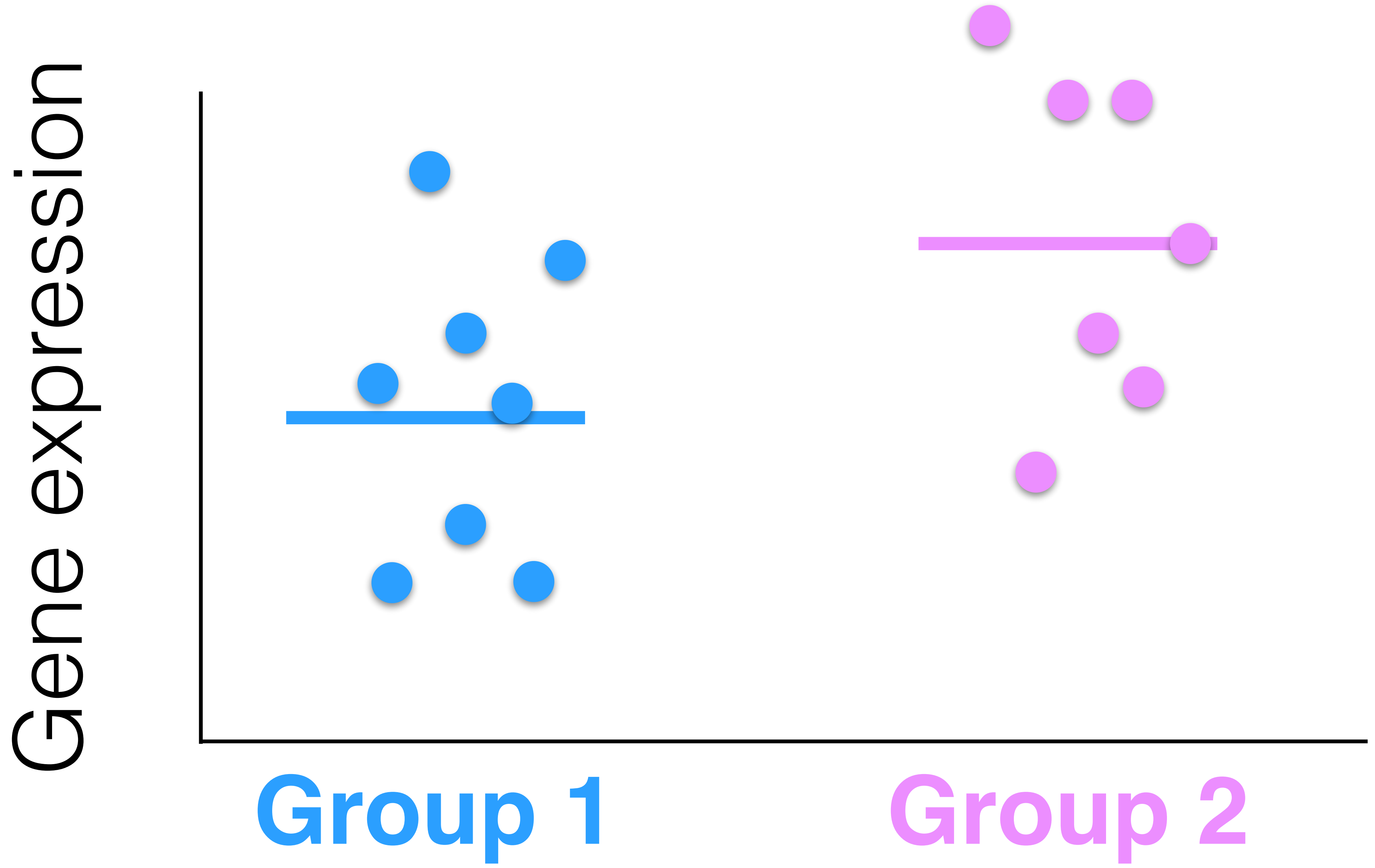


Different types of units

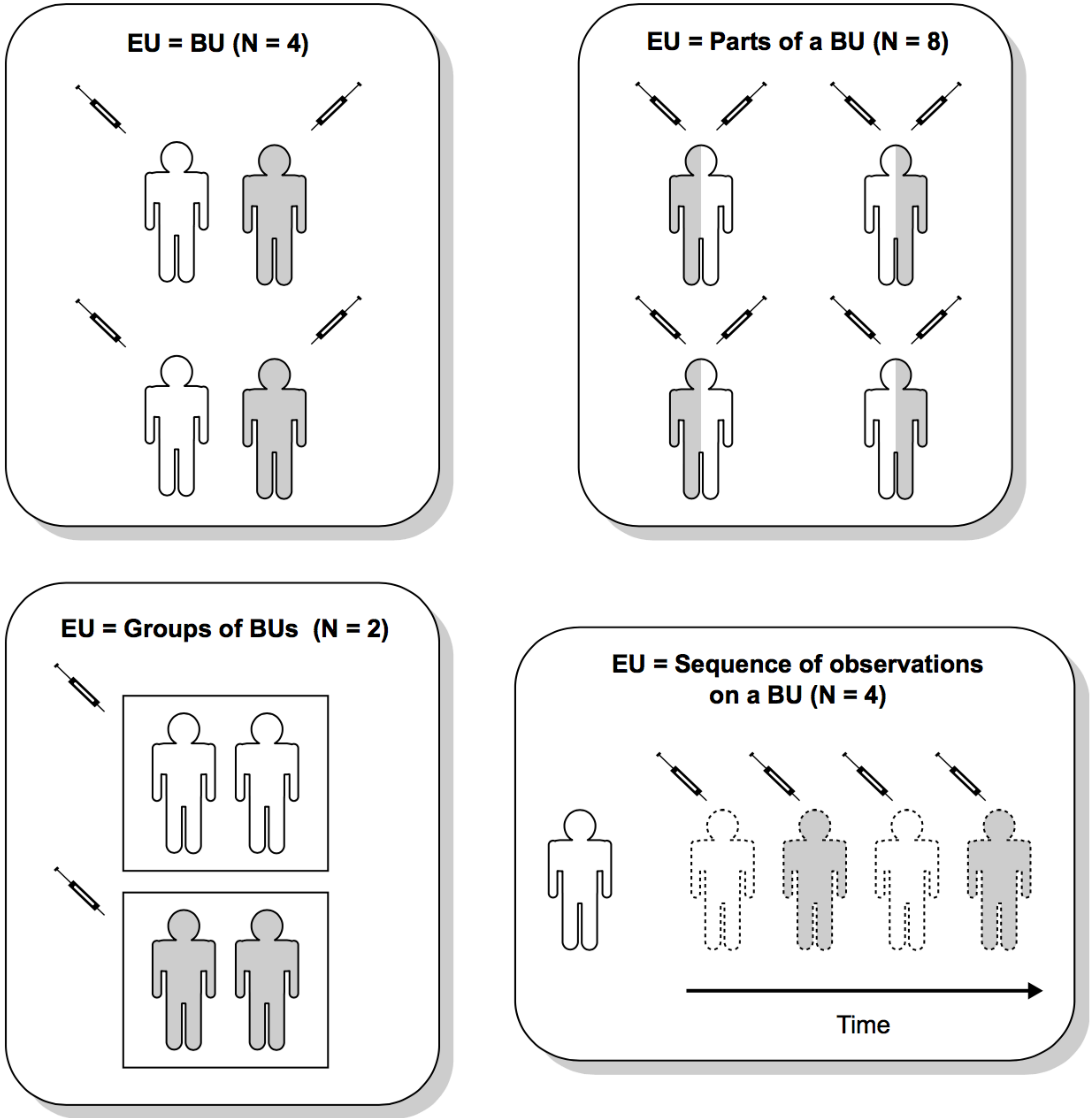
- Biological units (BU) - entities we want to make inferences about (e.g., animal, person)

- Experimental units (EU) - smallest entities that can be independently assigned to a treatment (e.g., animal, litter, cage, well)

- Observational units (OU) - entities at which measurements are made

[Lazic, 2016]

Biological vs experimental units


EU = BU (N = 4)
EU = Parts of a BU (N = 8)
EU = Groups of BUs (N = 2)
EU = Sequence of observations on a BU (N = 4)
Time

[Lazic, 2016]

Pseudoreplication

- “**Artificial inflation** of the sample size, that usually occurs when the biological unit of interest differs from the experimental unit or observational unit.”
- Only replication of experimental units is true replication

[Lazic, 2016]

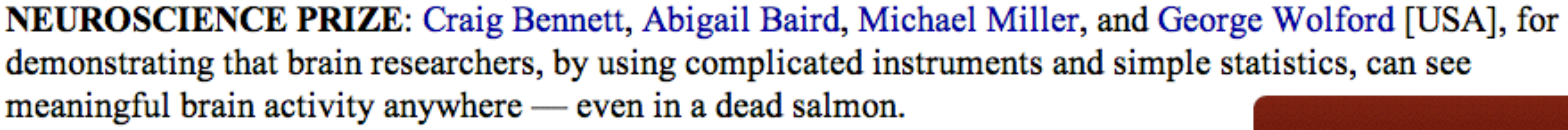
What is a p-value?

- The p-value is the probability of obtaining a test statistic *at least as extreme* as the one observed, *if the null hypothesis is true* (i.e., if there is no true signal in the data)

- Hence, if we get a p-value of **0.05**, it means that there is a **5%** chance of getting that extreme results even in the absence of real signal!

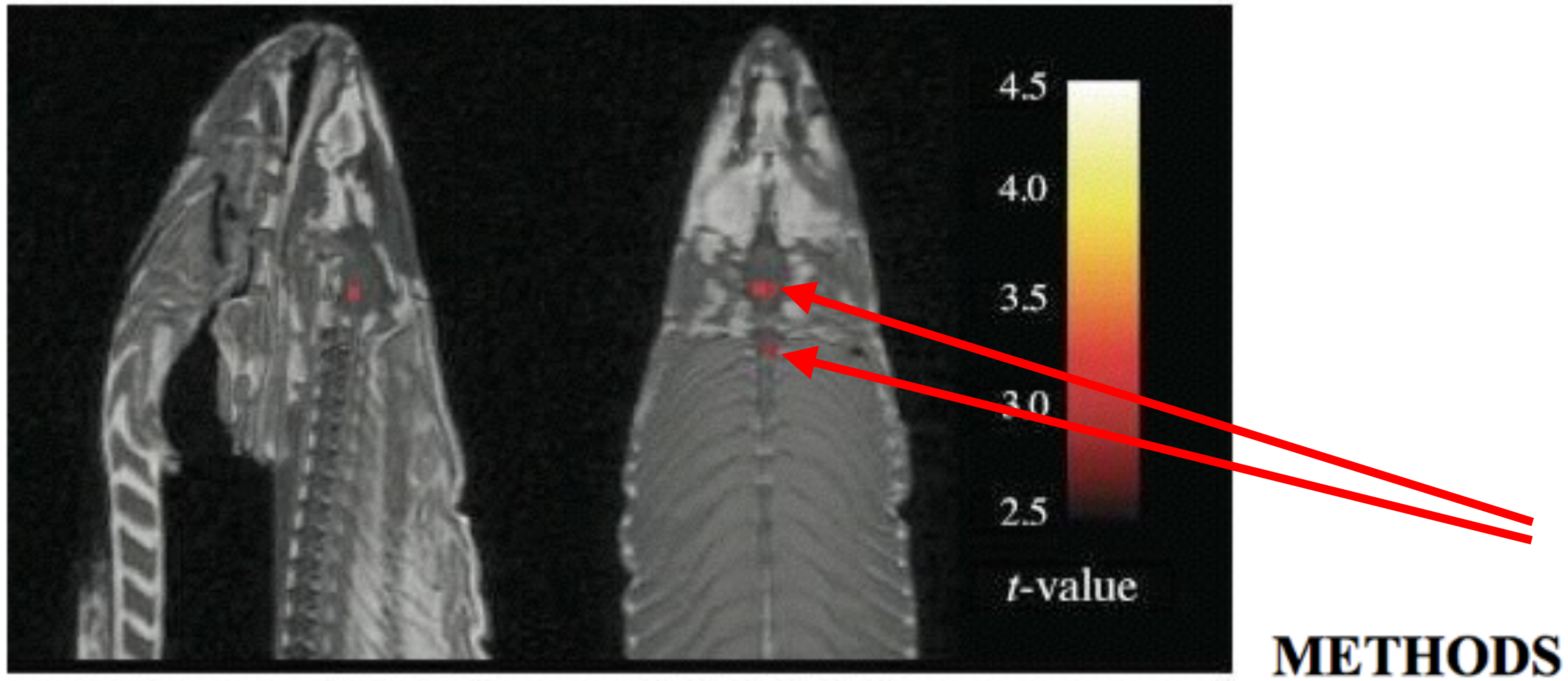
What does this mean for high-throughput studies?

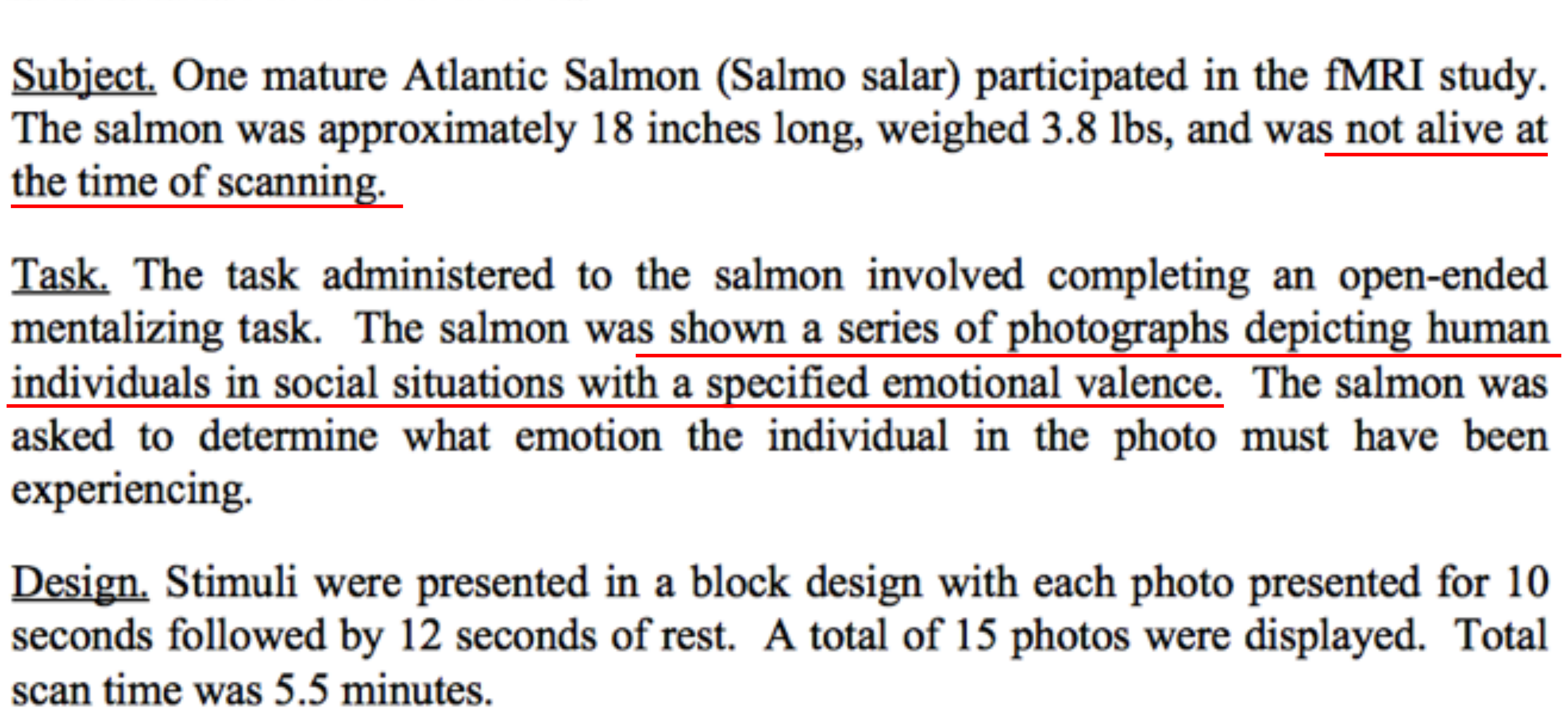
- Assume that we perform 10,000 tests (one for each gene)…
- … and that there is no true signal at all in the data
- Then we would expect to get around 500 p-values below 0.05
- Relying solely on p-values would be misleading!



NEUROSCIENCE PRIZE: Craig Bennett, Abigail Baird, Michael Miller, and George Wolford [USA], for demonstrating that brain researchers, by using complicated instruments and simple statistics, can see meaningful brain activity anywhere — even in a dead salmon.

IMPROBABLE RESEARCH





METHODS

Subject. One mature Atlantic Salmon (Salmo salar) participated in the fMRI study. The salmon was approximately 18 inches long, weighed 3.8 lbs, and was not alive at the time of scanning.

Task. The task administered to the salmon involved completing an open-ended mentalizing task. The salmon was shown a series of photographs depicting human individuals in social situations with a specified emotional valence. The salmon was asked to determine what emotion the individual in the photo must have been experiencing.

Design. Stimuli were presented in a block design with each photo presented for 10 seconds followed by 12 seconds of rest. A total of 15 photos were displayed. Total scan time was 5.5 minutes.

Craig M. Bennett, Abigail A. Baird, Michael B. Miller, and George L. Wolford, Journal of Serendipitous and Unexpected Results, vol. 1, no. 1, 2010, pp. 1-5

We need to change perspective

- Instead of limiting the false positive probability for *each individual test*, try to limit
  - the probability of obtaining *any* false positives (FWER)
  - the fraction of false positives among the significant genes (FDR)

Benjamini-Hochberg correction - controlling the FDR

- Assume we are performing N tests
- Intuition:
    - for each threshold α, we can estimate the expected number of false discoveries by αN
    - Compare this to the actual number of discoveries at that threshold (N_α)
    - Choose a so that $\alpha N/N_\alpha \leq 0.05$ (or another desired threshold)

Interpreting the FDR

- The FDR is a measure for a *set* of genes
- In a set of genes with FDR = 0.05, approximately 5% can be expected to be false discoveries
- However, we don’t know *which ones*! It could be the most significant!
- *q-values* are gene-wise significance measures (“adjusted p-values”) - the smallest FDR we have to accept in order to call the gene significant

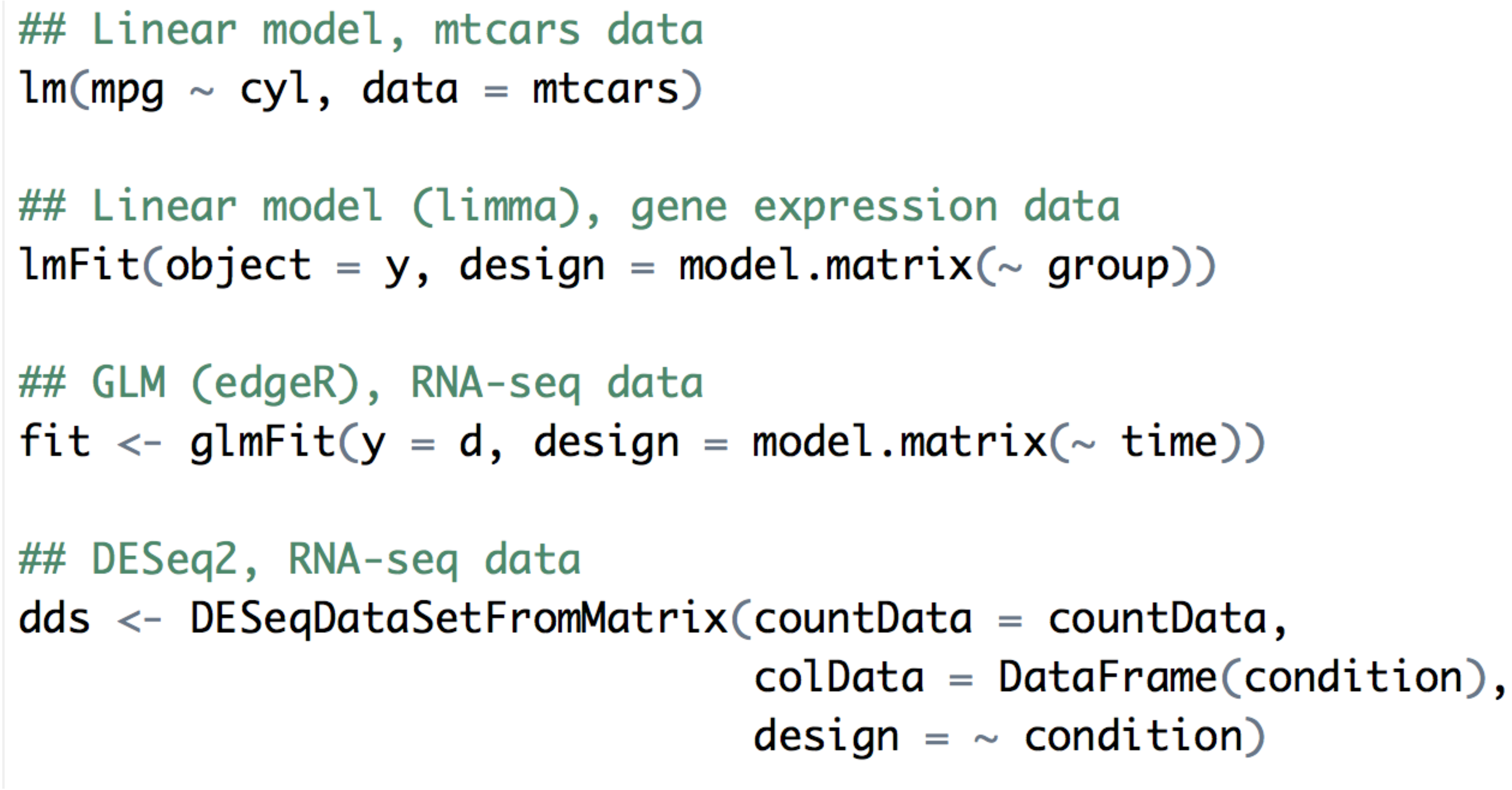
Model formulas and design matrices

- Testing is done separately for each gene
- We must tell the packages **which model** to fit (e.g. which predictors to use)
- The design does *not* follow “automatically” from having the sample annotation table - many different designs are often possible
- Model formulas in R:

$$\text{response variable} \;\sim\; \text{predictors}$$

- Fit a separate model for each gene - response variable changes. Specify only predictors

Examples



```
## Linear model, mtcars data
lm(mpg ~ cyl, data = mtcars)

## Linear model (limma), gene expression data
lmFit(object = y, design = model.matrix(~ group))

## GLM (edgeR), RNA-seq data
fit <- glmFit(y = d, design = model.matrix(~ time))

## DESeq2, RNA-seq data
dds <- DESeqDataSetFromMatrix(countData = countData,
                              colData = DataFrame(condition),
                              design = ~ condition)
```

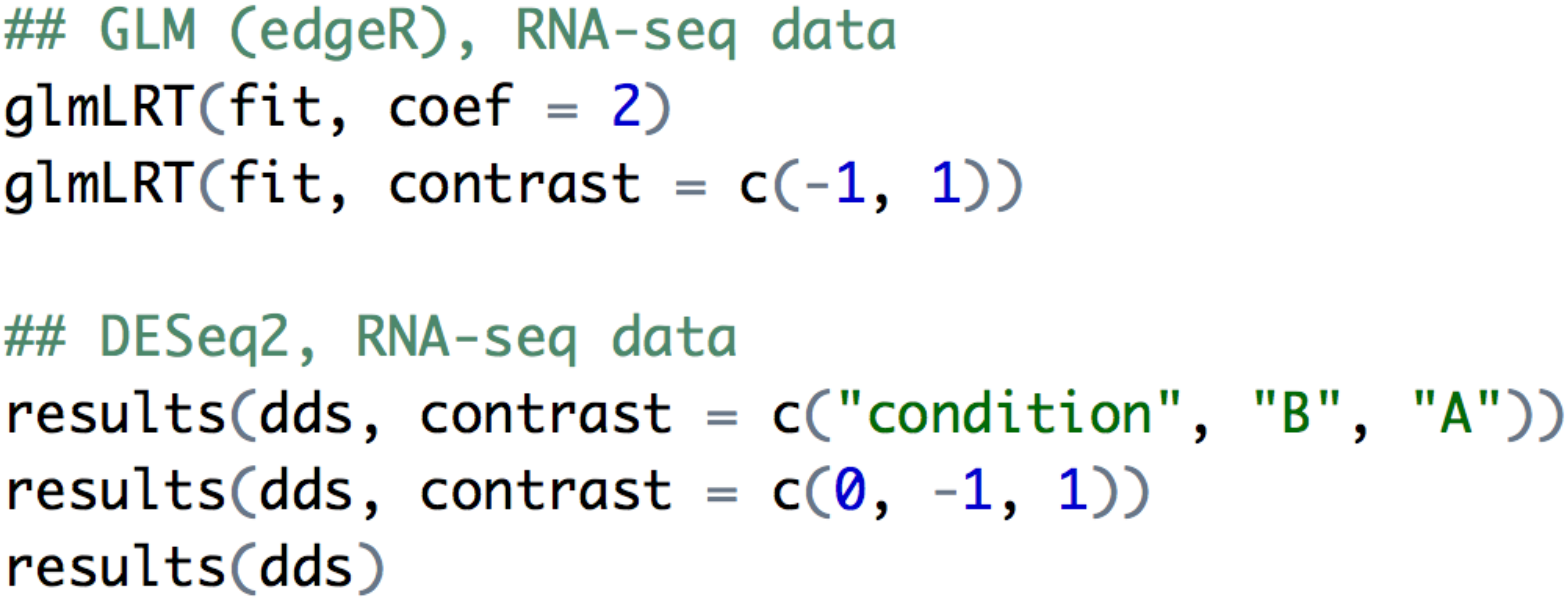
Testing and contrasts

- After fitting the model(s), we must decide *which* coefficient (or combination thereof) we want to apply a hypothesis test for.

- Combinations of coefficients are called *contrasts*.

- Design matrices can often be defined in many equivalent ways - important that the contrast is defined accordingly!

Examples



```r
## GLM (edgeR), RNA-seq data
glmLRT(fit, coef = 2)
glmLRT(fit, contrast = c(-1, 1))

## DESeq2, RNA-seq data
results(dds, contrast = c("condition", "B", "A"))
results(dds, contrast = c(0, -1, 1))
results(dds)
```

Model formulas and design matrices

- A design matrix contains the values of the predictor variables for each sample

coefficients

$$\begin{pmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \end{pmatrix} = \boxed{\begin{pmatrix} 1 & 0 \\ 1 & 0 \\ 1 & 0 \\ 1 & 1 \\ 1 & 1 \\ 1 & 1 \end{pmatrix}} \begin{pmatrix} \beta_0 \\ \beta_1 \end{pmatrix} + \begin{pmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \varepsilon_3 \\ \varepsilon_4 \\ \varepsilon_5 \\ \varepsilon_6 \end{pmatrix} = \boxed{X}\beta + \varepsilon$$

$$y_i = \beta_0 + \beta_1 x_i + \varepsilon_i$$

e.g.: (log) expression values for a given gene

Many ways of modeling the same expected values

- 1 predictor, 2 groups

	group 1	group 2
X	b0	b0 + b1

~X

	group 1	group 2
X	b0	b1

~0 + X

the coefficients mean different things in the different cases!

- 2 predictors, 2*2 groups

	Y	
X	b0	b0+b1
	b0+b2	b0+b1+ b2+b3

~X*Y

~X + Y + X:Y

	Y	
X	b0	b1
	b2	b3

~0 + XY

New variable, combining X and Y

	Y	
X	b0	b0+b1
	b0+b2	b0+b2+ b3

~X + X:Y

Model formulas and design matrices - example 1
One predictor, two levels (without intercept)

Sample table:

```
  sample treatment
1     s1   control
2     s2   control
3     s3   control
4     s4   treated
5     s5   treated
6     s6   treated
```

Design matrix:

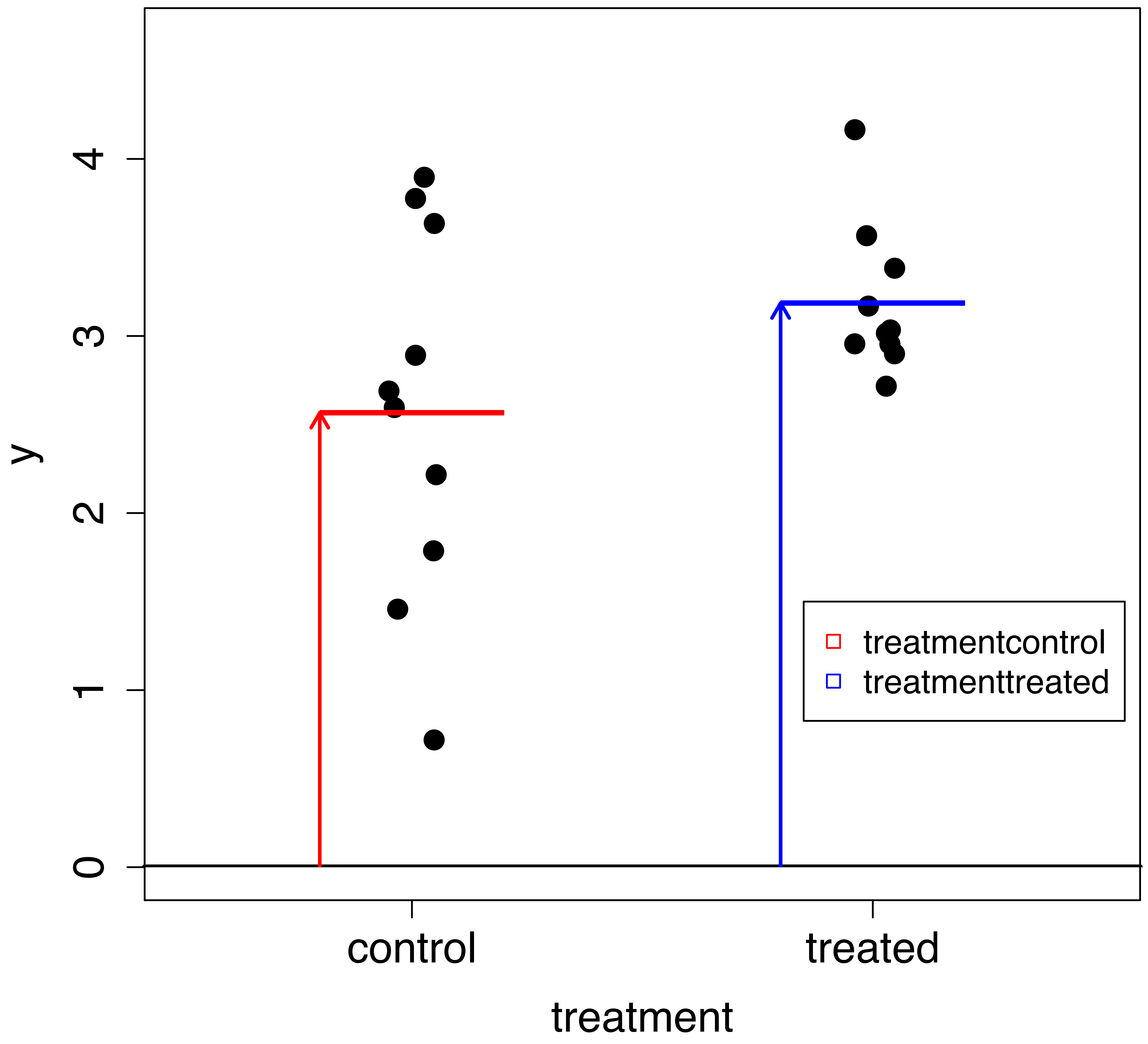
```
  treatmentcontrol treatmenttreated
1                1                0
2                1                0
3                1                0
4                0                1
5                0                1
6                0                1
```

Formula:

$$\sim 0 + \text{treatment}$$

Modeled values:

control	treated
treatmentcontrol	treatmenttreated


0
1
2
3
4
y
control
treated
treatment
□ treatmentcontrol
□ treatmenttreated

Model formulas and design matrices - example 1
One predictor, two levels (with intercept)

Sample table:

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  sample treatment
1     s1   control
2     s2   control
3     s3   control
4     s4   treated
5     s5   treated
6     s6   treated
```

Design matrix:

```
  (Intercept) treatmenttreated
1           1                0
2           1                0
3           1                0
4           1                1
5           1                1
6           1                1
```

Formula:

$$\sim \text{treatment}$$

Modeled values:

control	treated
1 * Intercept + **0 * treatmenttreated**	**1 * Intercept +** **1 * treatmenttreated**

Model formulas and design matrices - example 1
One predictor, two levels (with intercept)

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1     s1   control
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Modeled values:

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1 * Intercept + 0 * treatmenttreated	1 * Intercept + 1 * treatmenttreated

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6           1                1
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Modeled values:

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Model formulas and design matrices - example 1
One predictor, two levels (with intercept)

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  sample treatment
1     s1   control
2     s2   control
3     s3   control
4     s4   treated
5     s5   treated
6     s6   treated
```

Design matrix:

```
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1           1                0
2           1                0
3           1                0
4           1                1
5           1                1
6           1                1
```

Formula:

$$\sim \text{treatment}$$

Modeled values:

control	treated
1 * Intercept + **0 * treatmenttreated**	**1 * Intercept +** **1 * treatmenttreated**

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One predictor, two levels (with intercept)

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3           1                0
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5           1                1
6           1                1
```

Formula:

$$\sim \text{treatment}$$

Modeled values:

control	treated
1 * Intercept + 0 * treatmenttreated	1 * Intercept + 1 * treatmenttreated

Model formulas and design matrices - example 1
One predictor, two levels (with intercept)

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1     s1   control
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5     s5   treated
6     s6   treated
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Design matrix:

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3           1                0
4           1                1
5           1                1
6           1                1
```

Formula:

$$\sim \text{treatment}$$

Modeled values:

control	treated
1 * Intercept + **0 * treatmenttreated**	**1 * Intercept +** **1 * treatmenttreated**

Model formulas and design matrices - example 1
One predictor, two levels (with intercept)

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1     s1   control
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5           1                1
6           1                1
```

Formula:

$$\sim \text{treatment}$$

Modeled values:

control	treated
1 * Intercept + 0 * **treatmenttreated**	1 * Intercept + 1 * **treatmenttreated**

Model formulas and design matrices - example 1
One predictor, two levels (with intercept)

Sample table:

```
  sample treatment
1     s1   control
2     s2   control
3     s3   control
4     s4   treated
5     s5   treated
6     s6   treated
```

Design matrix:

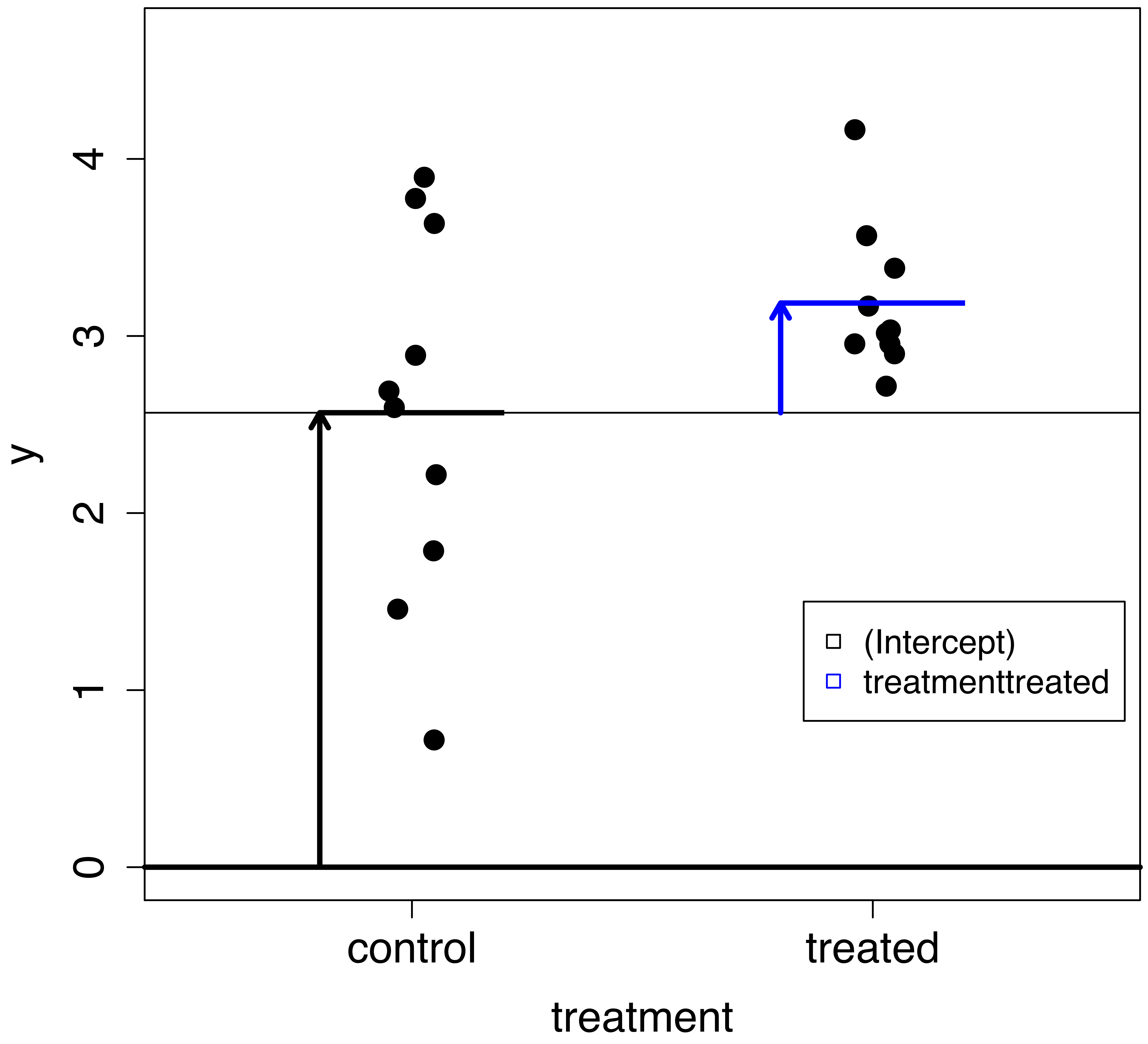
```
  (Intercept) treatmenttreated
1           1                0
2           1                0
3           1                0
4           1                1
5           1                1
6           1                1
```

Formula:

$$\sim \text{treatment}$$

Modeled values:

control	treated
Intercept	**Intercept +** **treatmenttreated**


4
3
2
1
0
y
control
treated
treatment
□ (Intercept)
□ treatmenttreated

Model formulas and design matrices - example 2
One continuous predictor

Sample table:

```
  sample age
1     s1  21
2     s2  12
3     s3  64
4     s4  44
5     s5  19
6     s6  26
```

Design matrix:

```
  (Intercept) age
1           1  21
2           1  12
3           1  64
4           1  44
5           1  19
6           1  26
```

Formula:

$$\sim \text{age}$$

Modeled values:

s1	s2	s3	s4	s5	s6
Intercept + 21 * age	**Intercept + 12 * age**	**Intercept + 64 * age**	**Intercept + 44 * age**	**Intercept + 19 * age**	**Intercept + 26 * age**

Model formulas and design matrices - example 3
One predictor, three levels

Sample table:

```
  sample treatment
1     s1   control
2     s2   control
3     s3    treatA
4     s4    treatA
5     s5    treatB
6     s6    treatB
```

Design matrix:

```
  (Intercept) treatmenttreatA treatmenttreatB
1           1               0               0
2           1               0               0
3           1               1               0
4           1               1               0
5           1               0               1
6           1               0               1
```

Formula:

$$\sim \text{treatment}$$

Modeled values:

control	treatA	treatB
Intercept	**Intercept +** **treatmenttreatA**	**Intercept +** **treatmenttreatB**

Model formulas and design matrices - example 4
One predictor, paired data (or two predictors)

Sample table:

```
  sample treatment
1     s1   control
2     s1   treated
3     s2   control
4     s2   treated
5     s3   control
6     s3   treated
```

Design matrix:

```
  (Intercept) samples2 samples3 treatmenttreated
1           1        0        0                0
2           1        0        0                1
3           1        1        0                0
4           1        1        0                1
5           1        0        1                0
6           1        0        1                1
```

Formula:

$$\sim \text{sample} + \text{treatment}$$

Modeled values:

	s1	s2	s3
control	**Intercept**	**Intercept +** **samples2**	**Intercept +** **samples3**
treated	**Intercept +** **treatmenttreated**	**Intercept +** **samples2 +** **treatmenttreated**	**Intercept +** **samples3 +** **treatmenttreated**

Model formulas and design matrices - example 4
One predictor, paired data (or two predictors)

Sample table:

```
  genotype treatment
1        A   control
2        A   control
3        A   treated
4        A   treated
5        B   control
6        B   control
7        B   treated
8        B   treated
```

Design matrix:

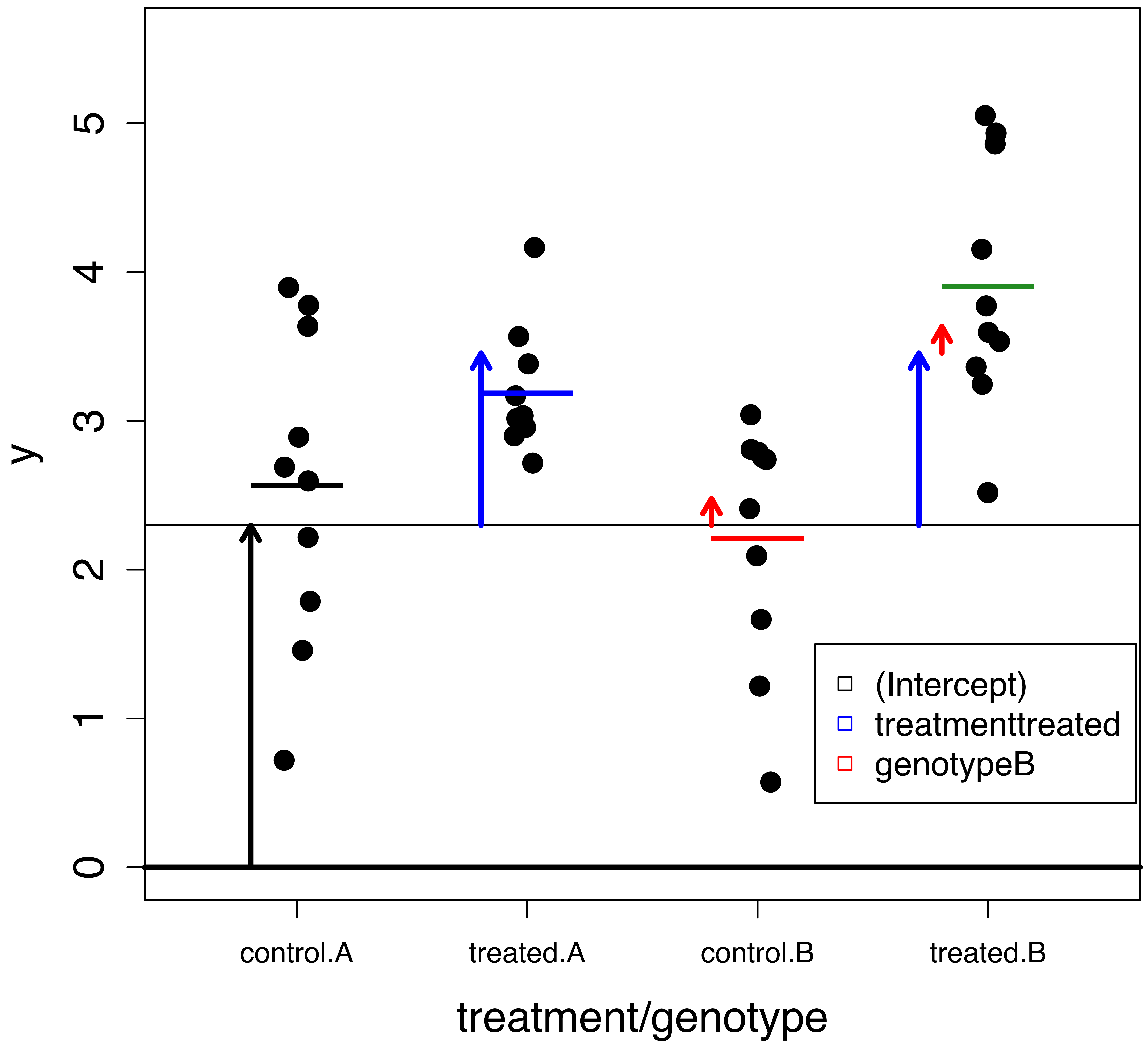
```
  (Intercept) genotypeB treatmenttreated
1           1         0                0
2           1         0                0
3           1         0                1
4           1         0                1
5           1         1                0
6           1         1                0
7           1         1                1
8           1         1                1
```

Formula:

$$\sim \text{genotype} \; + \; \text{treatment}$$

Modeled values:

	genotype A	genotype B
control	**Intercept**	**Intercept + genotypeB**
treated	**Intercept + treatmenttreated**	**Intercept + genotypeB + treatmenttreated**


y
0
1
2
3
4
5
□ (Intercept)
□ treatmenttreated
□ genotypeB
control.A
treated.A
control.B
treated.B
treatment/genotype

Model formulas and design matrices - example 5
Two predictors, with interaction

Sample table:

```
  genotype treatment
1        A   control
2        A   control
3        A   treated
4        A   treated
5        B   control
6        B   control
7        B   treated
8        B   treated
```

Design matrix:

```
  (Intercept) genotypeB treatmenttreated genotypeB:treatmenttreated
1           1         0                0                          0
2           1         0                0                          0
3           1         0                1                          0
4           1         0                1                          0
5           1         1                0                          0
6           1         1                0                          0
7           1         1                1                          1
8           1         1                1                          1
```

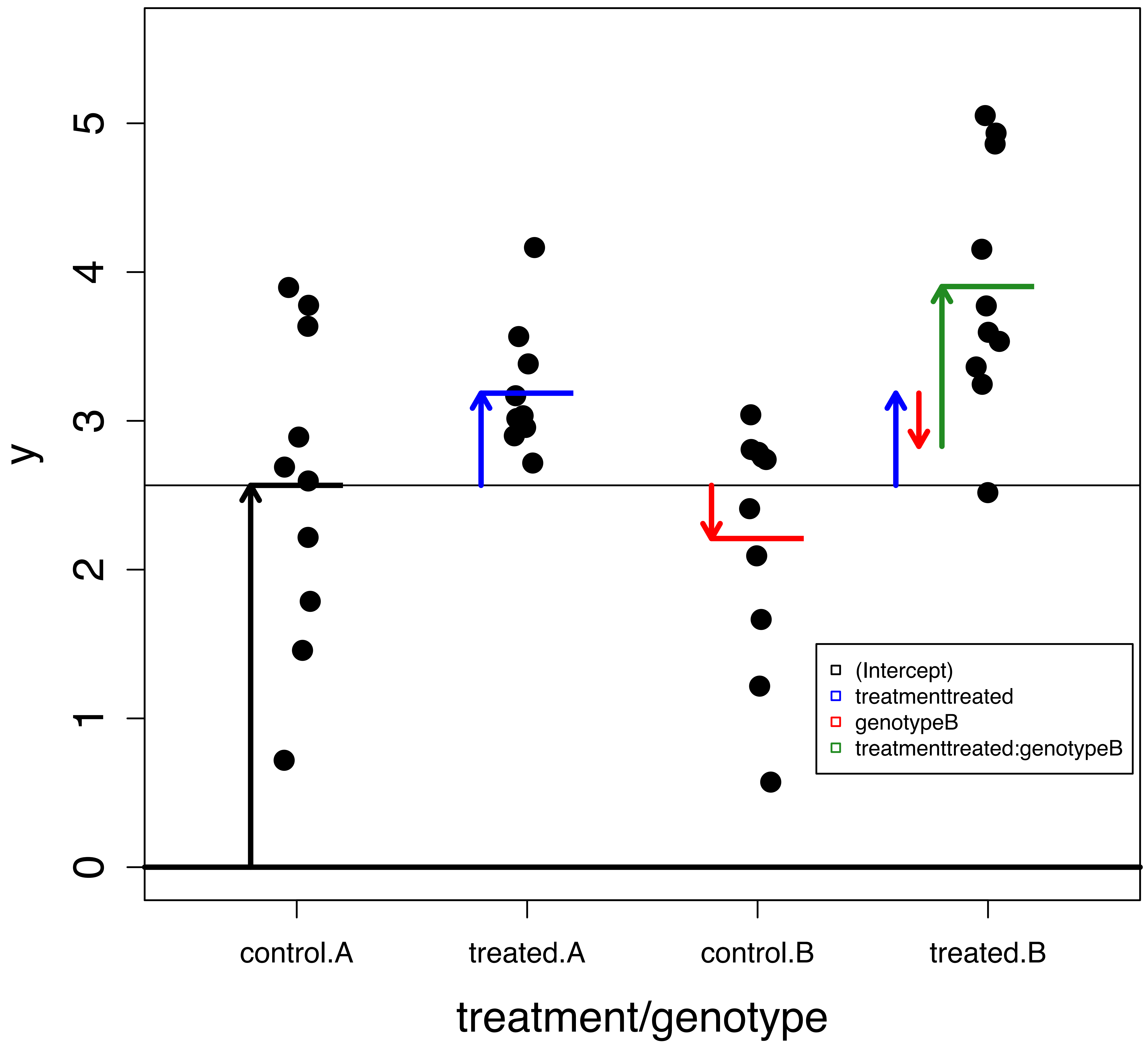
Formula:

$$\sim \text{genotype} * \text{treatment}$$

$$\sim \text{genotype} + \text{treatment} + \text{genotype:treatment}$$

Modeled values:

	genotype A	genotype B
control	**Intercept**	**Intercept + genotypeB**
treated	**Intercept + treatmenttreated**	**Intercept + genotypeB + treatmenttreated + genotypeB:treatmenttreated**


y
0
1
2
3
4
5
control.A
treated.A
control.B
treated.B
treatment/genotype
□ (Intercept)
□ treatmenttreated
□ genotypeB
□ treatmenttreated:genotypeB

Model formulas and design matrices - example 6
Two predictors, with interaction

Sample table:

```
    treat.gt
1  control.A
2  control.A
3  treated.A
4  treated.A
5  control.B
6  control.B
7  treated.B
8  treated.B
```

Design matrix:

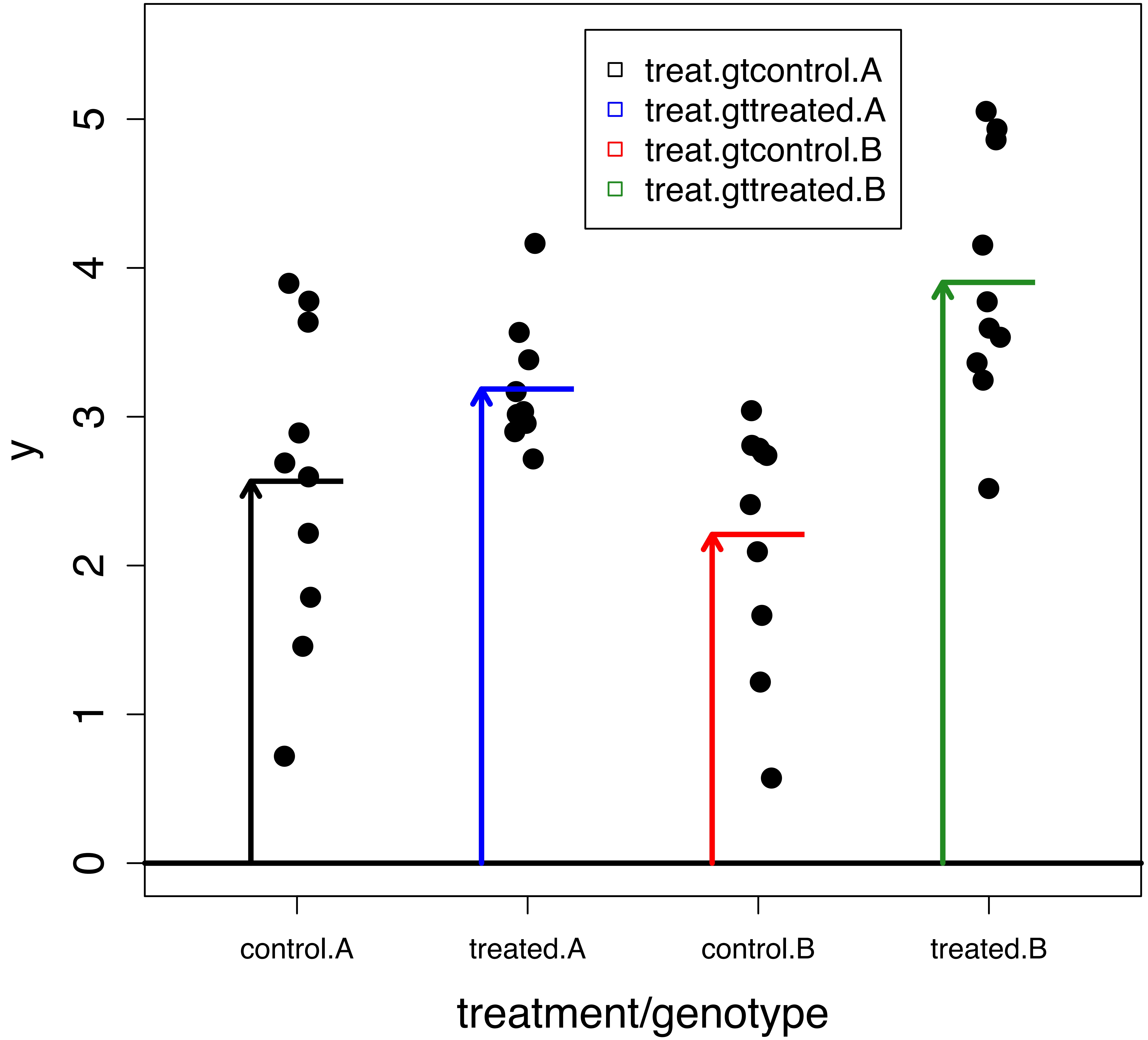
```
  treat.gtcontrol.A treat.gttreated.A treat.gtcontrol.B treat.gttreated.B
1                 1                 0                 0                 0
2                 1                 0                 0                 0
3                 0                 1                 0                 0
4                 0                 1                 0                 0
5                 0                 0                 1                 0
6                 0                 0                 1                 0
7                 0                 0                 0                 1
8                 0                 0                 0                 1
```

Formula:

$$\sim 0 + \text{treat.gt}$$

Modeled values:

	genotype A	genotype B
control	**treat.gtcontrol.A**	**treat.gtcontrol.B**
treated	**treat.gttreated.A**	**treat.gttreated.B**



□ treat.gtcontrol.A
□ treat.gttreated.A
□ treat.gtcontrol.B
□ treat.gttreated.B
y
0
1
2
3
4
5
control.A
treated.A
control.B
treated.B
treatment/genotype

References

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- Schurch et al.: How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? RNA (2016)
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