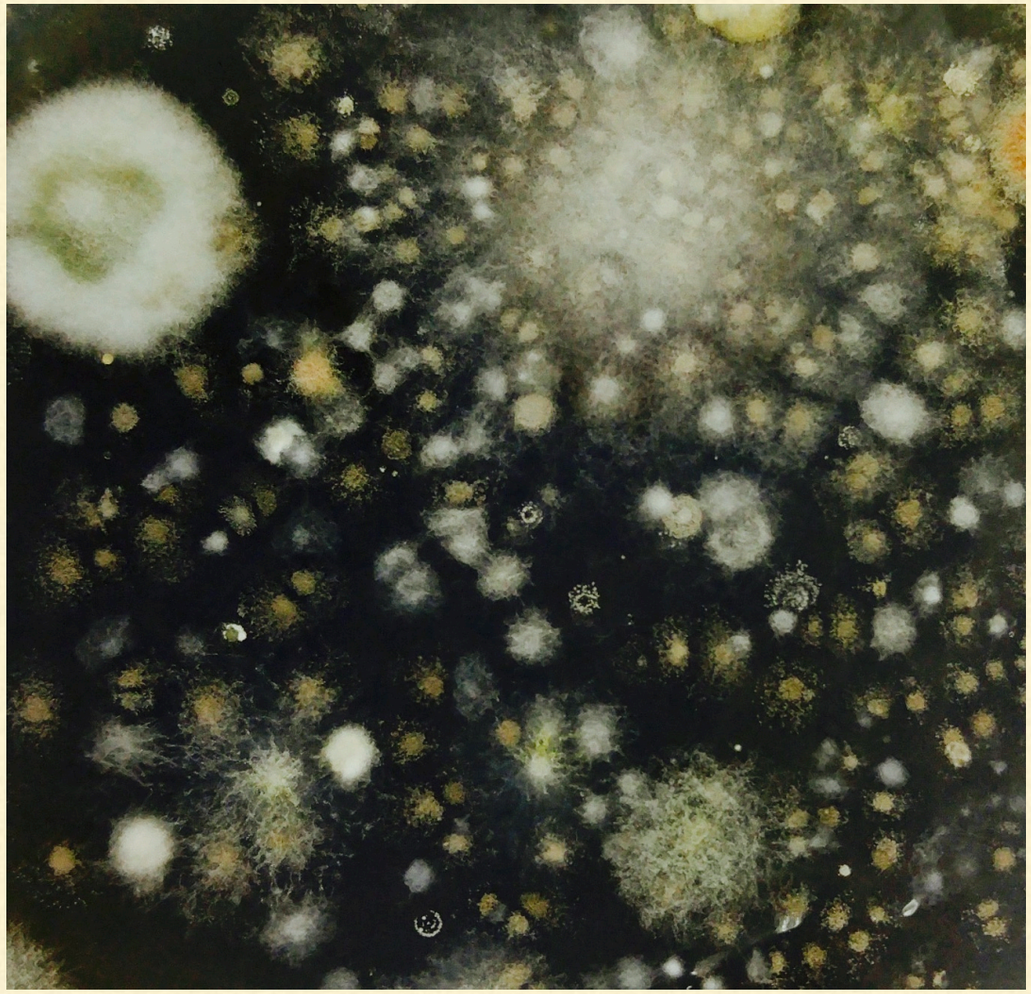


MODELING MICROBIOME DATA

Statistical Diversity Lab @ University of Washington

Amy Willis — @AmyDWillis — Assistant Professor

David Clausen — @davidandacat — PhD Candidate

Sarah Teichman — @sarah_teichman — PhD Candidate

Photo credit: T.D. Berry, Whitman lab, UW Madison

"How do I rigorously analyze my data?"

–Everyone, all the time

"It depends."

–Stat Div Lab, all the time

DECIDING ON A MODEL

- Your scientific questions should guide you in choosing your model
  - Many studies look at multiple models
  - These can answer *the same* or *different* questions
- What type of data you have may also constrain you

There is not **one** way to model/analyse your data!
You need to decide what is important to you!

DECIDING ON A MODEL

- Different models make different assumptions…
  - … and will lead you to different conclusions

LEARNING OBJECTIVES

- Learning objectives

1. ~~Learn all the models~~
2. ~~Understand all their assumptions~~
3. ~~Resolve all confusion about statistical analysis of microbiome data~~

LEARNING OBJECTIVES

- Learning objectives

1. Learn *more* about *some* models
2. Understand *some* of the *most important* assumptions and limitations of *some* methods
3. Develop some facility using software to fit models
4. Leave with more questions than ever

THE PLAN

- Modeling with microbiome data
  - Abundance
    - Multiple lecture + labs
  - Diversity:
    - Multiple lectures + lab
  - Presence/absence, trees, etc… next week!
- Misc
- Questions

THE PEP TALK

MODELING ABUNDANCE

ANALYSIS

- There are many different data/sequencing types that can be used to model "abundance"
  - amplicon - count tables
  - shotgun - coverage, proportion data…
  - qPCR / ddPCR - counts/concentrations…
- The *type of data* you have impacts the *approach you need*

SCENARIO

ABSOLUTE ABUNDANCE DATA
e.g. qPCR (16S or taxon-specific); ddPCR

ABSOLUTE ABUNDANCE	MICROBE A	MICROBE B	MICROBE C
ENVIRO 1	5	5	20
ENVIRO 2	10	10	40

↓ observe

# COPIES OBS'D/UL	MICROBE A	MICROBE B	MICROBE C	TOTAL
ENVIRO 1	**4**	**5**	**18**	**27**
ENVIRO 2	**9**	**11**	**37**	**57**

Can compare rows to rows, and columns to columns

SCENARIO

MOST HIGH-THROUGHPUT SEQUENCING DATA

~~compositional data~~

e.g. 16S counts

e.g. genome/MAG coverages

ABSOLUTE ABUNDANCE	MICROBE A	MICROBE B	MICROBE C
ENVIRO 1	5	5	20
ENVIRO 2	10	10	40

observe

# OBSERVED	MICROBE A	MICROBE B	MICROBE C	TOTAL
ENVIRO 1	**499**	**500**	**2001**	**3000**
ENVIRO 2	**250**	**251**	**1010**	**1511**

SCENARIO

PROPORTION DATA

e.g., shotgun data processed w metaphlan

ABSOLUTE ABUNDANCE	MICROBE A	MICROBE B	MICROBE C
ENVIRO 1	5	5	20
ENVIRO 2	10	10	40

↓ observe

# OBSERVED	MICROBE A	MICROBE B	MICROBE C	TOTAL
ENVIRO 1	1.01 / 6 = **0.168**	1/6 = **0.167**	3.99 / 6 = **0.665**	1
ENVIRO 2	0.99 / 6 = **0.165**	0.99 / 6 = **0.165**	4.02 / 6 = **0.67**	1

HOW DO YOU KNOW?

- You can't tell your data type from the tables alone
- You need some understanding of
  - what your technology is doing
  - and how it works

COMPARISONS WITH HTS DATA

- Can compare counts within a sample*, e.g. In Sample 1
  - 500 counts from Taxon B
  - 2001 counts from Taxon C
- Cannot compare counts across sample, e.g. for Taxon A
  - 499 from Enviro 1
  - 250 from Enviro 2

*with some caveats, like always — more later

COMPARISONS WITH HTS DATA

- I recommend against transforming sequences to proportions, and avoiding bioinformatics pipelines that only give you proportions
- This is not necessary!
- It loses information about precision!
- Good statistical methods model precision

*with some caveats, like always — more later

MODELING ABUNDANCE

- **Modeling absolute abundances**
- Modeling counts (via relative abundance parameters)
- Modeling absolute abundance parameters from relative

ABSOLUTE ABUNDANCE DATA

- qPCR and ddPCR data can usually be modeled with techniques you can learn in Applied Stats 101

- **Linear models** most generally look like

$$\text{mean outcome}_i = \beta_0 + \beta_1 X_{i1} + \ldots + \beta_p X_{ip}$$

ABSOLUTE ABUNDANCE DATA

- Examples:

$$\text{mean bacterial load}_i = \beta_0 + \beta_1 \times \mathbf{1}_{\text{person } i \text{ is on antibiotics}\}}$$

  - $\hat{\beta}_0$ is an estimate of the mean/average/expected bacterial load for people not on antibiotics

  - $\hat{\beta}_1$ is an estimate of the difference in mean bacterial load between people who are versus aren't on antibiotics

ABSOLUTE ABUNDANCE DATA

mean bacterial load$_i = \beta_0 + \beta_1 \mathbf{1}_{\text{person } i \text{ is on antibiotics}\}} + \beta_2(\text{age}_i - 40)$

- $\hat{\beta}_0$ is an estimate of the mean bacterial load for 40 y.o.'s people *not* on antibiotics
- $\hat{\beta}_1$ is an estimate of the difference in mean bacterial load between people of the same age who *are* versus *aren't* on antibiotics
- $\hat{\beta}_2$ is an estimate of the difference in mean bacterial load between people who differ in age by 1 year who have the same antibiotics use

ABSOLUTE ABUNDANCE DATA

$$\text{mean bacterial load}_i = \beta_0 + \beta_1 \mathbf{1}_{\text{person } i \text{ is on antibiotics}\}} + \beta_2(\text{age}_i - 40)$$

$$+\beta_3 \times \mathbf{1}_{\text{person } i \text{ is on antibiotics}\}} \times (\text{age}_i - 40)$$

- $\hat{\beta}_0$ is an estimate of the mean bacterial load for 40 y.o.'s people *not* on antibiotics
- $\hat{\beta}_1$ is an estimate of the difference in mean bacterial load between 40 y.o.'s who *are* versus *aren't* on antibiotics
- $\hat{\beta}_2$ is an estimate of the difference in mean bacterial load between people who differ in age by 1 year who *aren't* on antibiotics
- $\hat{\beta}_3$ is an estimate of the difference in mean bacterial load between people who differ in age by 1 year who *are* on antibiotics, compared to between people who differ in age by 1 year who *aren't* on antibiotics

ABSOLUTE ABUNDANCE DATA

- Step 1: decide on your model
- Step 2: figure out how to fit it

ABSOLUTE ABUNDANCE DATA

- Step 1: decide on your model

$$\text{mean bacterial load}_i = \beta_0 + \beta_1 \mathbf{1}_{\text{person } i \text{ is on antibiotics}\}} + \beta_2 \mathbf{1}_{\text{person } i\text{'s sample is from sputum}\}}$$

- Step 2: figure out how to fit it

```
> my_data %>%
+   lm(ddpcr ~ Treatment + `Sample Type`, data = .)

Call:
lm(formula = ddpcr ~ Treatment + `Sample Type`, data = .)

Coefficients:
        (Intercept)          TreatmentON
             996530              -409238
`Sample Type`Sputum
            1006955
```

ABSOLUTE ABUNDANCE DATA

- Step 1: decide on your model

$$\text{mean bacterial load}_i = \beta_0 + \beta_1 \mathbf{1}_{\text{person } i \text{ is on antibiotics}\}} + \beta_2 \mathbf{1}_{\text{person } i\text{'s sample is from sputum}\}}$$

$$+ \beta_3 \mathbf{1}_{\text{person } i \text{ is on antibiotics}\}} \mathbf{1}_{\text{person } i\text{'s sample is from sputum}\}}$$

- Step 2: figure out how to fit it

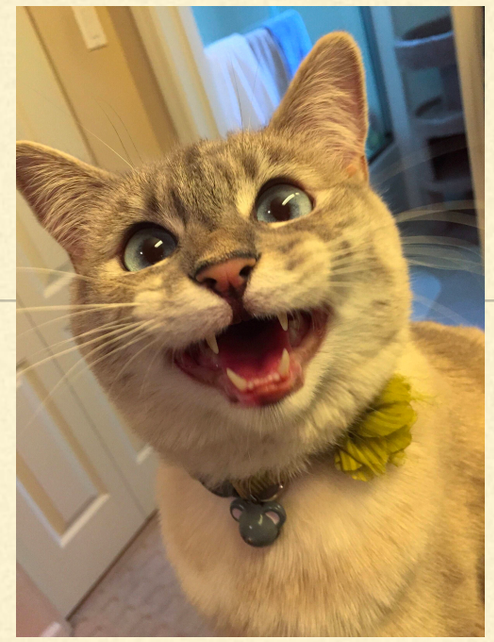
```
> my_data %>%
+   lm(ddpcr ~ Treatment * `Sample Type`, data = .)

Call:
lm(formula = ddpcr ~ Treatment * `Sample Type`, data = .)

Coefficients:
                     (Intercept)                      TreatmentON
                          968202                        -234550
          `Sample Type`Sputum  TreatmentON:`Sample Type`Sputum
                         1063610                        -349375
```

ACCESSING `LM` LAB

Get pumped!

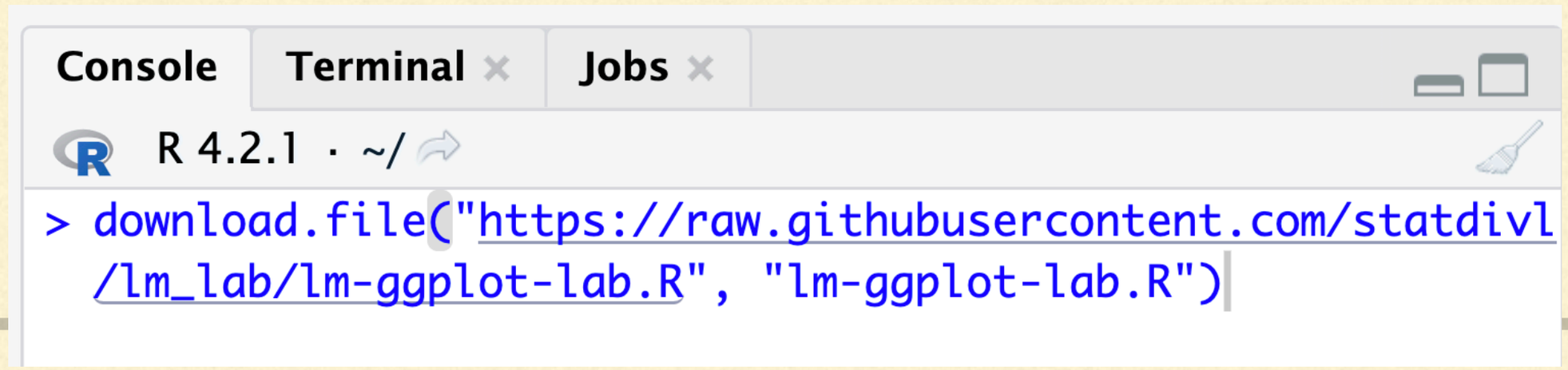


1. Go to schedule on Wiki to Sunday afternoon, click on "Labs"

2. Copy the command under the lab we're working on

lm lab:
download.file("https://raw.githubusercontent.com/statdivlab/stamps2022/main/Sunday-afternoon/labs/lm_lab/lm-ggplot-lab.R", "lm-ggplot-lab.R")

3. Run this command in your RStudio Server console



MODELING ABUNDANCE

- Modeling absolute abundances
- **Modeling counts (via relative abundance parameters)**
- Modeling absolute abundance parameters from relative

MODELING COUNTS

- Common goal:
  - Determine which taxa are present in greater abundance in one group compared to another

- *"Differential abundance [is] a category subject to some controversy in part on account of the fact that no unambiguous definitions of 'differential' or 'abundance' are widely agreed upon."*

MODELING COUNTS

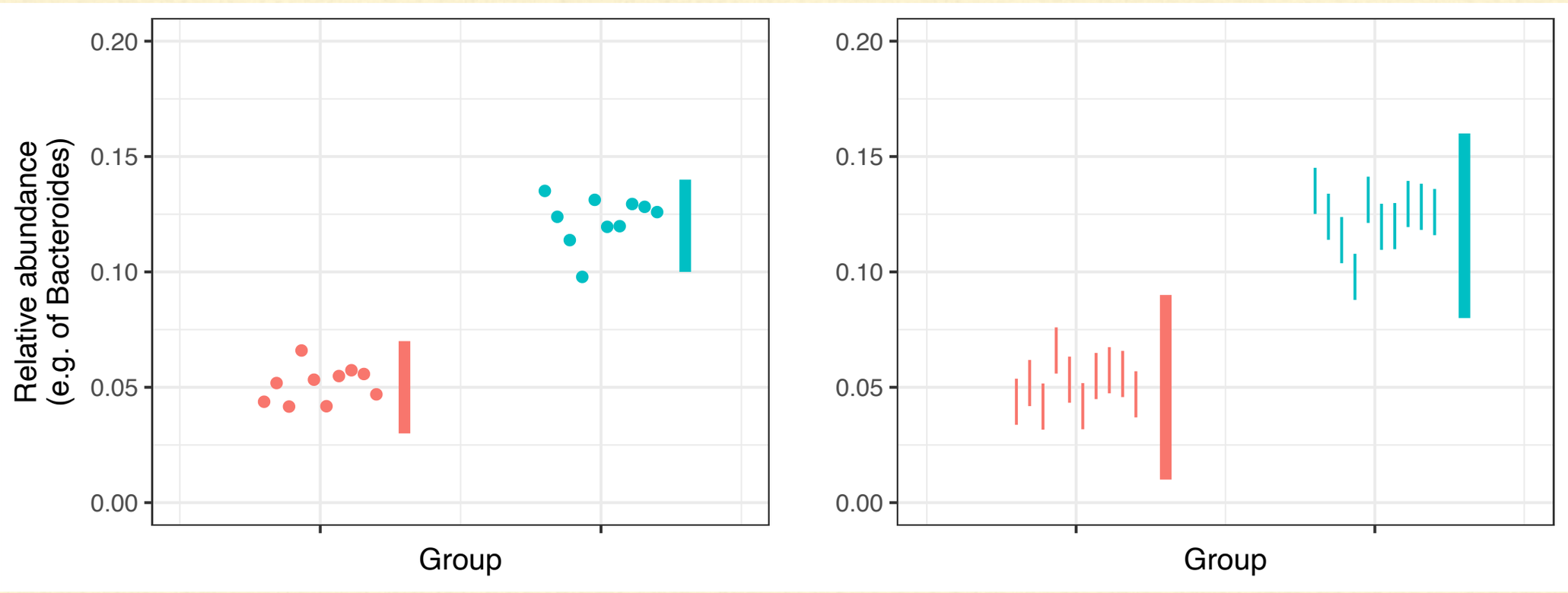
- Many methods exist
  - ALDEx
  - ANCOM
  - corncob
  - DESeq2
  - edgeR
  - metagenomeSeq
  - MaAsLin
  - LEfSE
  - limma voom
  - Wilcoxon on proportions
  - t-tests on proportions
- multiple versions of almost all methods; multiple options for almost all methods

MODELING COUNTS

- Many methods exist
  - ALDEx
  - ANCOM
  - corncob
  - DESeq2
  - edgeR
  - metagenomeSeq
  - MaAsLin
  - LEfSE
  - limma voom
  - Wilcoxon on proportions
  - t-tests on proportions
- multiple versions of almost all methods; multiple options for almost all methods

SAMPLE ≠ POPULATION

- Observed relative abundance ≠ true relative abundance
- Any statistical test for the microbiome needs to account for this *measurement error*



CORNCOB

COmpositional RegressioN for Correlated Observations with the Beta-binomial

Bryan Martin, UW Statistics

Daniela Witten, UW Statistics

- Models **relative abundance**
  - Connects count data to relative abundances
  - Models relative abundance with covariates
- Hypothesis testing for changes in
  - relative abundance
  - variance in abundance

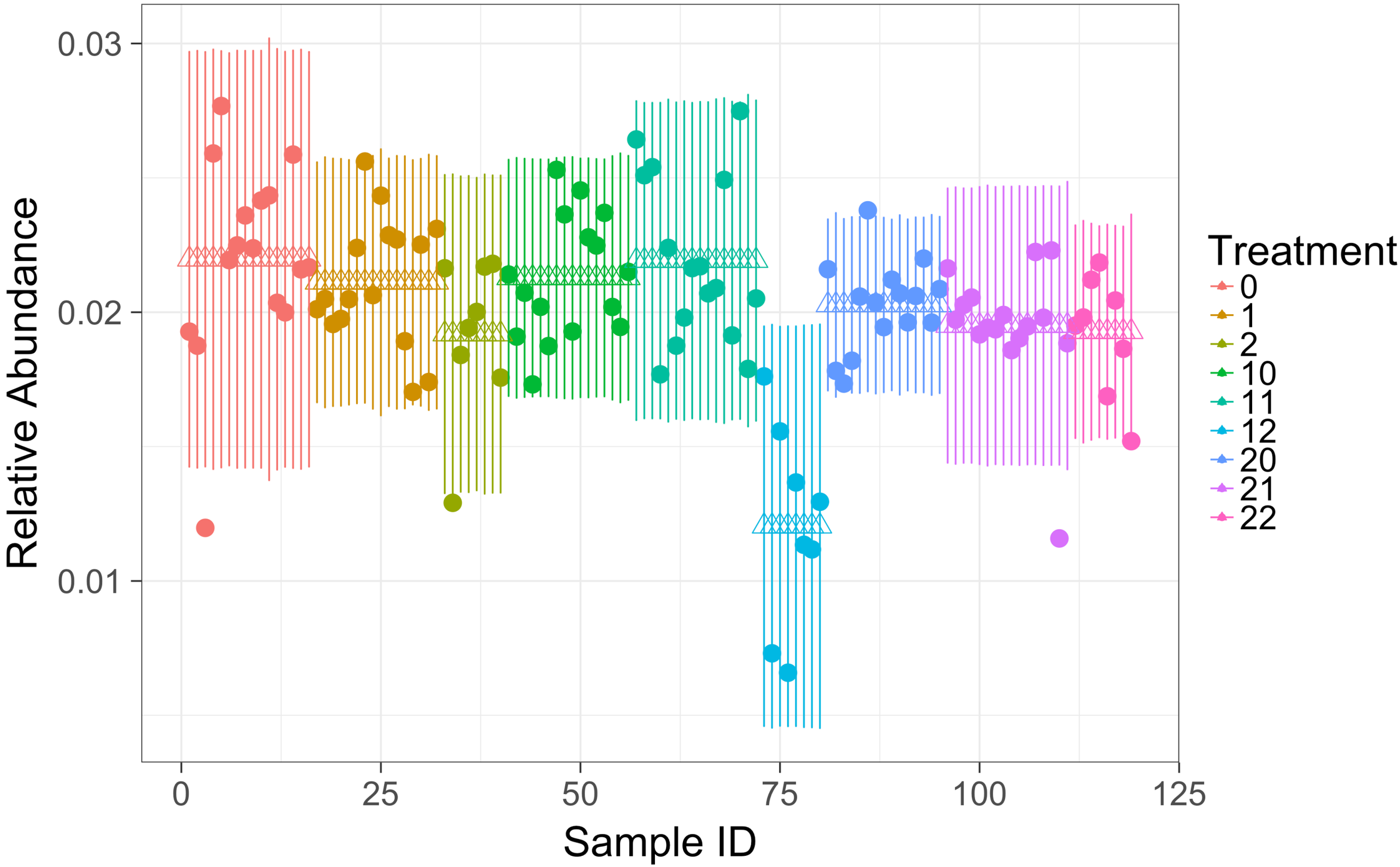
CORNCOB

COmpositional RegressioN for Correlated Observations with the Beta-binomial

- "The relative abundance of *S. aureus* is significantly lower in the treatment group (95% CI for $\beta_{treatment}$: (-4.74, -2.91), q = 0.003, see Methods)."

- "Methods: All phylum-level relative abundances were modeled using 🌽corncob🌽* with a logit-link for mean and dispersion. Differential abundance (DA) was modeled as a linear function of age and treatment group. Differential variability was modeled as a function of treatment group. The parametric Wald test was used to test DA hypotheses. …"

* Emojis optional



Data from Whitman et. al, (2015) *ISME*

CORNCOB

COmpositional RegressioN for Correlated Observations with the Beta-binomial

- Addresses measurement error issue
- Adjusts for different sequencing depths
- Suitable with multiple covariates, various experimental designs
- Mean and variance testing
- All taxa/genes; inbuilt FDR control
- No need to choose a psuedocount

BETA-BINOMIAL DISTRIBUTION

$$W_i \mid Z_i, M_i \sim \text{Binomial}(M_i, Z_i)$$

$$Z_i \sim \text{Beta}(a_{1i}, a_{2i})$$

- n = samples, indexed by i = 1, …, n
- W_i = # of individuals observed in the taxon/gene of interest
- M_i = total # of counts observed
- Z_i = the (latent) relative abundance in sample i

BETA-BINOMIAL DISTRIBUTION

$$W_i \mid Z_i, M_i \sim \text{Binomial}(M_i, Z_i)$$

$$Z_i \sim \text{Beta}(a_{1i}, a_{2i})$$

what you need

- n = samples, indexed by i = 1, …, n
- W_i = # of individuals observed in the taxon/gene of interest
- M_i = total # of counts observed
- Z_i = the (latent) relative abundance in sample i

LINKING ABUNDANCE TO COVARIATES

1. Parameters

$$\mu_i = \frac{a_{1,i}}{a_{1,i} + a_{2,i}},$$

"(latent) relative abundance"

$$\phi_i = \frac{1}{a_{1,i} + a_{2,i} + 1}$$

"within sample correlation"
"absolute abundance overdispersion"

2. Link to covariates

μ_i is a function of $\mathbf{X}_i,\ \boldsymbol{\beta}$

ϕ_i is a function of $\mathbf{X}_i^*,\ \boldsymbol{\beta}^*$

LINKING ABUNDANCE TO COVARIATES

1. Parameters

$$\mu_i = \frac{a_{1,i}}{a_{1,i} + a_{2,i}},$$

"(latent) relative abundance"

$$\phi_i = \frac{1}{a_{1,i} + a_{2,i} + 1}$$

"within sample correlation"
"absolute abundance overdispersion"

2. Link to covariates

what you need

μ_i is a function of $\mathbf{X}_i$ $\boldsymbol{\beta}$

ϕ_i is a function of $\mathbf{X}_i^*$ $\boldsymbol{\beta}^*$

```
Call:
bbdml(formula = OTU.1 ~ Day + Amdmt, phi.formula = ~Day, data = soil_full)


Coefficients associated with abundance:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) -0.78562    0.02507 -31.336  < 2e-16 ***
Day1         0.35077    0.03807   9.214 2.31e-15 ***
Day2         0.14267    0.02389   5.971 2.88e-08 ***
Amdmt1       0.03466    0.02436   1.423    0.158
Amdmt2       0.19374    0.04120   4.702 7.44e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Coefficients associated with dispersion:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  -5.6957     0.2971 -19.173   <2e-16 ***
Day1          1.1279     0.4366   2.584   0.0111 *
Day2         -0.7231     0.4292  -1.685   0.0948 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Log-likelihood: -1056.1
```

CORNCOB

COmpositional RegressioN for Correlated Observations with the Beta-binomial

- Coefficients are on the logit relative abundance scale
  - *Negative* coefficients indicate *decreased* estimated abundance
- Usually we are interested in testing for differential abundance, controlling for the effect of the covariate on variability

C🌽RNC🌽B AND DESEQ2

- **Similarities**
  - Easy to use
  - Use counts to assess precision of estimates (no rarefying)
  - Benjamini-Hochberg adjustment for multiple comparisons
  - Dispersion parameter for overdispersion
  - Tests for differential abundance

C🌽RNC🌽B AND DESEQ2

- Designed for microbiome count data
- Models relative abundance & overdispersion
- Different structure for different taxa
- Models zeros without psuedocounts
- Easy to diagnose model misspecification

- Designed for RNAseq data
- Models relative abundance only
- Constrained dispersion
- Individual microbes are assumed independent
- Geometric mean can't handle zeroes without psuedocounts
- Not so easy to diagnose model misspecification problems

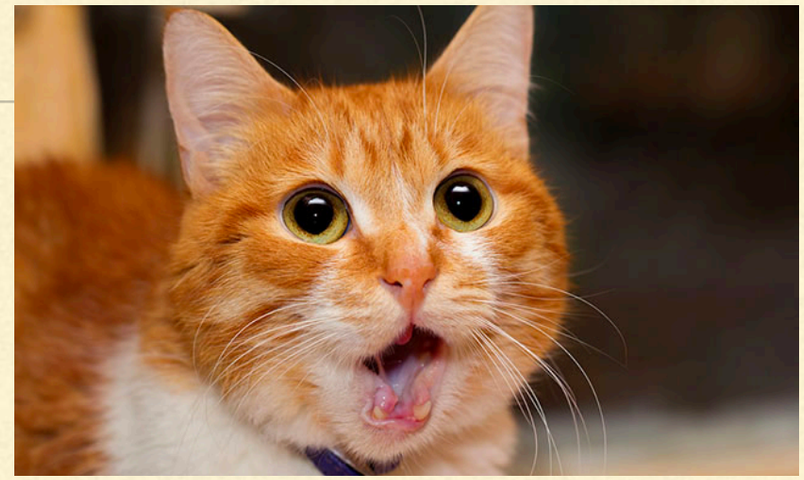
SUMMARY: CORNCOB

- Modeling and hypothesis testing for relative abundances using count data
- Adjusts for different sequencing depth
- Valid hypothesis testing with small sample sizes (use Beta-binomial bootstrap for hypothesis test)
- False discovery rate control for testing many taxa

🌽🌽 github.com/bryandmartin/corncob 🌽🌽

🎉🎉 Martin, Witten & Willis, 2020, *Annals of Applied Statistics* 🎉🎉

ACCESSING `CORNCOB` LAB



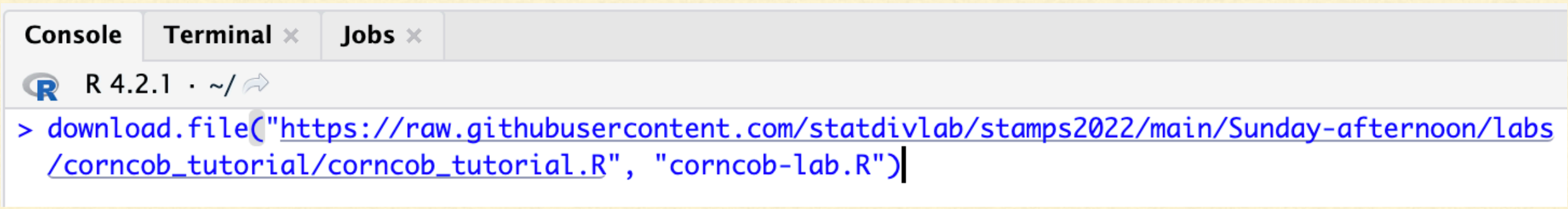
Get pumped!

1. Go to schedule on Wiki to Sunday afternoon, click on "Labs"

2. Copy the command under the lab we're working on

```
corncob lab:
download.file("https://raw.githubusercontent.com/statdivlab/stamps2022/main/Sunday-afternoon/labs/corncob_tutorial/corncob_tutorial.R", "corncob-lab.R")
```

3. Run this command in your RStudio Server console



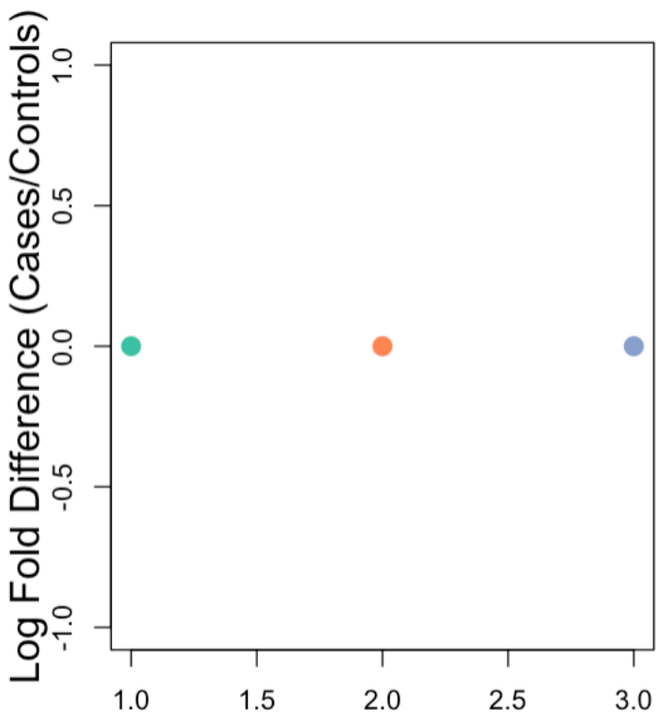
MODELING ABUNDANCE

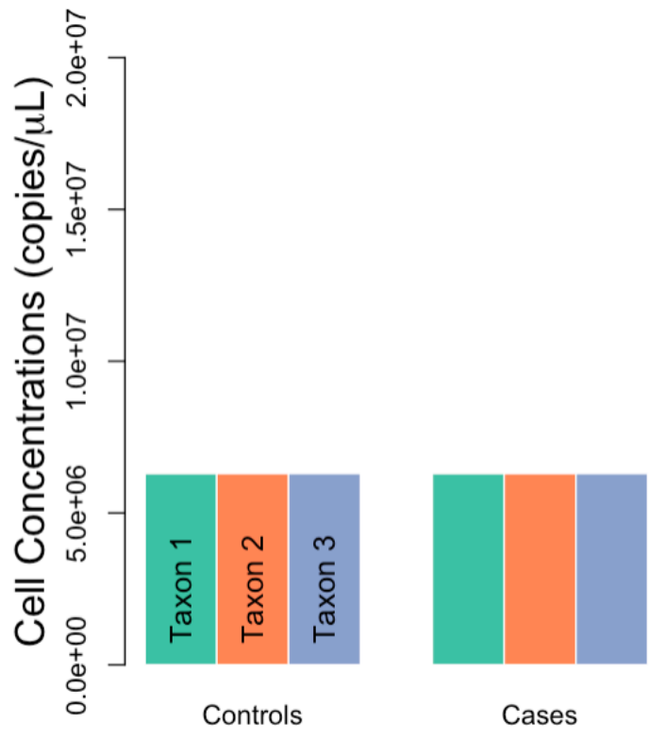
- Modeling absolute abundances
- Modeling counts (via relative abundance parameters)
- **Modeling absolute abundance parameters from relative**

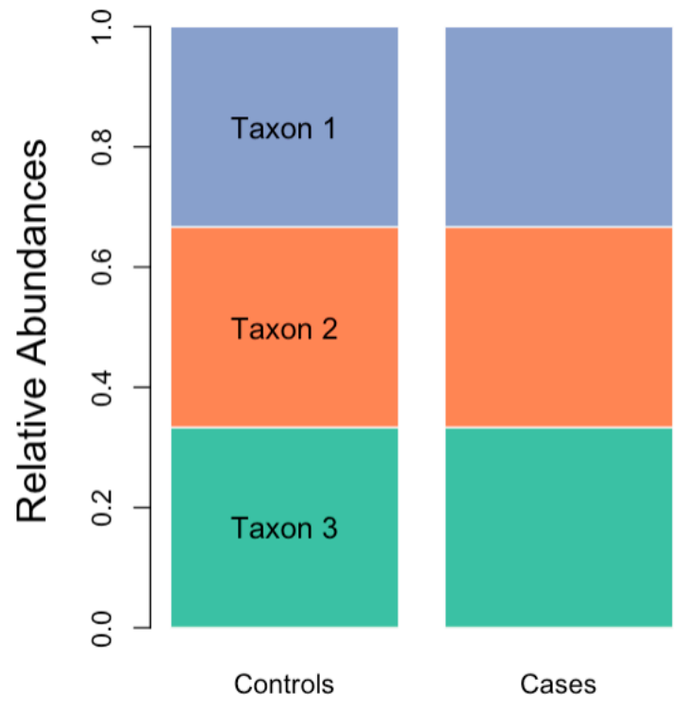
RADEMU

- radEmuAbPill
  - Using **r**elative **a**bundance **d**ata
    - to **e**stimate **mu**ltiplicative differences in **ab**solute abundances
      - with **p**artially **i**dentified **l**og-**l**inear models
- Basic idea
  - We observe "relative abundance" data (e.g., #reads by taxon)
  - We'd like to say something about an "absolute" quantity (like cell concentration)
  - Can we?

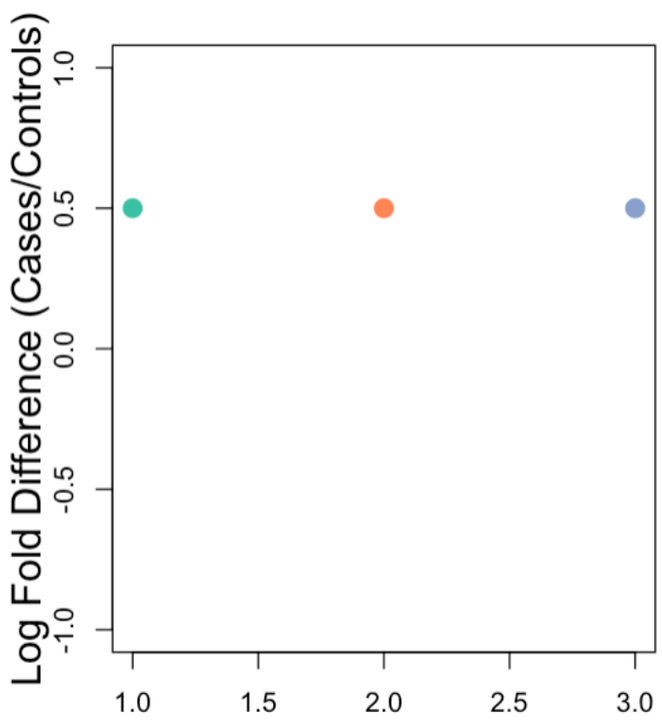
FOLD DIFFERENCES

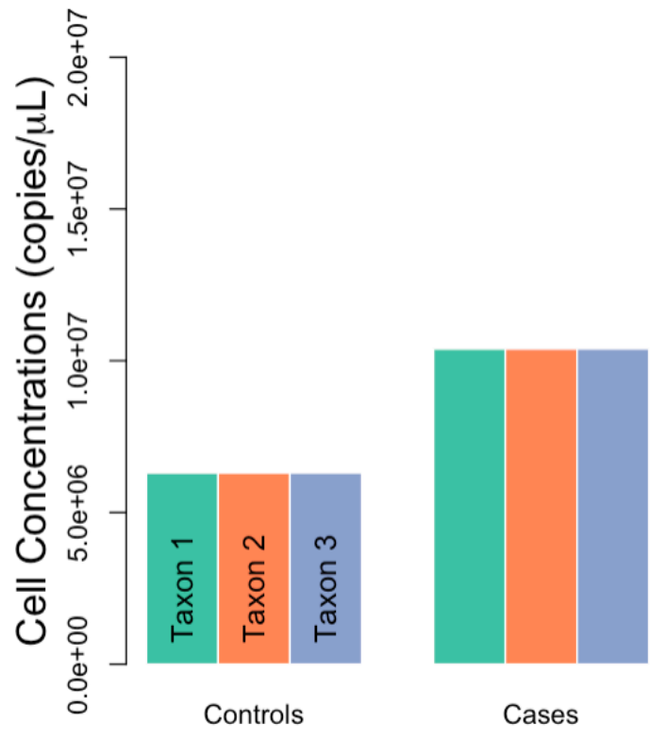


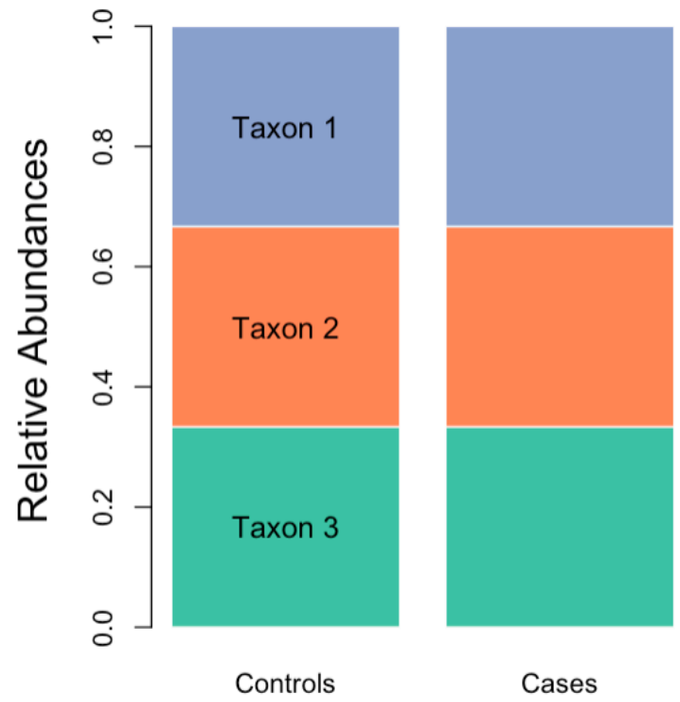




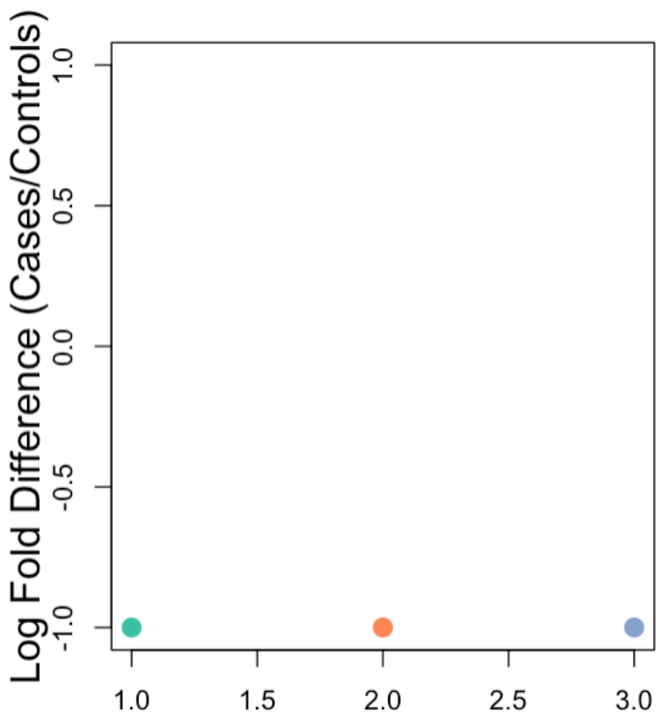
FOLD DIFFERENCES

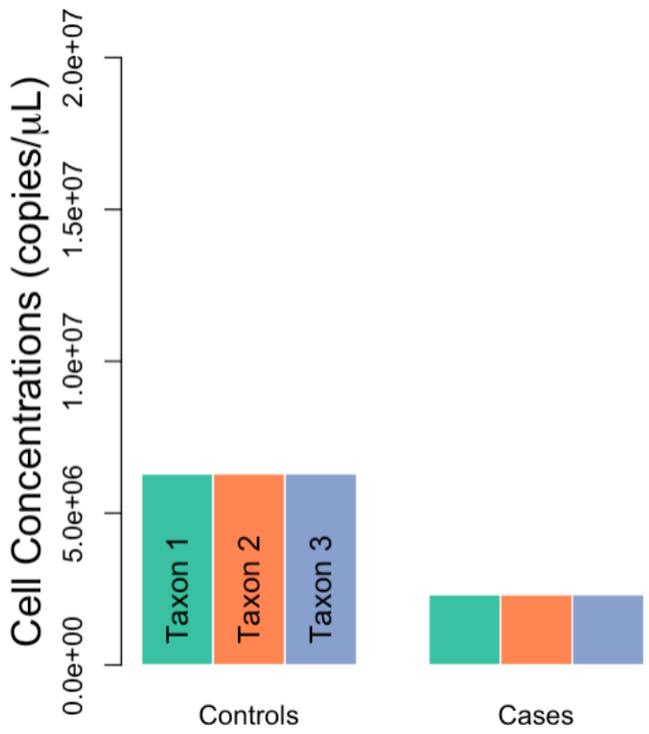


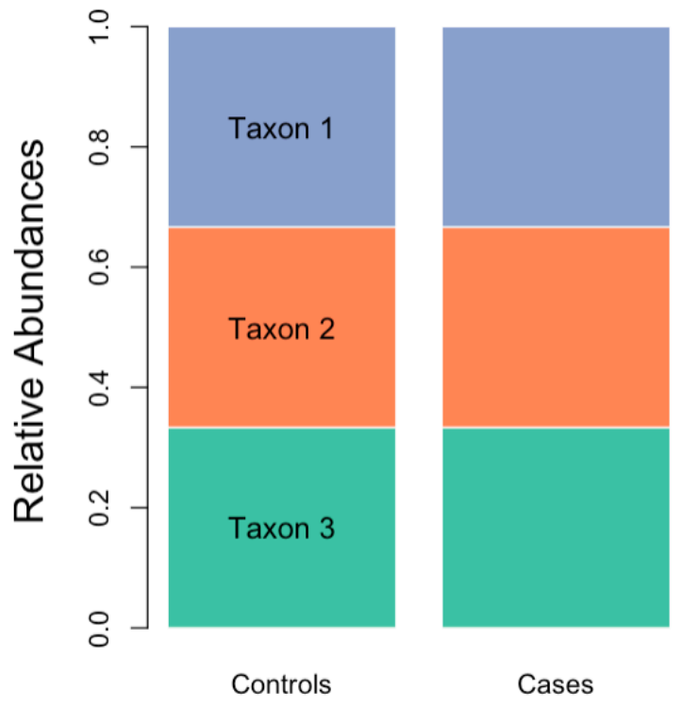




FOLD DIFFERENCES

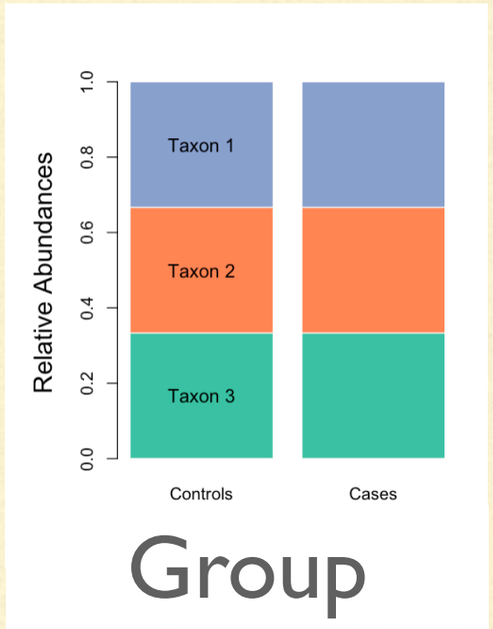


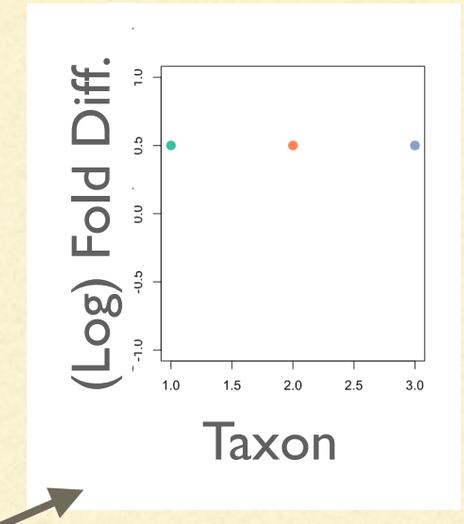




STARTING FROM PROPORTIONS

We observe

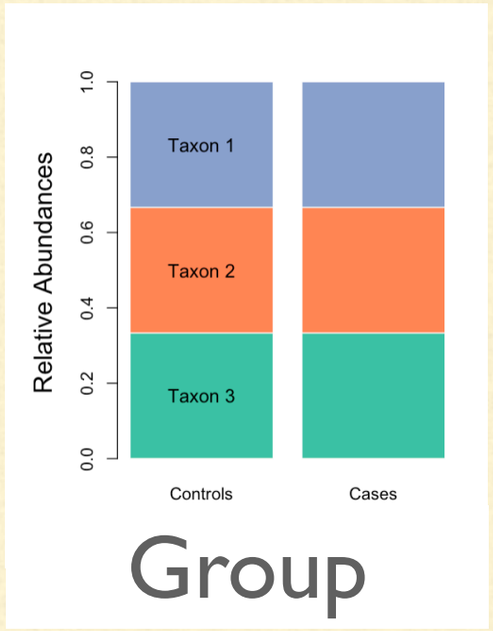




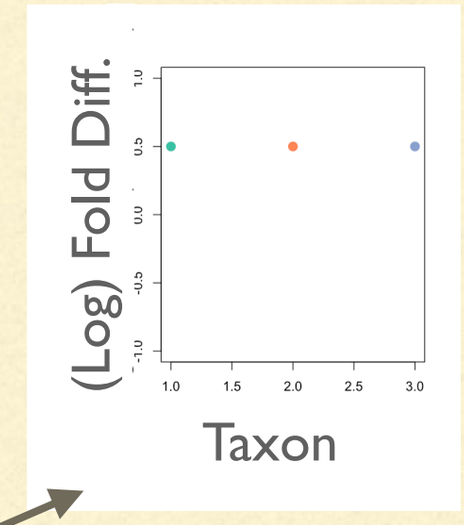
Consistent with this

STARTING FROM PROPORTIONS

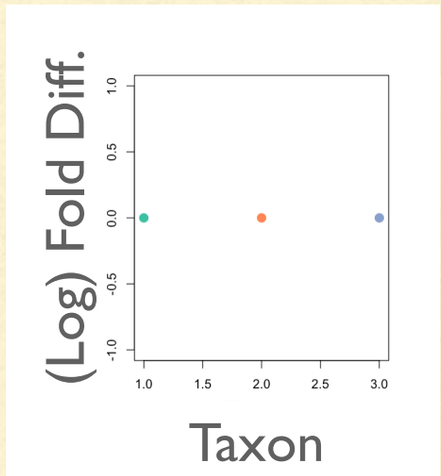
We observe



Consistent with this

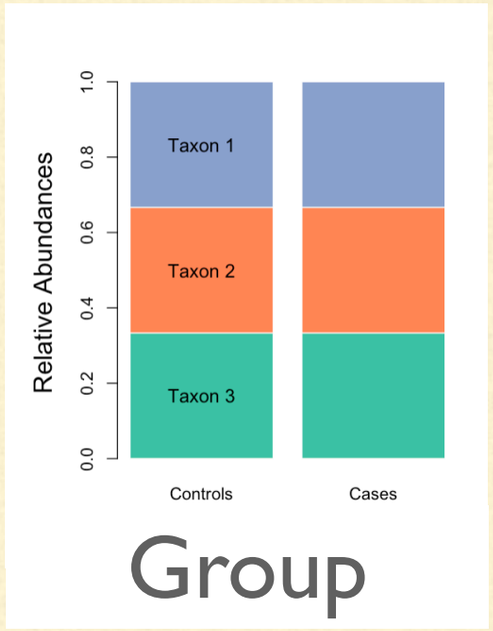


Or this

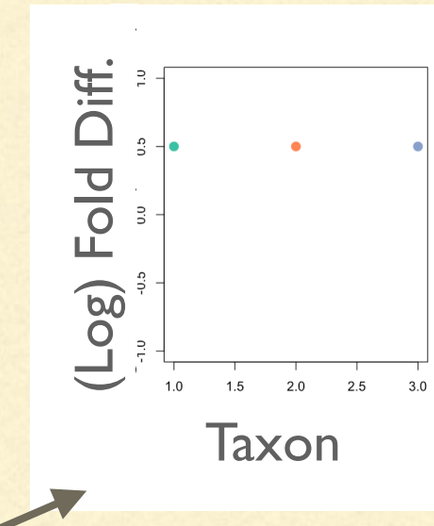


STARTING FROM PROPORTIONS

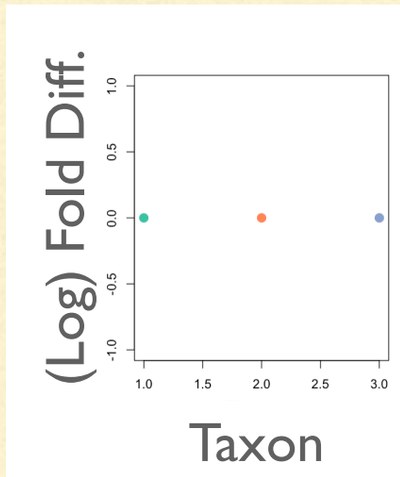
We observe



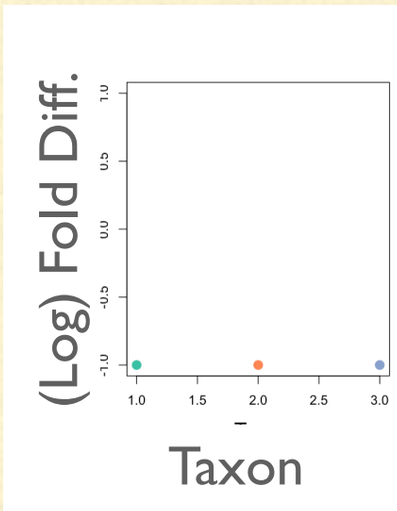
Consistent with this



Or this

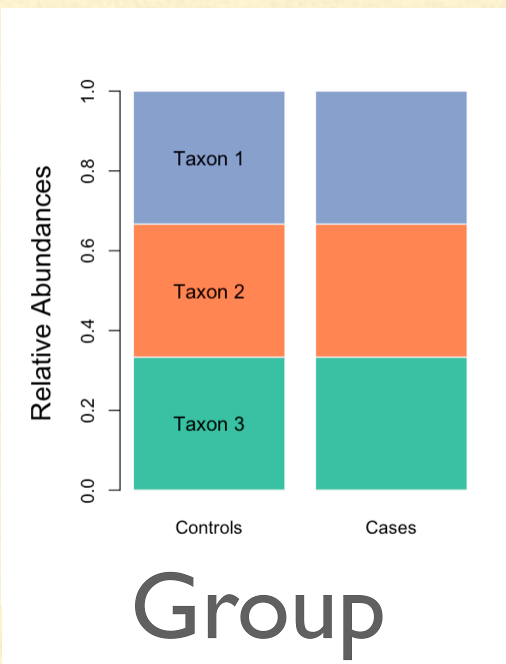


Or this

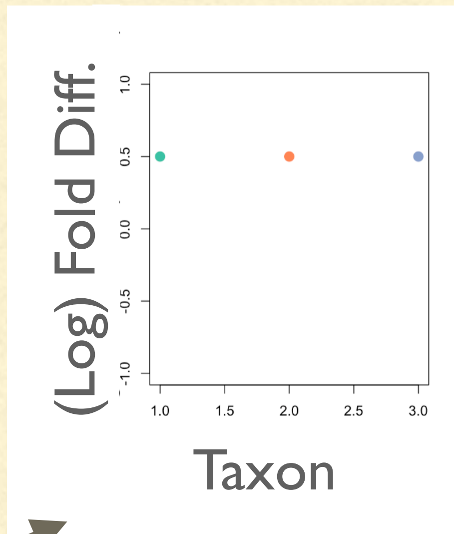


STARTING FROM PROPORTIONS

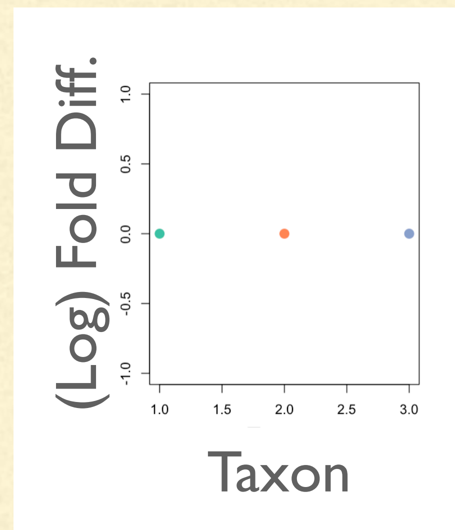
We observe



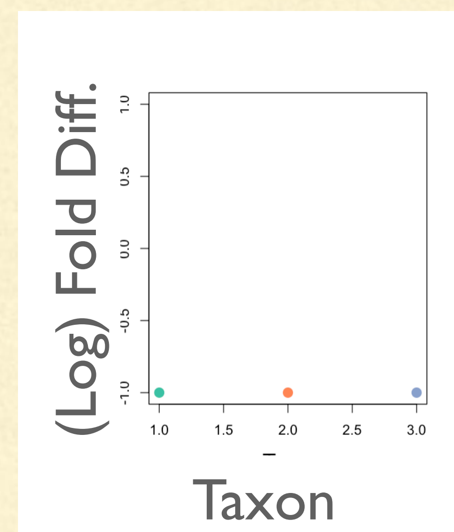
Consistent with this



Or this



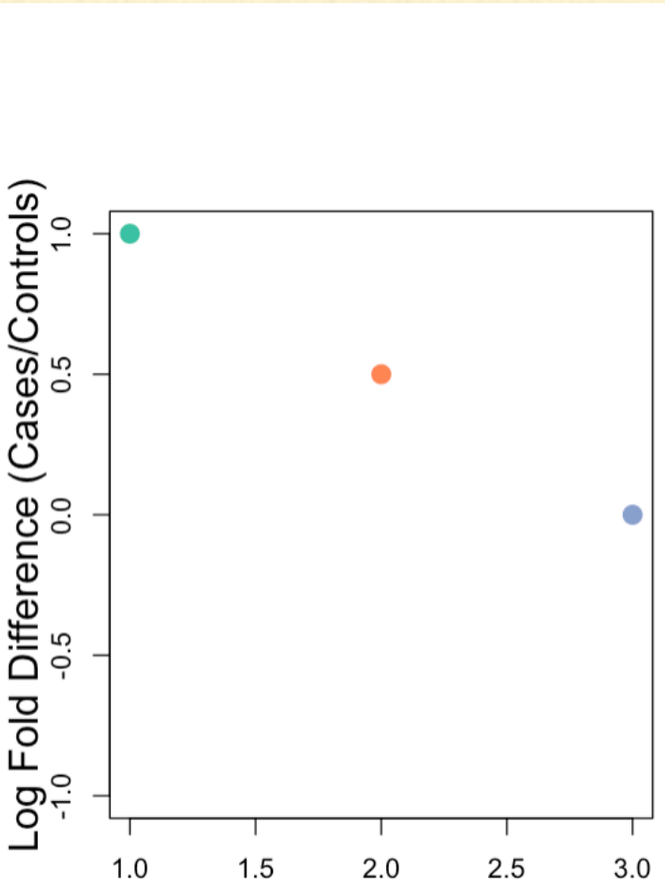
Or this

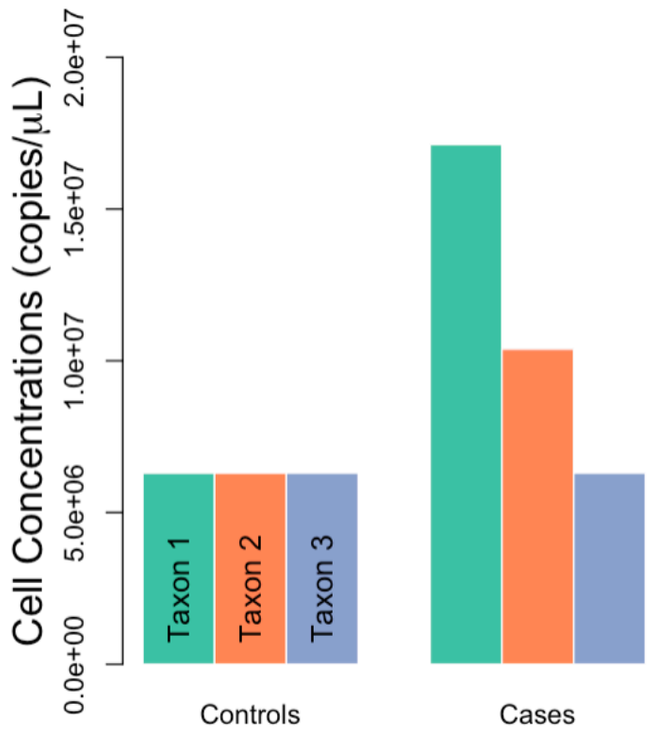


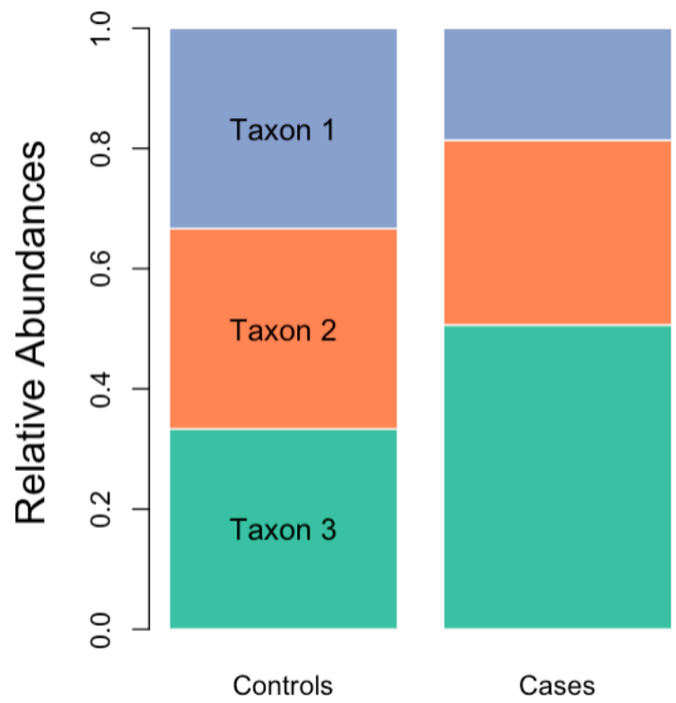
Or…

…

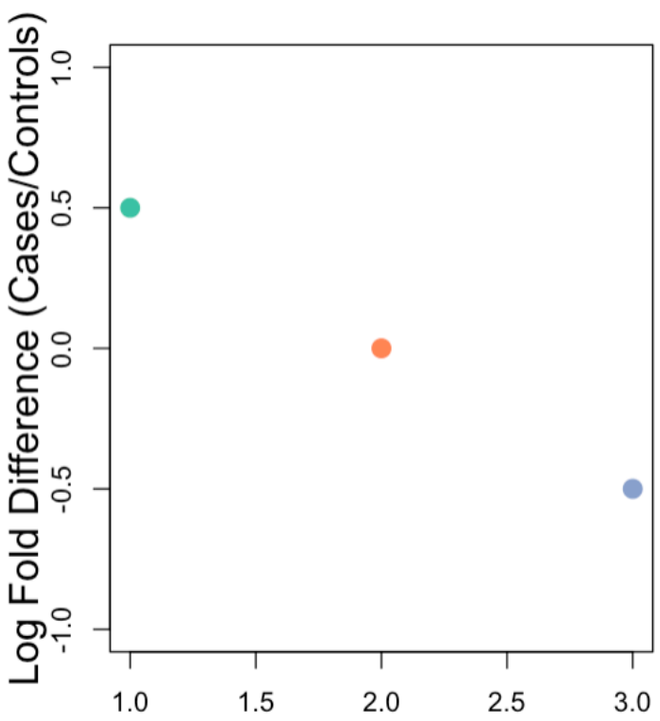
FOLD DIFFERENCES

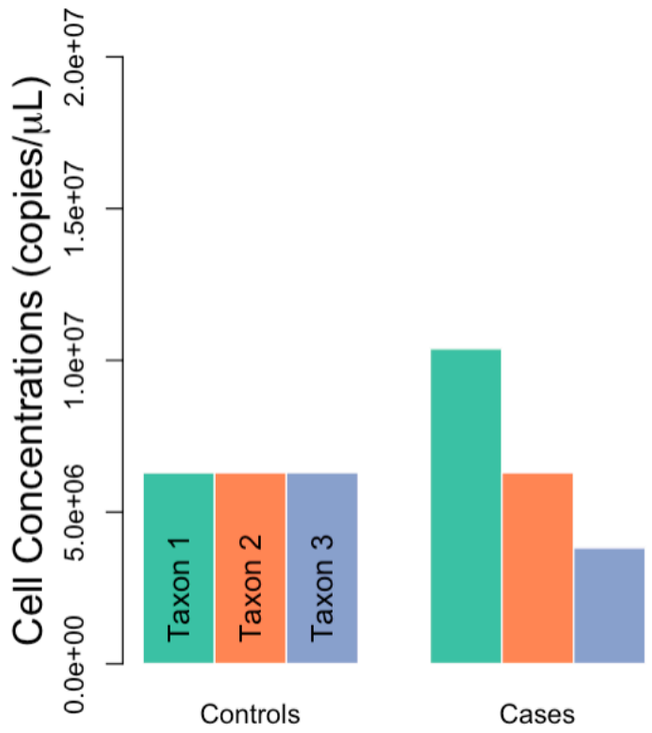


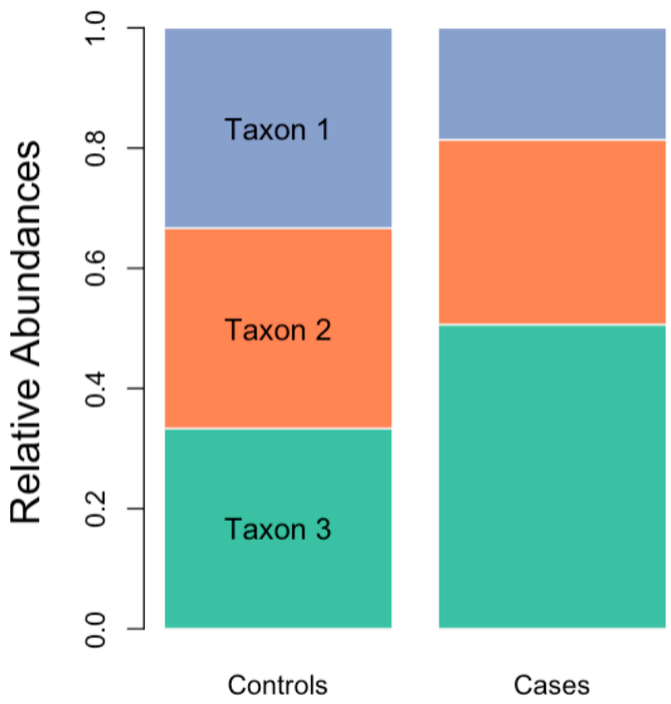




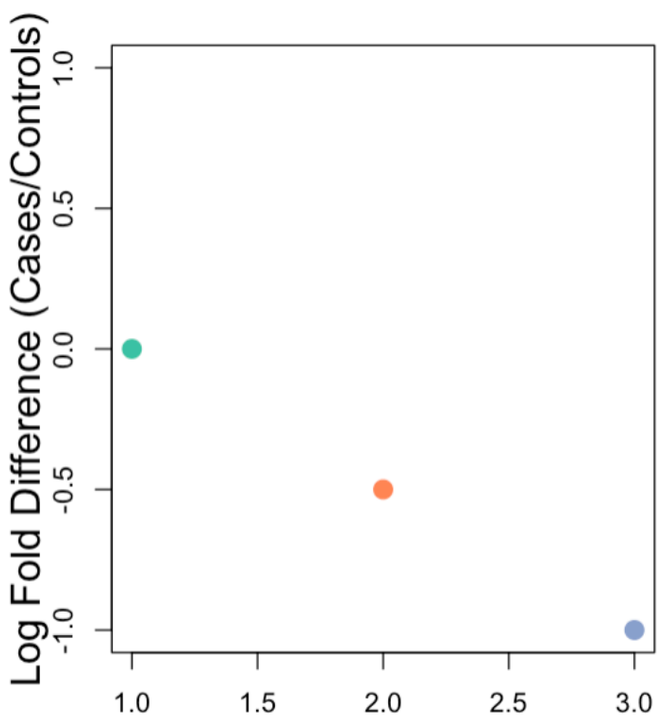
FOLD DIFFERENCES

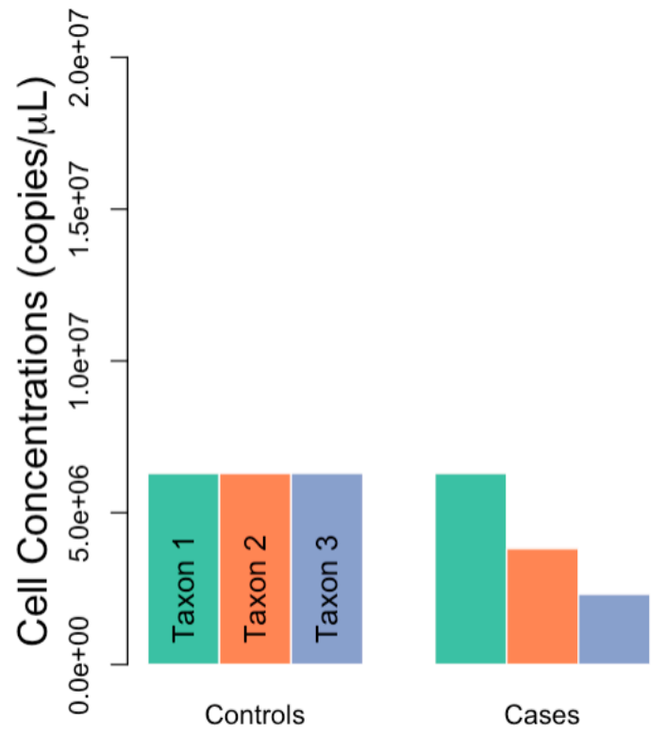


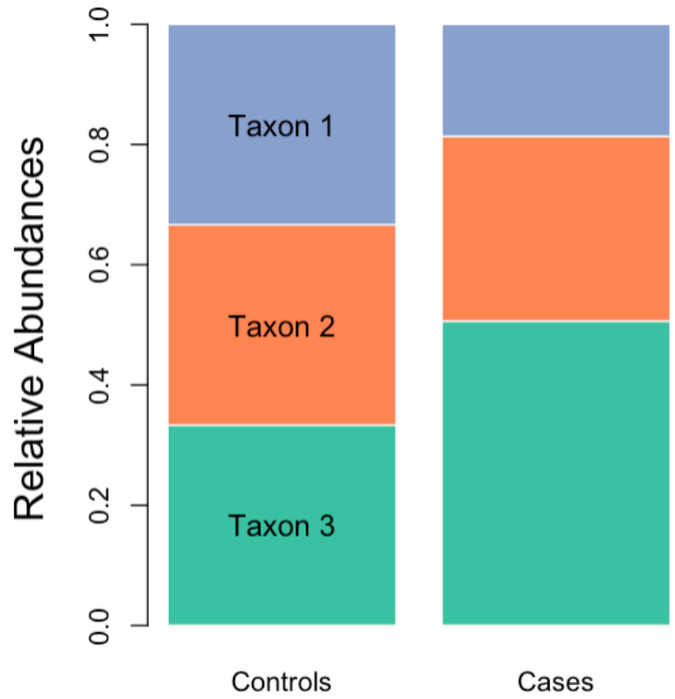




FOLD DIFFERENCES

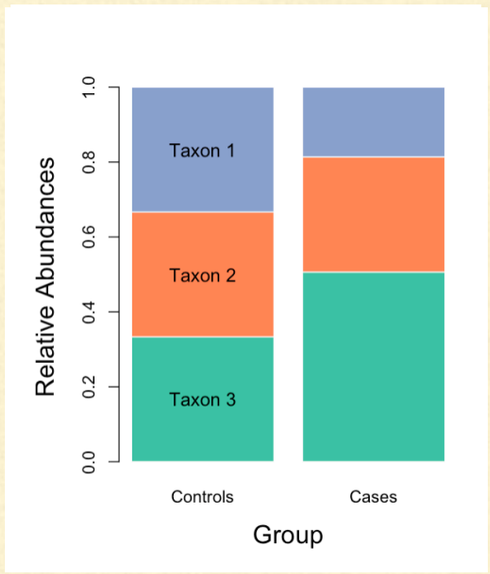




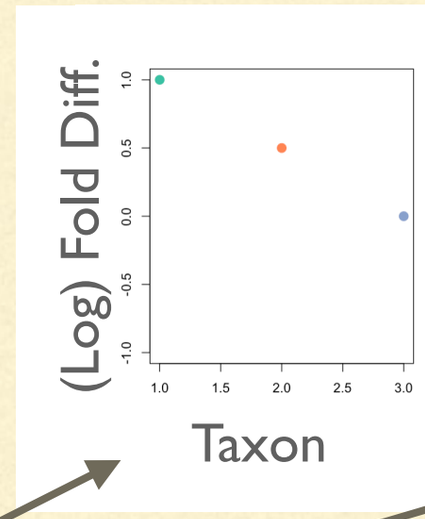


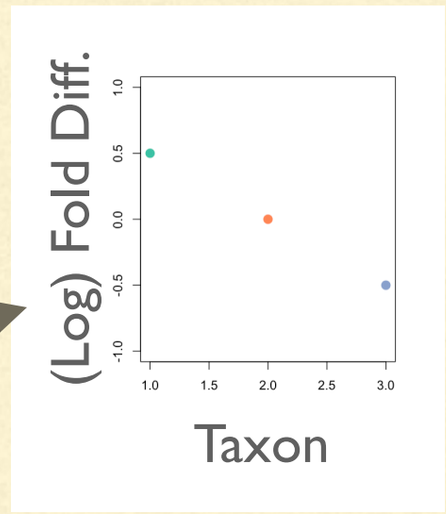
STARTING FROM PROPORTIONS

We observe

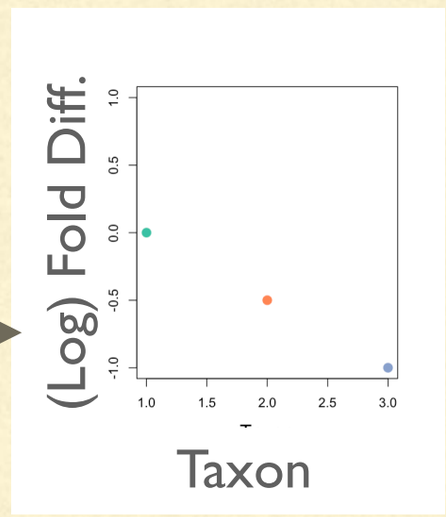


Consistent with this





Or this



Or this

Or…

…

RADEMU AGAIN

- This pattern holds in general
- We can use this to estimate fold differences* in absolute abundance from sequencing data
  - *Up to a location shift

RADEMU AGAIN

MATH FACT!

- This pattern holds in general
- We can use this to estimate fold differences* in absolute abundance from sequencing data
  - *Up to a location shift

RADEMU AGAIN

MATH FACT!

e.g., $\frac{\text{mean cell conc. F. prauznitzii in cases}}{\text{mean cell conc. F. prauznitzii in controls}}$

- This pattern holds in general
- We can use this to estimate fold differences* in absolute abundance from sequencing data
  - *Up to a location shift

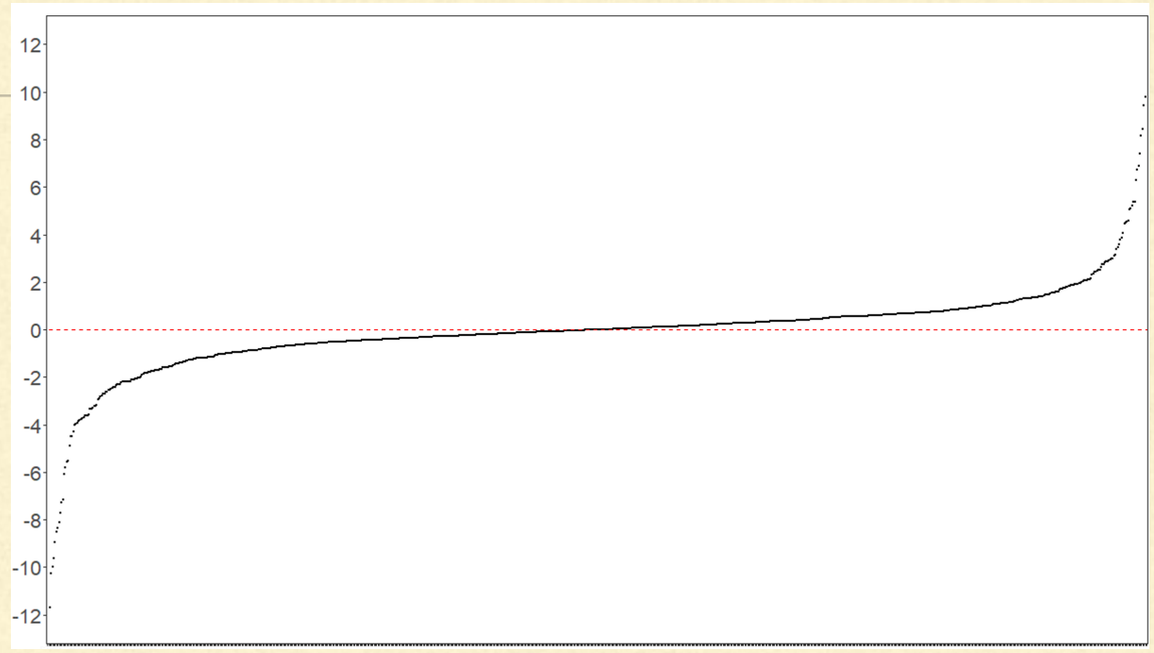
RADEMU: EXAMPLE

- Wirbel et al. (2019): meta-analysis of 4 case-control studies of association between gut microbiome and colorectal cancer
    - Cases = participants diagnosed with colorectal cancer
    - Controls = participants without a colorectal cancer diagnosis
    - Published data: mOTU tables
        - mOTU = “metagenomic OTU”
        - Clustering based on a set of highly conserved marker genes
- Our goal: characterize which mOTUs unusually over/under-represented in cases

Wirbel, Jakob, et al. "Meta-analysis of fecal metagenomes reveals global microbial signatures that are specific for colorectal cancer." *Nature Medicine* 25.4 (2019): 679-689.

RADEMU: EXAMPLE

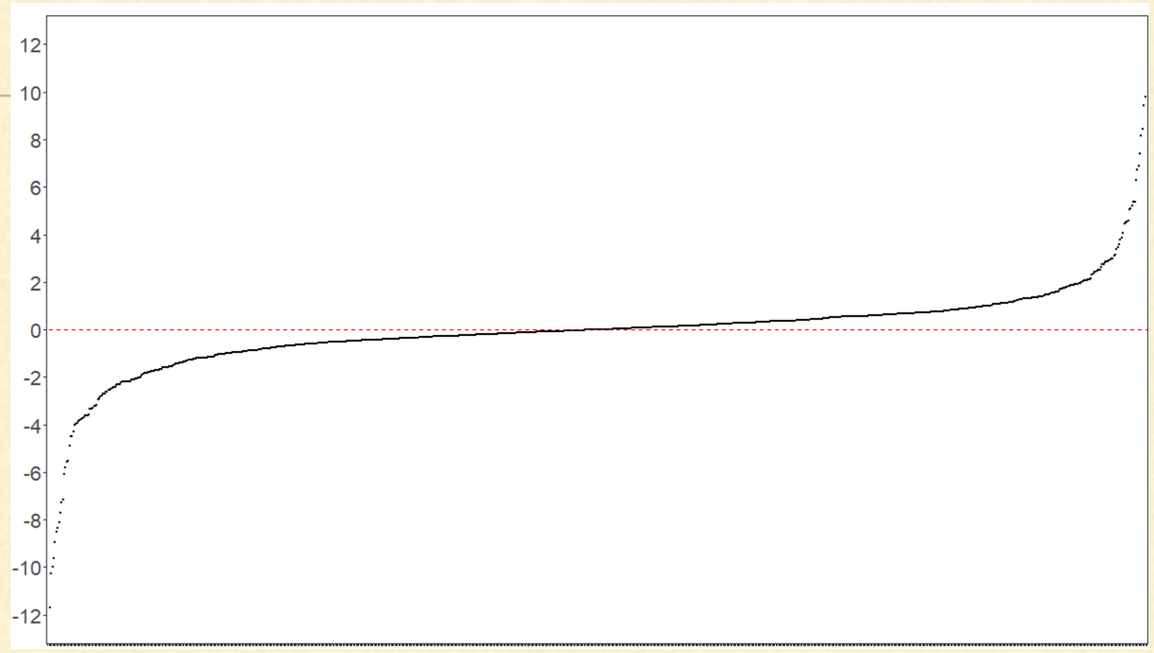
- Here's some output from a model fitted on a colorectal cancer case-control dataset



  - What's on y-axis?
  - $\log \frac{\text{mean cell conc. taxon j in cases}}{\text{mean cell conc. taxon j in controls}}$
    - but constrained: we force median to be zero
    - **This changes interpretation!**

RADEMU: EXAMPLE

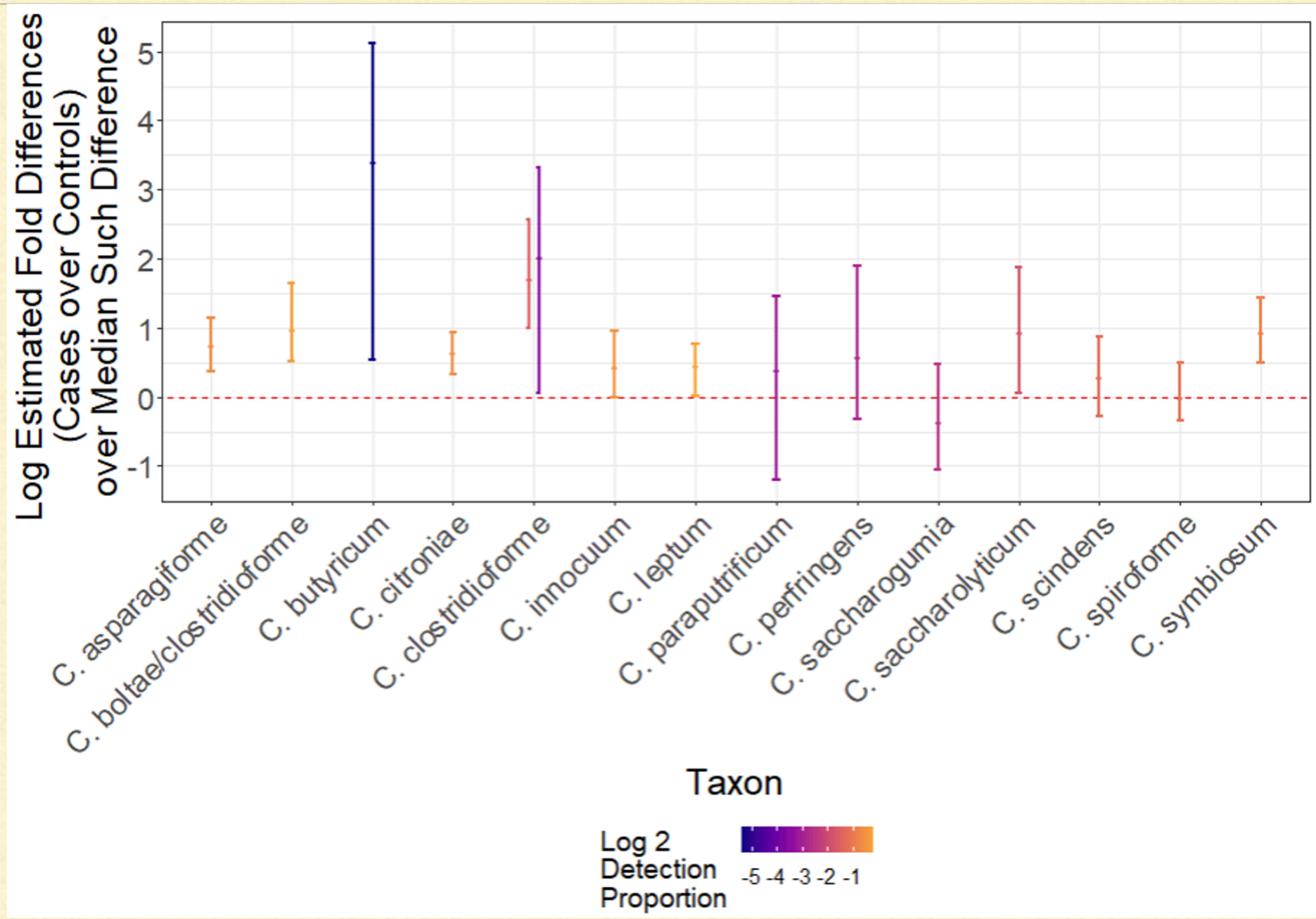
- Here's some output from a model fitted on a colorectal cancer case-control dataset



  - What's on y-axis?
  - $\log \frac{\text{mean cell conc. taxon j in cases}}{\text{mean cell conc. taxon j in controls}}$
    - but constrained: we force median to be zero
    - **This changes interpretation!**

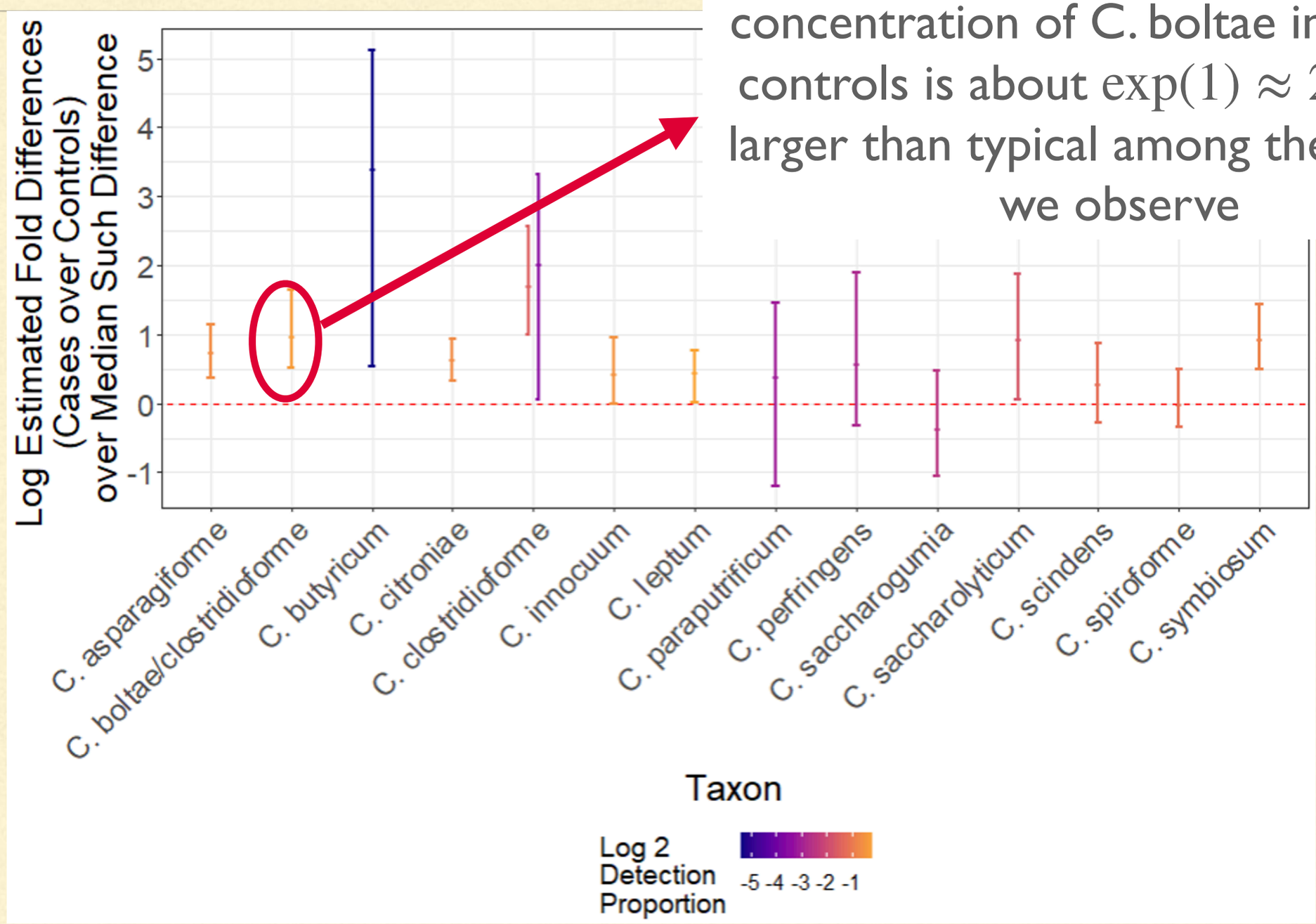
How different are cell concentrations across groups… relative to the *typical* difference?

RADEMU: EXAMPLES



RADEMU: EXAMPLES

We estimate that the ratio of mean concentration of C. boltae in cases to controls is about $\exp(1) \approx 2.7$ times larger than typical among the mOTUs we observe

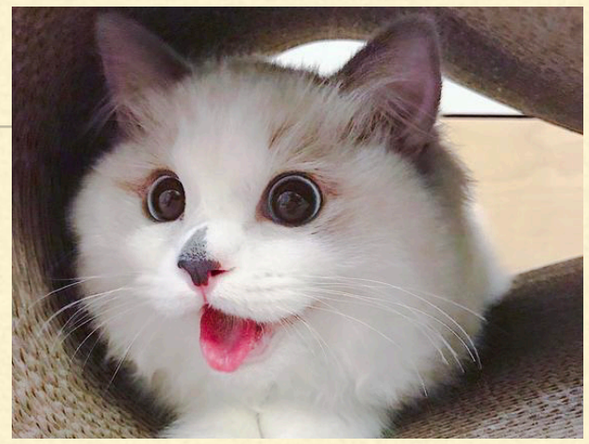


RADEMU: ASSUMPTIONS

- What assumptions does radEmu make (that you might consider caring about)?
  - Most important:
    - expected counts* are proportional to mean × efficiencies
      - Multiplicative over/under-detection of taxa not a problem
      - Contamination: could be a problem!

*not actually necessary for the outcome to be a count (coverage would also work, for example)

ACCESSING `RADEMU` LAB



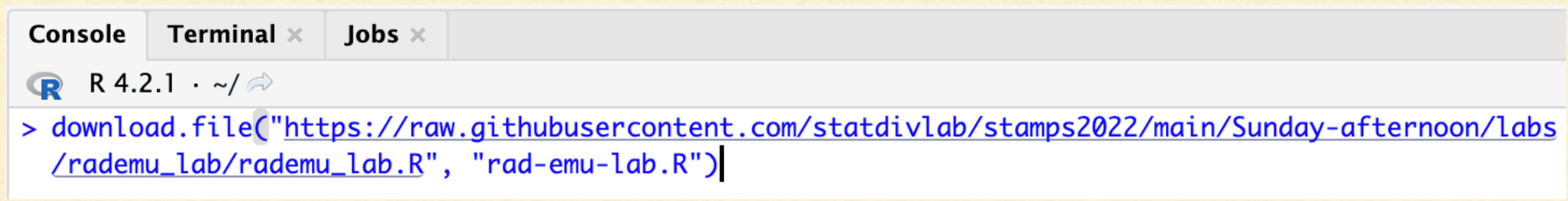
Get pumped!

1. Go to schedule on Wiki to Sunday afternoon, click on “Labs”

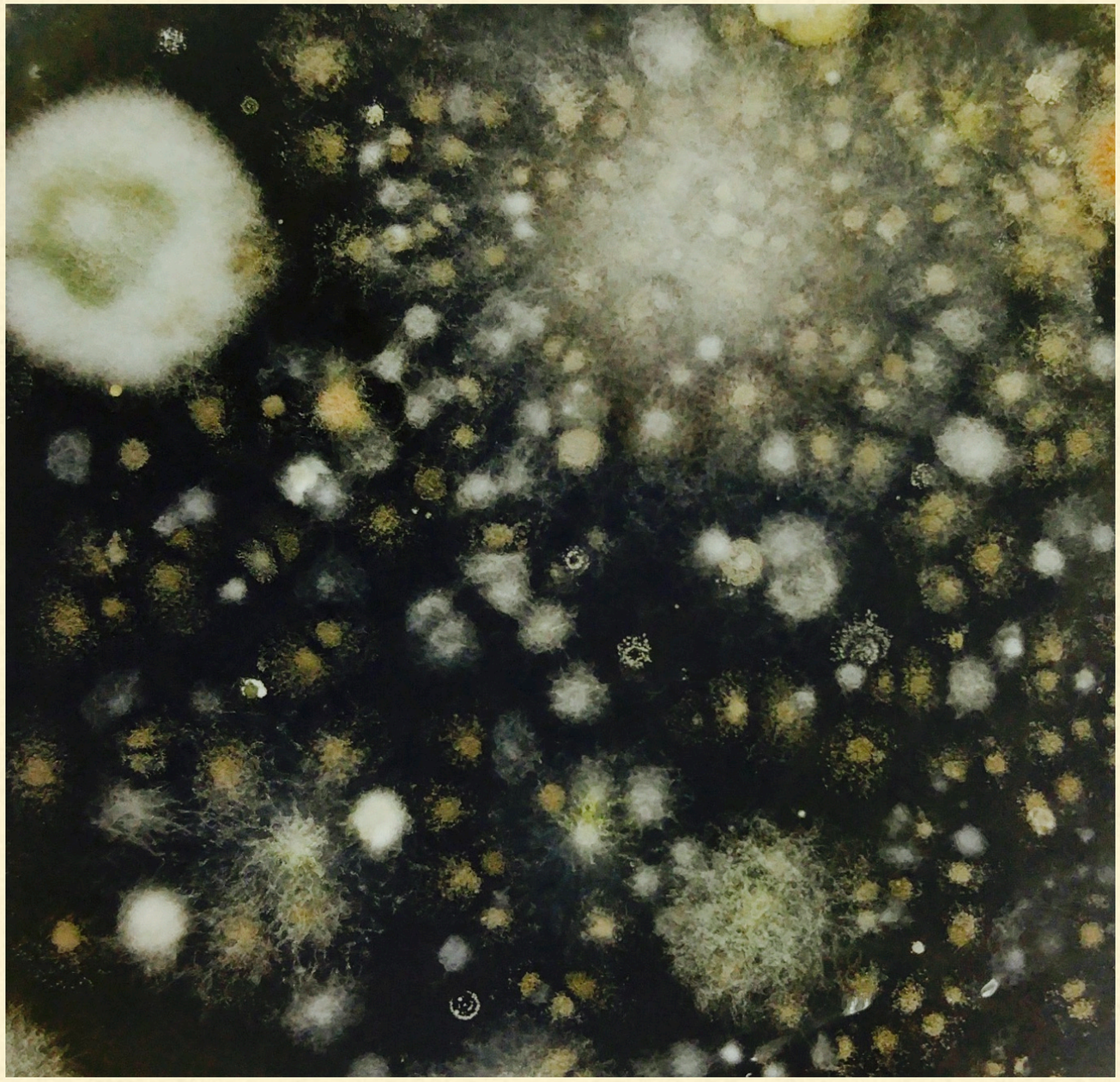
2. Copy the command under the lab we’re working on

```
rad emu lab:
download.file("https://raw.githubusercontent.com/statdivlab/stamps2022/main/Sunday-afternoon/labs/rademu_lab/rademu_lab.R", "rad-emu-lab.R")
```

3. Run this command in your RStudio Server console



MODELING DIVERSITY



DIVERSITY

- Low dimensional summaries of entire communities
  - α-diversity: one community
    - e.g., species richness, Shannon diversity
  - β-diversity: multiple communities
    - e.g., UniFrac, Bray-Curtis, Jaccard
    - Usually based on distances

DIVERSITY & PARAMETERS

- There are multiple choices to make when talking about diversity
  - Which taxonomic level? (strain/species/genus...)
  - Which diversity parameter?
  - Which estimate of the diversity parameter?

DIVERSITY & PARAMETERS

- There are multiple choices to make when talking about diversity
  - Which taxonomic level? (strain/species/genus...)
  - **Which diversity parameter?**
  - Which estimate of the diversity parameter?

ALPHA DIVERSITY

- Suppose we have C groups in our environment in proportions p_1, p_2, …, p_C

- Any function of
  - p_1, p_2, …, p_C OR
  - p_1, p_2, …, p_C and ~~some info about relationships amongst groups~~ phylogeny

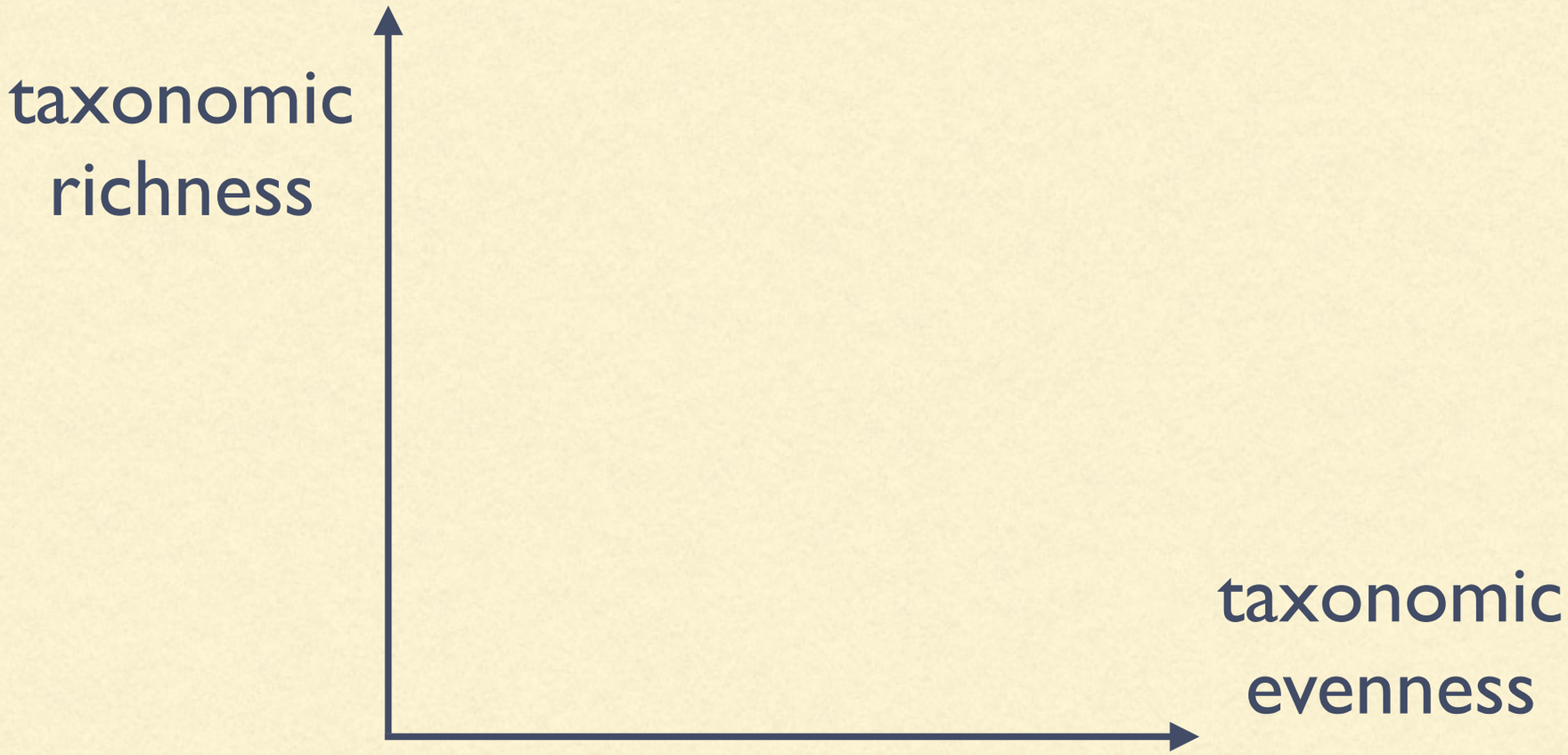
is a valid α-diversity parameter

ALPHA DIVERSITY

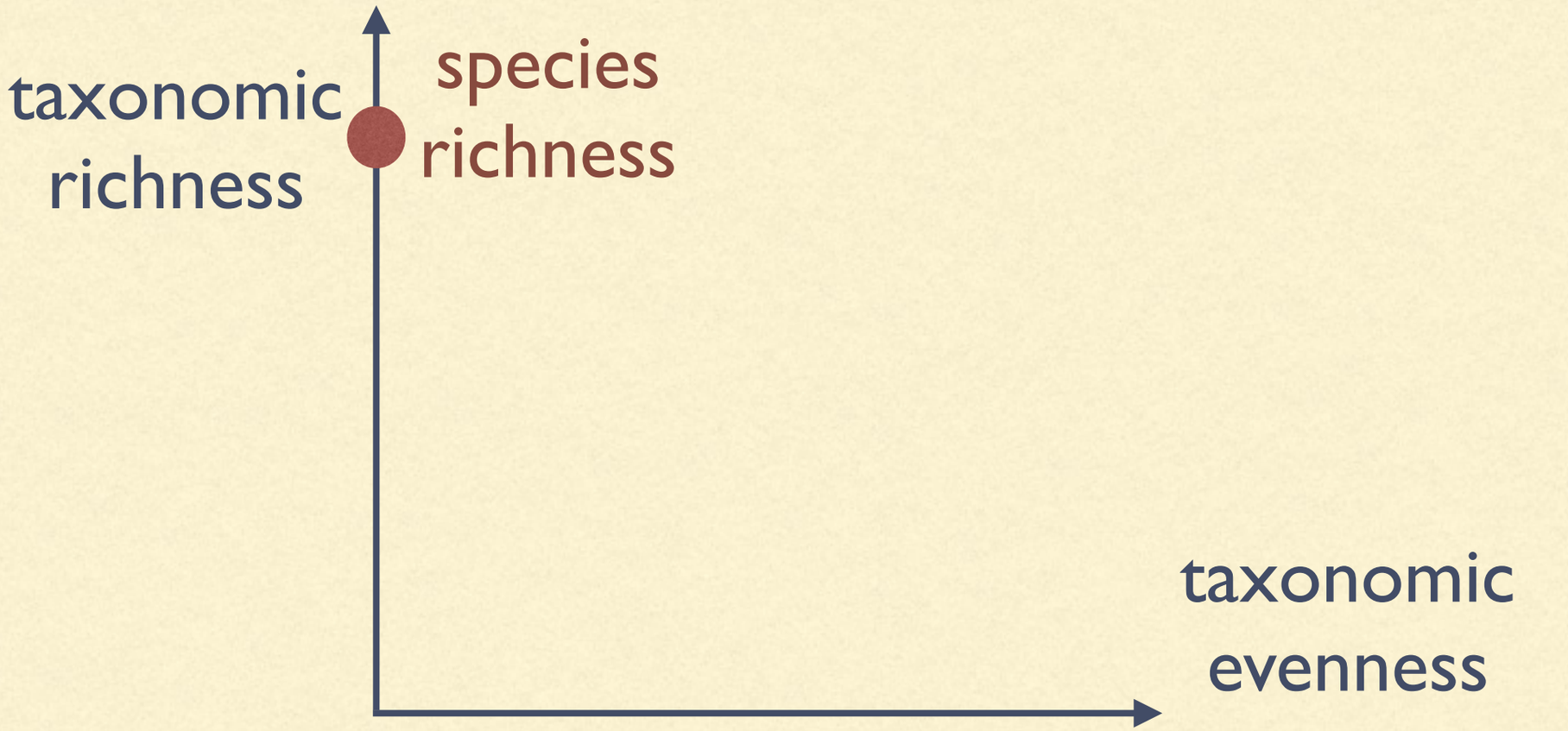
- Some examples of α-diversity measures include
  - Species richness: C
  - Simpson's index: $\sum_{i=1}^{C} p_i^2$
  - Shannon diversity: $-\sum_{i=1}^{C} p_i \ln p_i$
  - Shannon's E: $\frac{-\sum_{i=1}^{C} p_i \ln p_i}{\ln C}$

YOUR CHOICE

- Think: What difference do you want to highlight?



YOUR CHOICE



YOUR CHOICE

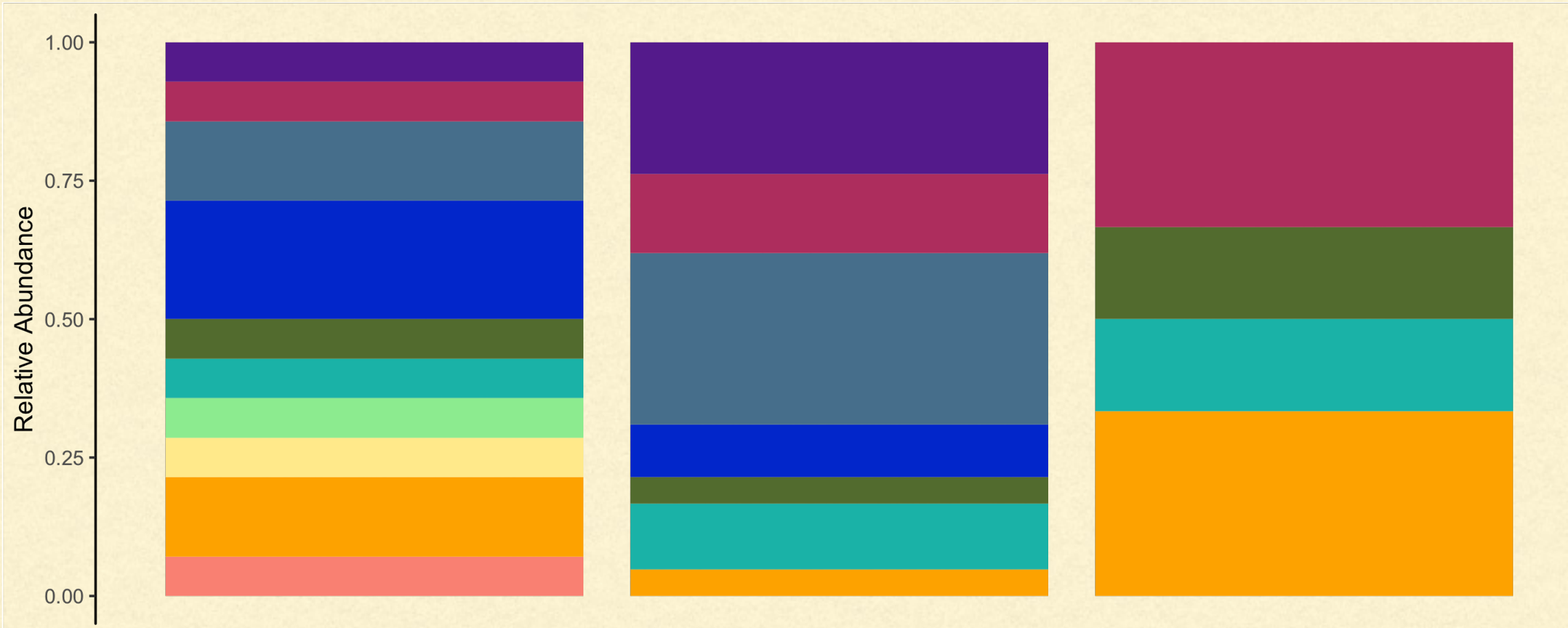

taxonomic
richness
species
richness
Shannon's E
taxonomic
evenness

YOUR CHOICE


taxonomic
richness
species
richness
Shannon
taxonomic
evenness
Shannon's E

YOUR CHOICE

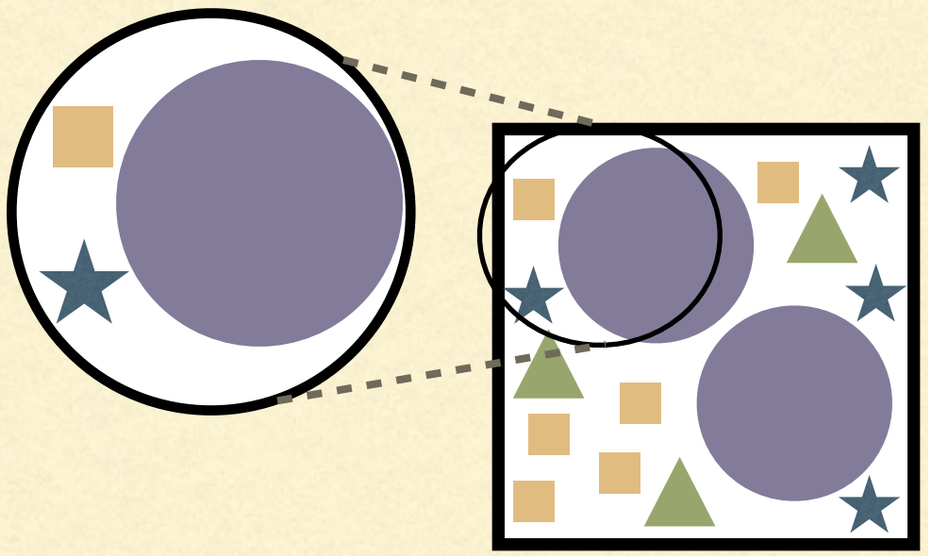
This is a question of *parameter choice:*
Which parameter highlights the differences I care about?



Richness	10	7	4
Shannon	2.21	1.75	1.33
Evenness	0.96	0.90	0.96
Simpson's	0.88	0.80	0.72

THE PROBLEM

- In practice, we don't observe the entire community, just a sample from it
  - we don't know C or $p_1, p_2, \ldots, p_C$



- **We need to *estimate* them using the data we collected**

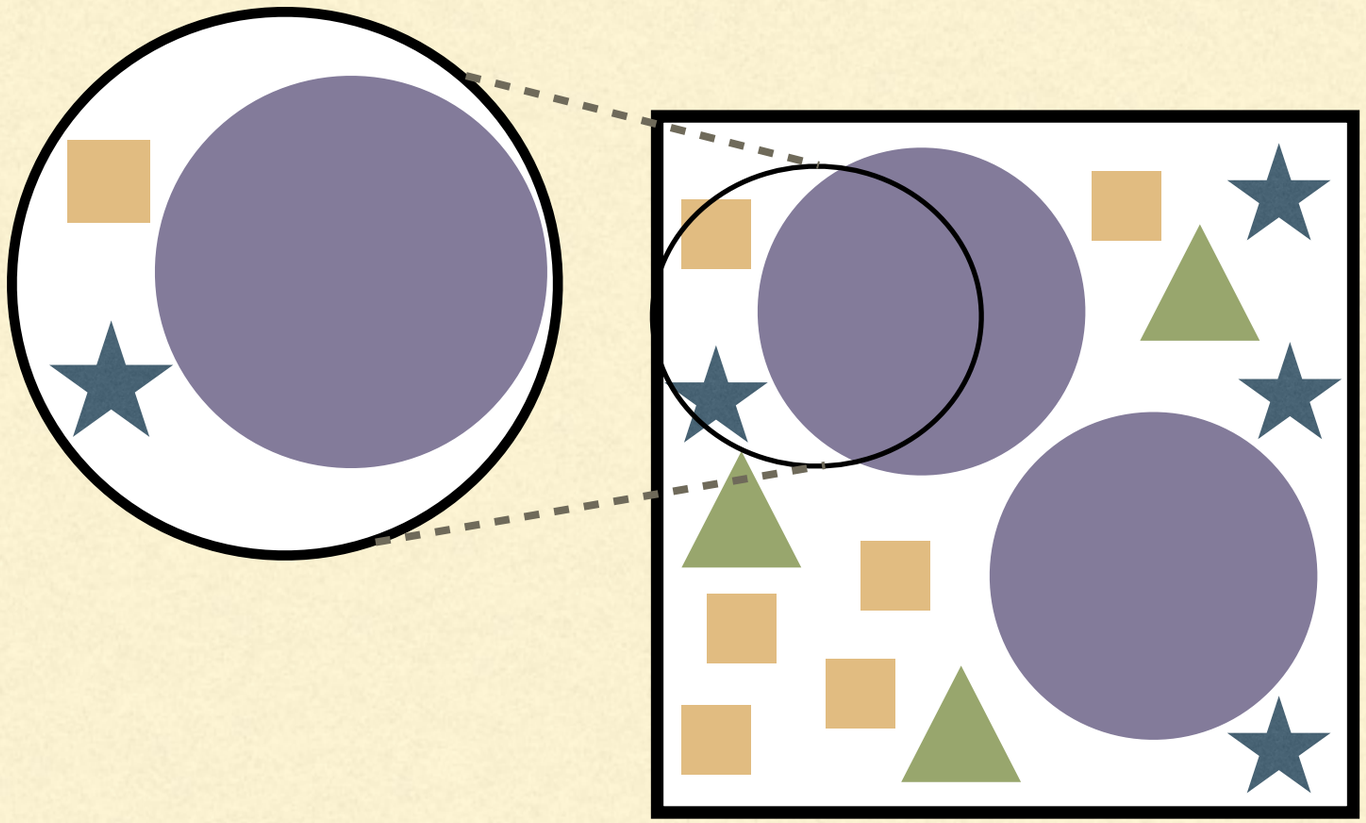
naive

THE "~~CLASSICAL~~" APPROACH

- Substitute the observed abundances $\hat{p}_1, \ldots, \hat{p}_c$ for the unknown, true abundances $p_1, p_2, \ldots, p_c$ and pretend nothing happened
  - e.g. Estimate the richness with: $c = \#\{i : \hat{p}_i \neq 0\}$
  - e.g. Estimate the Simpsons index: $\sum_{i=1}^{c} \hat{p}_i^2$

ONE PROBLEM (OF MANY)

- Species richness: plug-in estimate *underestimates*
- Simpson: estimate *overestimates*
- ~~Need new indices~~
- Need new estimators



HOW TO FIX

- Two things are wrong here:
  - bias (under/overestimation)
  - variance (how big are the error bars — you'll never be exactly right)

SPECIES RICHNESS

- The "species problem": how many species were missing from the sample

- Idea

  - If many rare species in sample, likely there are many missing species

  - If few rare species in sample, likely there are few missing species

  - Use data on rare species to predict # missing species

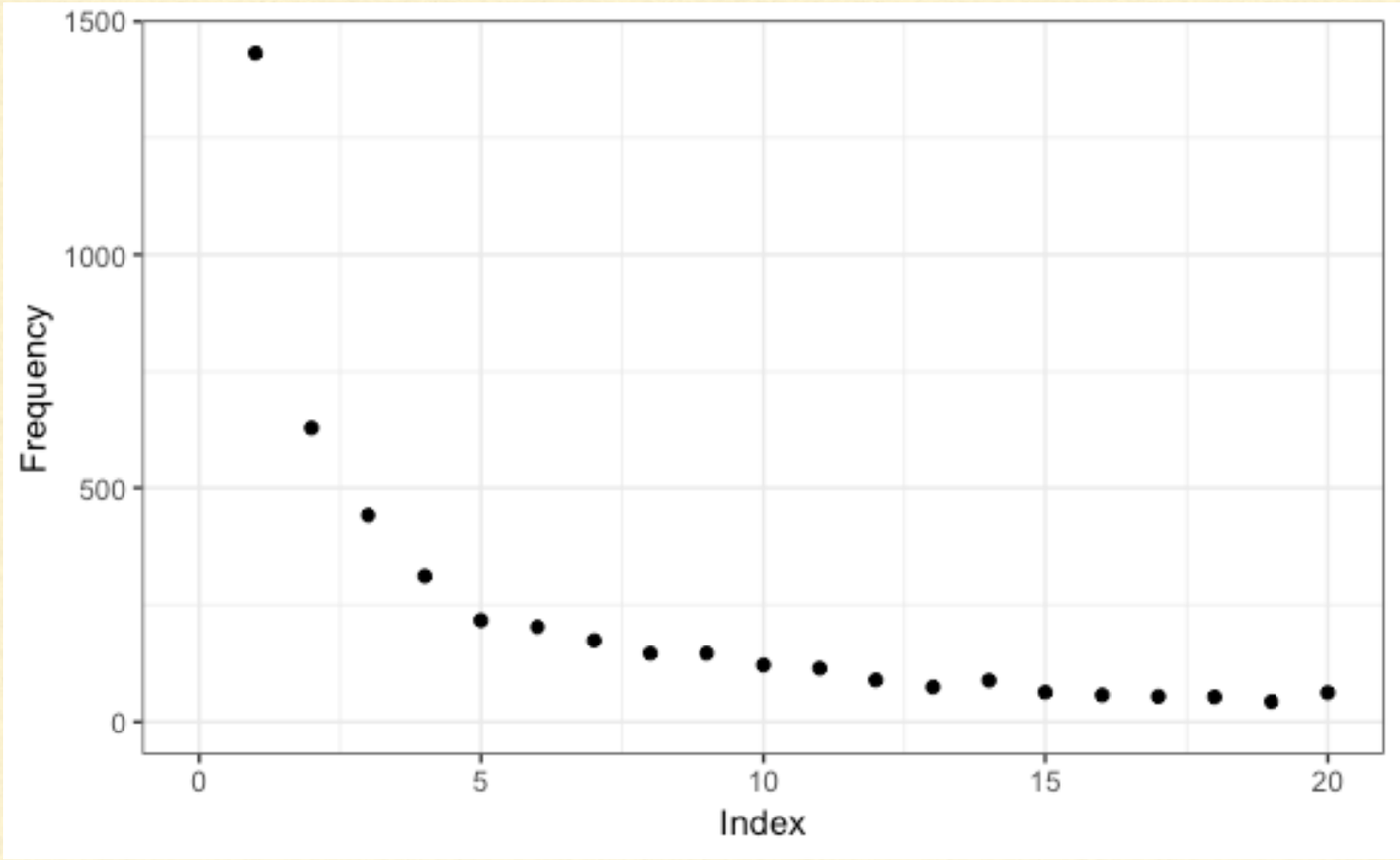
SPECIES RICHNESS ESTIMATION

- The necessary data for richness is the **frequency counts**
- f_j = number of species observed j times
- f_1 = singletons,
- f_2 = doubletons, …
- *e.g. 1431 strains observed once*

```
> library(phyloseq)
> library(magrittr)
> library(breakaway)
> data("GlobalPatterns")
> GlobalPatterns %>%
+   otu_table %>%
+   build_frequency_count_tables %>%
+   head(1)
$CL3
      Index Frequency
 [1,]     1      1431
 [2,]     2       629
 [3,]     3       442
 [4,]     4       311
 [5,]     5       217
 [6,]     6       203
 [7,]     7       174
 [8,]     8       146
 [9,]     9       146
[10,]    10       121
[11,]    11       114
[12,]    12        89
[13,]    13        74
[14,]    14        88
```

SPECIES RICHNESS ESTIMATION

- Idea: extend the pattern in $f_1, f_2, f_3 \ldots$ to f_0



- Rare taxa are most informative for missing taxa

SPECIES RICHNESS ESTIMATION

- Good options
  - breakaway::breakaway() - Kemp models
  - breakaway::chao_bunge() - Negative binomial model
  - breakaway::objective_bayes_*() - mixed Poisson
  - CatchAll - mixed Poisson

- Bad options
  - anything involving rarefaction
  - QIIME2: chao1; scikitbio…
  - R:vegan::…

SPECIES RICHNESS ESTIMATION

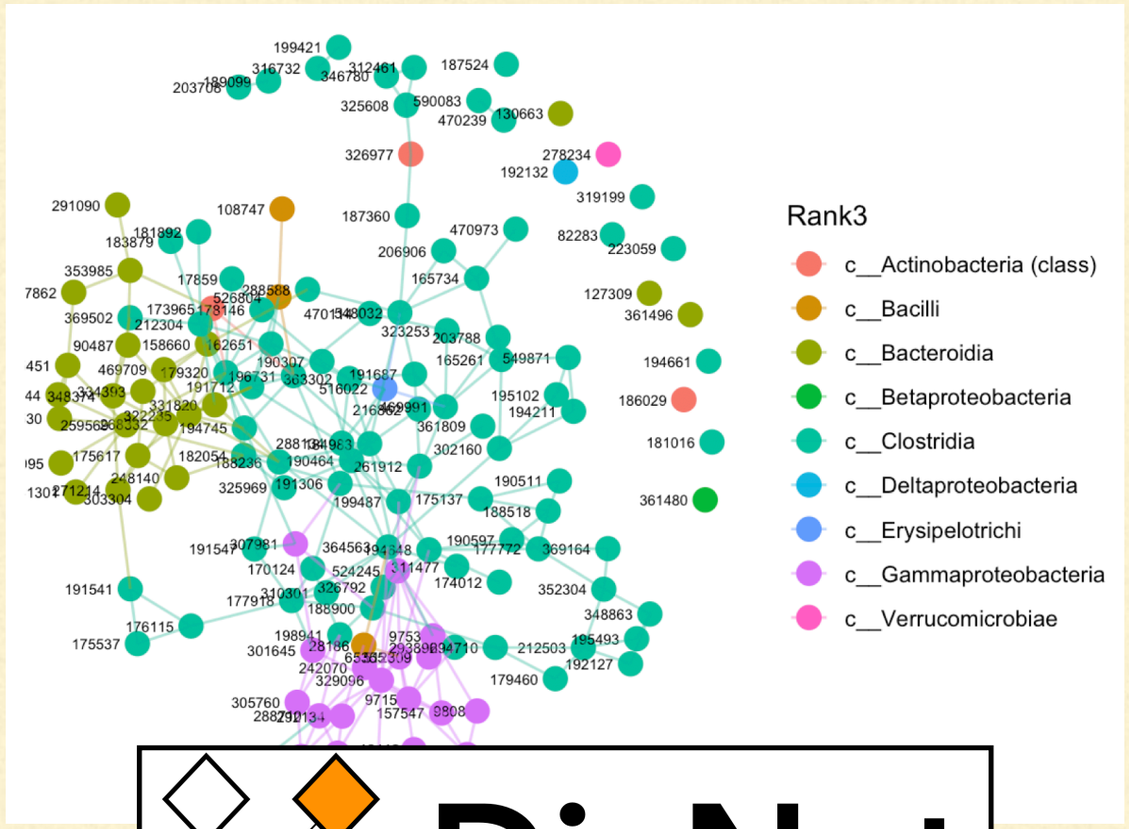
- "Chao1 diversity index" is not an index — it's an estimate of species richness, and it's based on the questionable assumption that

all species have the same abundance

- Large negative bias; very high variance
- Should not be used

ALPHA DIVERSITY: SHANNON & SIMPSON

- Slightly different approach:
  - Share strength across multiple samples to estimate C and $p_1, p_2, \ldots, p_C$, then use network models to get variance



github.com/adw96/DivNet

DivNet

BIAS AND DIVERSITY

- Alternative approach that I loathe: rarefaction
- Idea:
  - Discover more diversity with more sequencing
  - Can't directly compare samples with different depths
  - Randomly throw away reads until all samples have same depth
- Better idea: **Statistical estimation that accounts for different sequencing depths!**

BIAS AND DIVERSITY

- Alternative approach that I loathe: rarefaction

PLOS | COMPUTATIONAL BIOLOGY

BROWSE PUBLISH ABOUT

OPEN ACCESS PEER-REVIEWED

RESEARCH ARTICLE

Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible

Paul J. McMurdie, Susan Holmes

Published: April 3, 2014 • https://doi.org/10.1371/journal.pcbi.1003531

epth

- Better idea: **Statistical estimation that accounts for different sequencing depths!**

BIAS AND DIVERSITY

- Alternative approa

PLOS | COMPUTATIO BIOLOGY

OPEN ACCESS PEER-REVIEWED

RESEARCH ARTICLE

Waste Not, Want Not: W

Paul J. McMurdie, Susan Holmes

Published: April 3, 2014 • https://doi.org/10.

Microbiome

Home About Articles Submission Guidelines

Research | Open Access

Normalization and microbial differential abundance strategies depend upon data characteristics

Sophie Weiss, Zhenjiang Zech Xu, Shyamal Peddada, Amnon Amir, Kyle Bittinger, Antonio Gonzalez, Catherine Lozupone, Jesse R. Zaneveld, Yoshiki Vázquez-Baeza, Amanda Birmingham, Embriette R. Hyde and Rob Knight

Microbiome 2017 **5**:27

https://doi.org/10.1186/s40168-017-0237-y | © The Author(s). 2017

Received: 9 October 2015 | **Accepted:** 27 January 2017 | **Published:** 3 March 2017

- Better idea: **Statis**
  different seque

BIAS AND DIVERSITY

- Alternative approa

DIVERSITY

- Very useful summary of (high-dimensional) compositional data… in many settings!

- A change in diversity: a useful *first question*

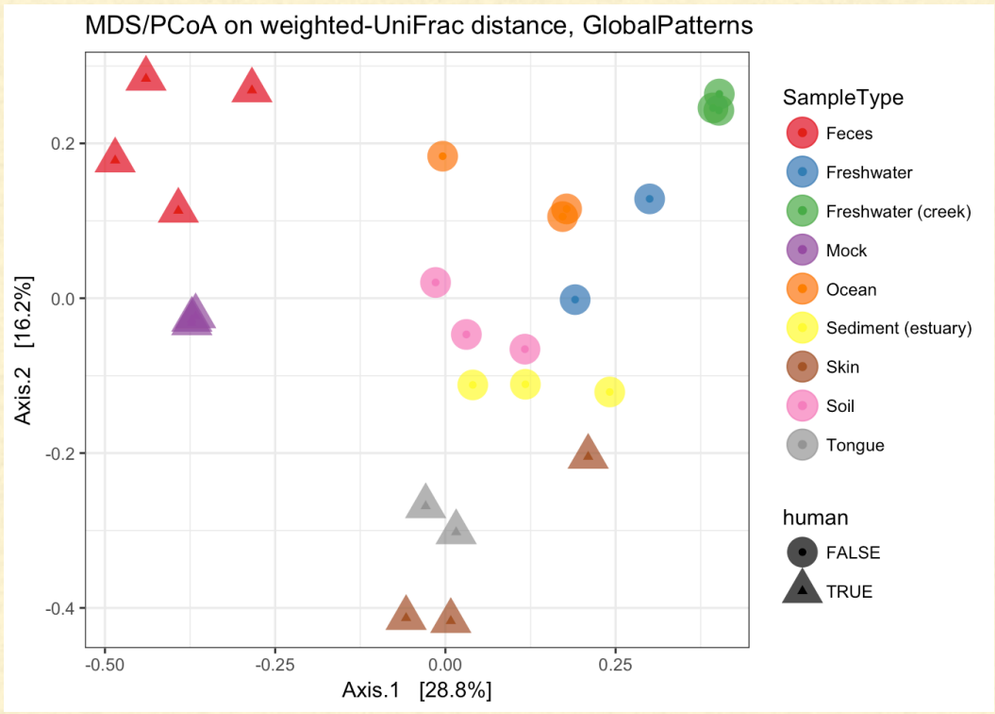
THOUGHTS ON BETA DIVERSITY

BETA DIVERSITY

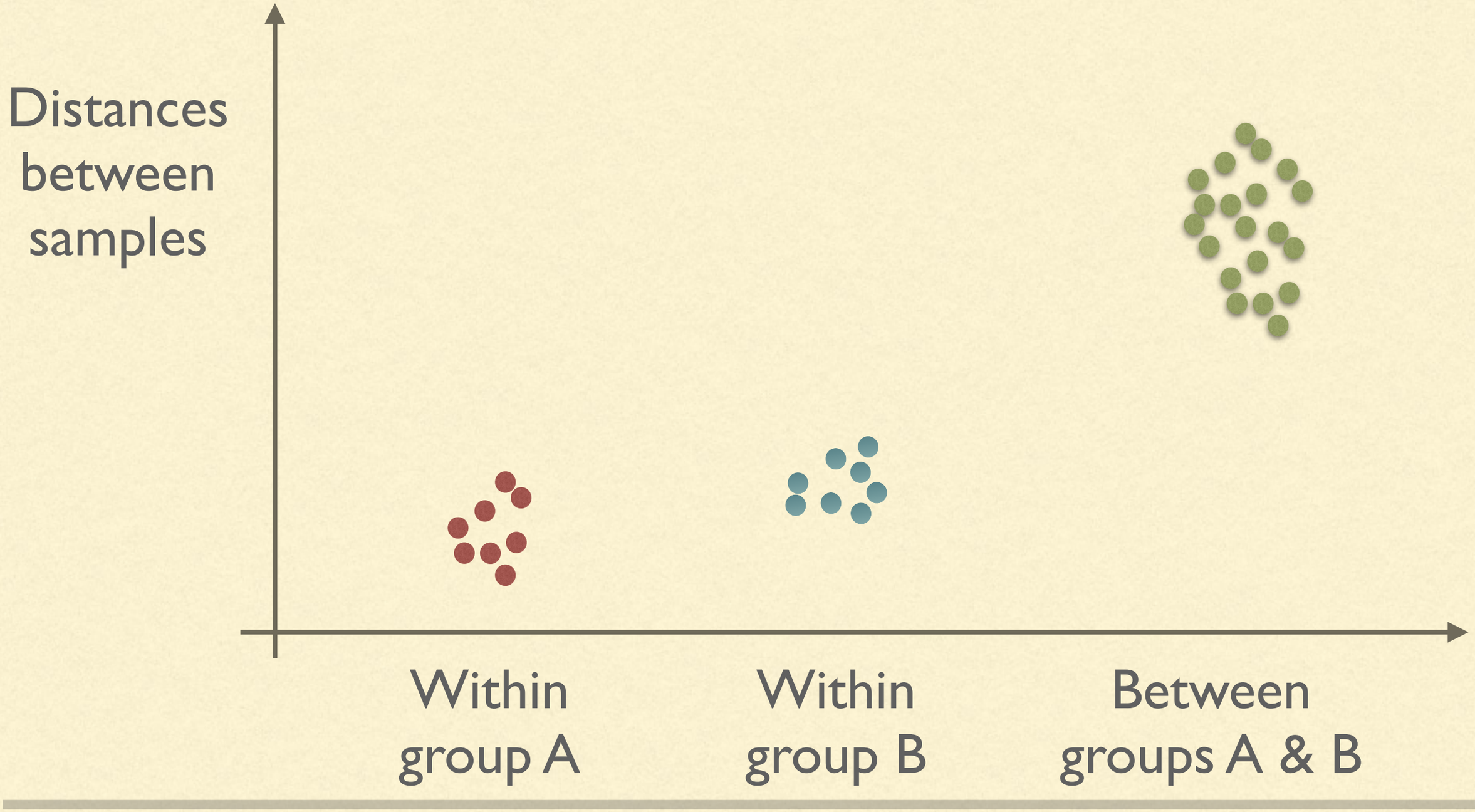
- Community 1: $p_1^{(1)}, p_2^{(1)}, \ldots, p_C^{(1)}$; Community 2: $p_1^{(2)}, p_2^{(2)}, \ldots, p_C^{(2)}$
  - β-diversity parameters are usually distances between compositional vectors
- Bray-Curtis: $\beta_{BC} = 1 - \sum_{i=1}^{C} \min(p_i^{(1)}, p_i^{(2)})$
- Jaccard: $\beta_J =$ % taxa not shared
- UniFrac: Weights phylogeny

DIVERSITY: EXPLORATORY

- Sometimes diversity is analysed as an exploratory tool
  - e.g., ordination
  - *However:* consider not doing an ordination
    - Difficult to interpret (& often over-interpreted)
    - Is there a more informative way to plot your data?



ALTERNATIVE VIZ



DIVERSITY: HYPOTHESIS TESTING

- Other times you want to do inference on beta diversities
  - e.g., H_0: dissimilarity within two communities is same as dissimilarity across
  - e.g., H_0: communities A & B have same dissimilarity as communities A & C
- But (yet again): *before running a test, ask "am I sure testing this hypothesis answers a meaningful scientific question?"*

HYPOTHESIS TESTING FOR DIVERSITY

- Common approach: PERMANOVA
- Best solution = ask *"do I really want to do this test?"*
- Better solution = use error bars
  - breakaway::betta(); DivNet::testDiversity
- (Bad solution = rarefy)

ACCESSING `DIVERSITY` LAB

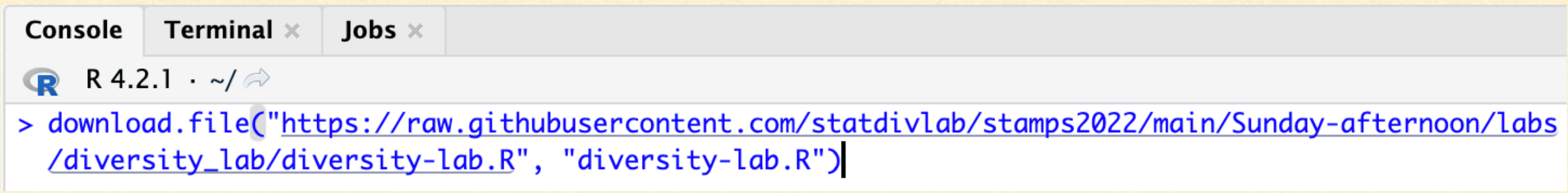
Get pumped!

1. Go to schedule on Wiki to Sunday afternoon, click on "Labs"

2. Copy the command under the lab we're working on

```
diversity lab:
download.file("https://raw.githubusercontent.com/statdivlab/stamps2022/main/Sunday-afternoon/labs/diversity_lab/diversity-lab.R", "diversity-lab.R")
```

3. Run this command in your RStudio Server console



CLOSING THOUGHTS

DIVERSITY

- If you really care about diversity, we recommend using
  - breakaway for species richness
  - DivNet for Shannon/Simpson diversity
  - ~~DivNet for weighted UniFrac/Bray-Curtis/Aitchison~~ asking yourself why you care about β-diversity

PEOPLE WORRY ABOUT THE WRONG THINGS

- Examples
  - Is data compositional?
  - Where to rarefy to?
  - Which beta diversity metric?

PEOPLE DON'T WORRY ABOUT THE RIGHT THINGS

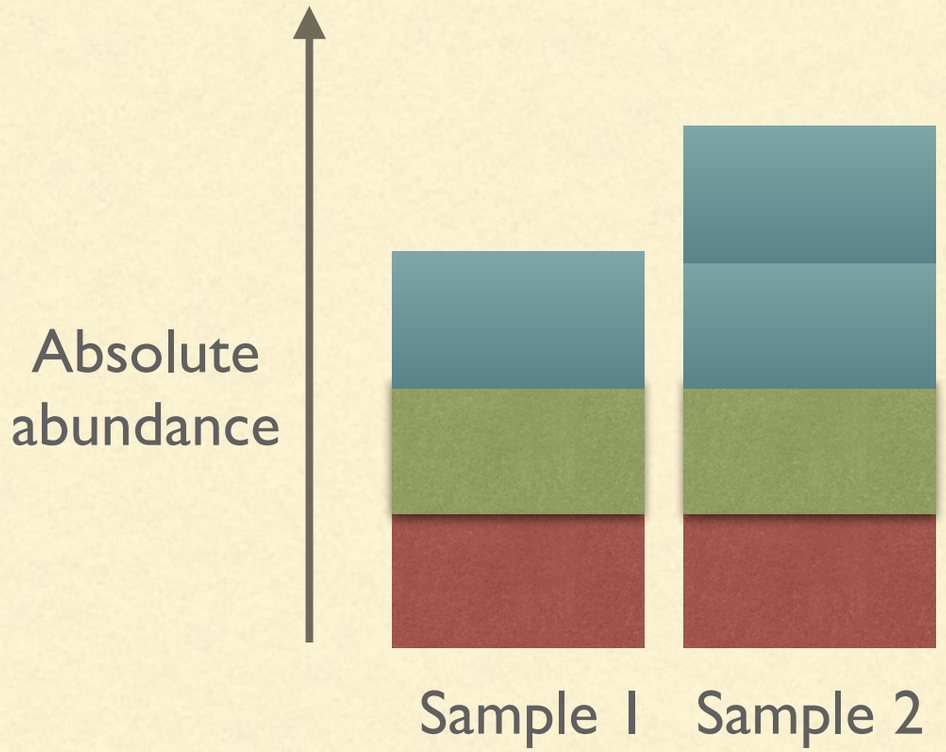
- Examples
  - Am I analyzing something I care about?
  - Can I estimate what I care about?

RELATIVE ABUNDANCE

- Limitations of relative abundance
  - Relative abundance of all taxa change when only one relative abundance changes

LIMITATIONS OF RELATIVE ABUNDANCE

- Relative abundance of all taxa change even when only one relative abundance changes
  - Not "spurious" but misleading
  - **0.33** / **0.33** / **0.33**
  - **0.25** / **0.25** / **0.50**
- This is an inherent limitation of this type of analysis



LIMITATIONS OF RELATIVE ABUNDANCE

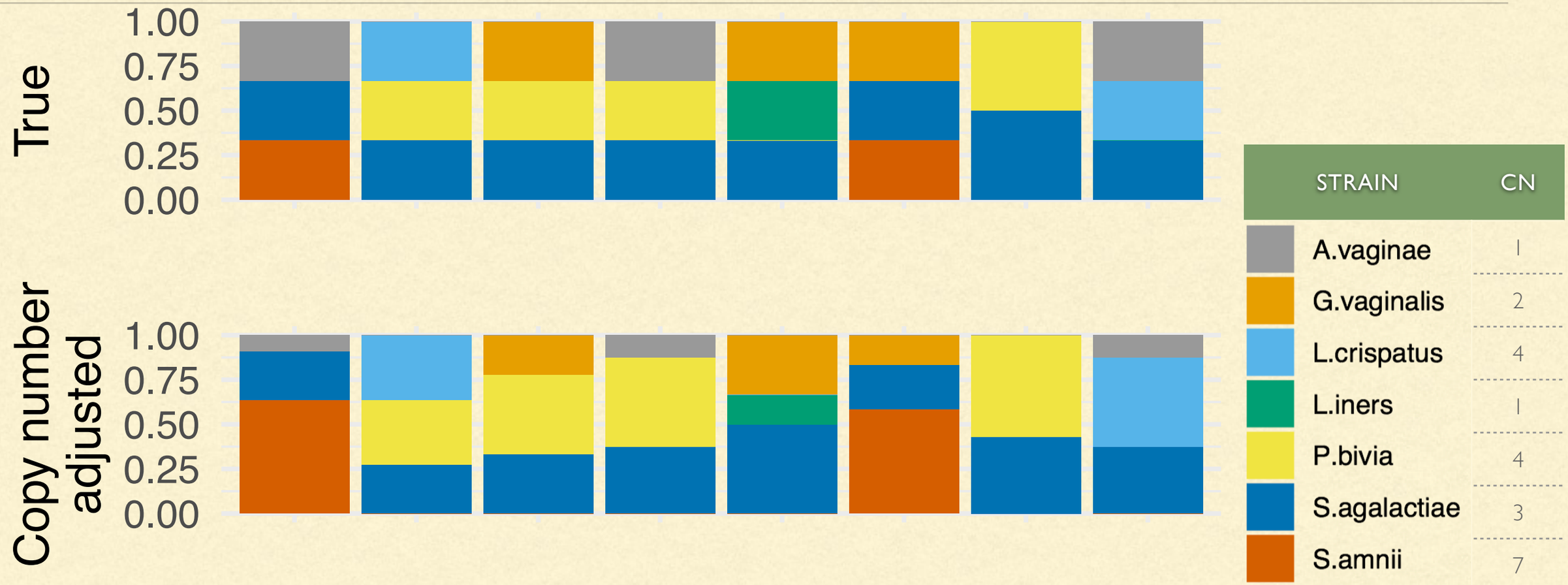
- Can we even estimate true relative abundance from sequencing data?
- Does our sequencing technology give us information about relative abundance?
  - Is observed relative abundance proportional to actual relative abundance?

DECONVOLUTION OF BIOLOGY AND SEQUENCING

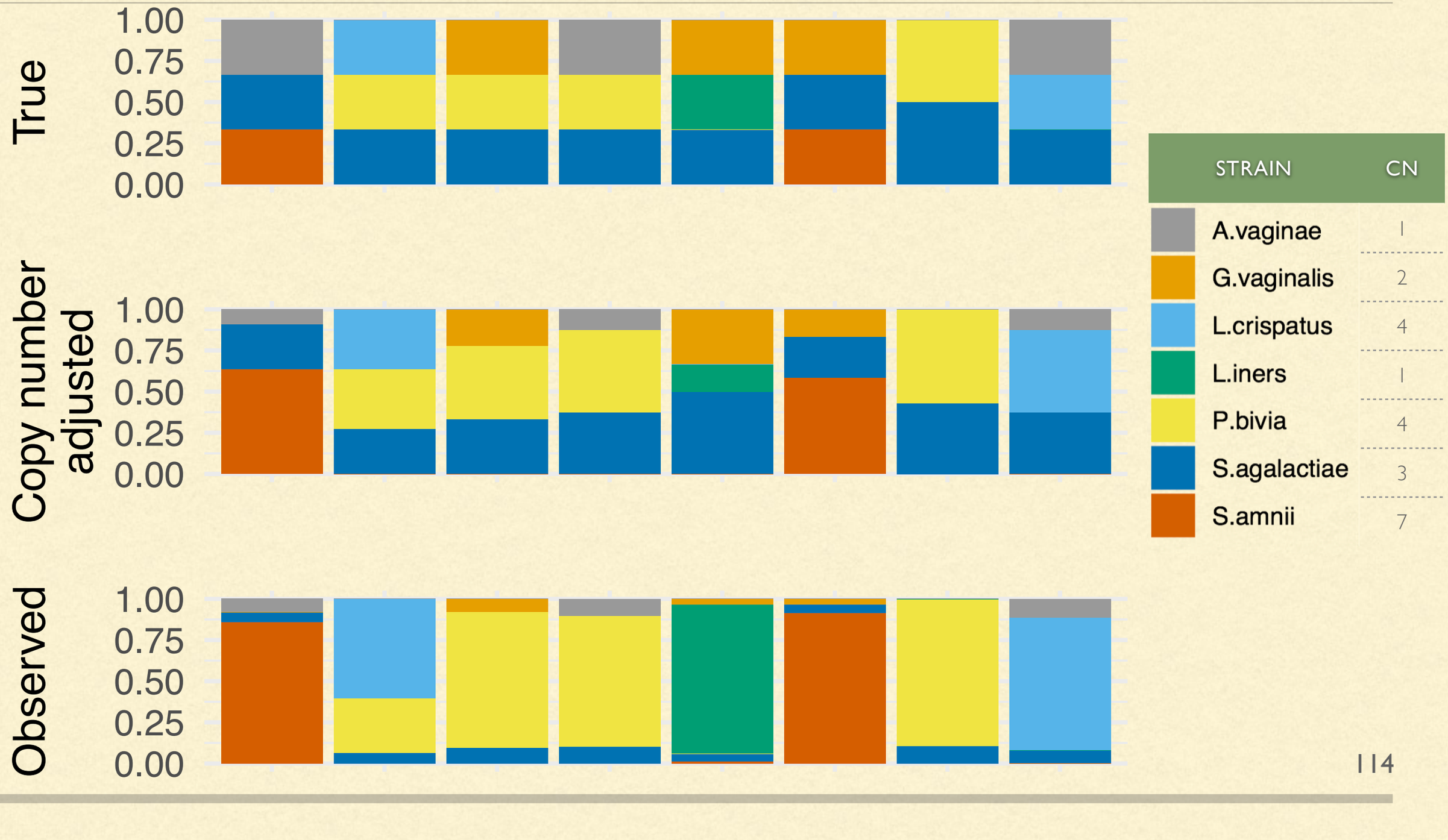
- Mock communities
- Artificially constructed communities of known composition
- Commonly used to benchmark sequencing and bioinformatics pipelines

WHAT DO WE OBSERVE?

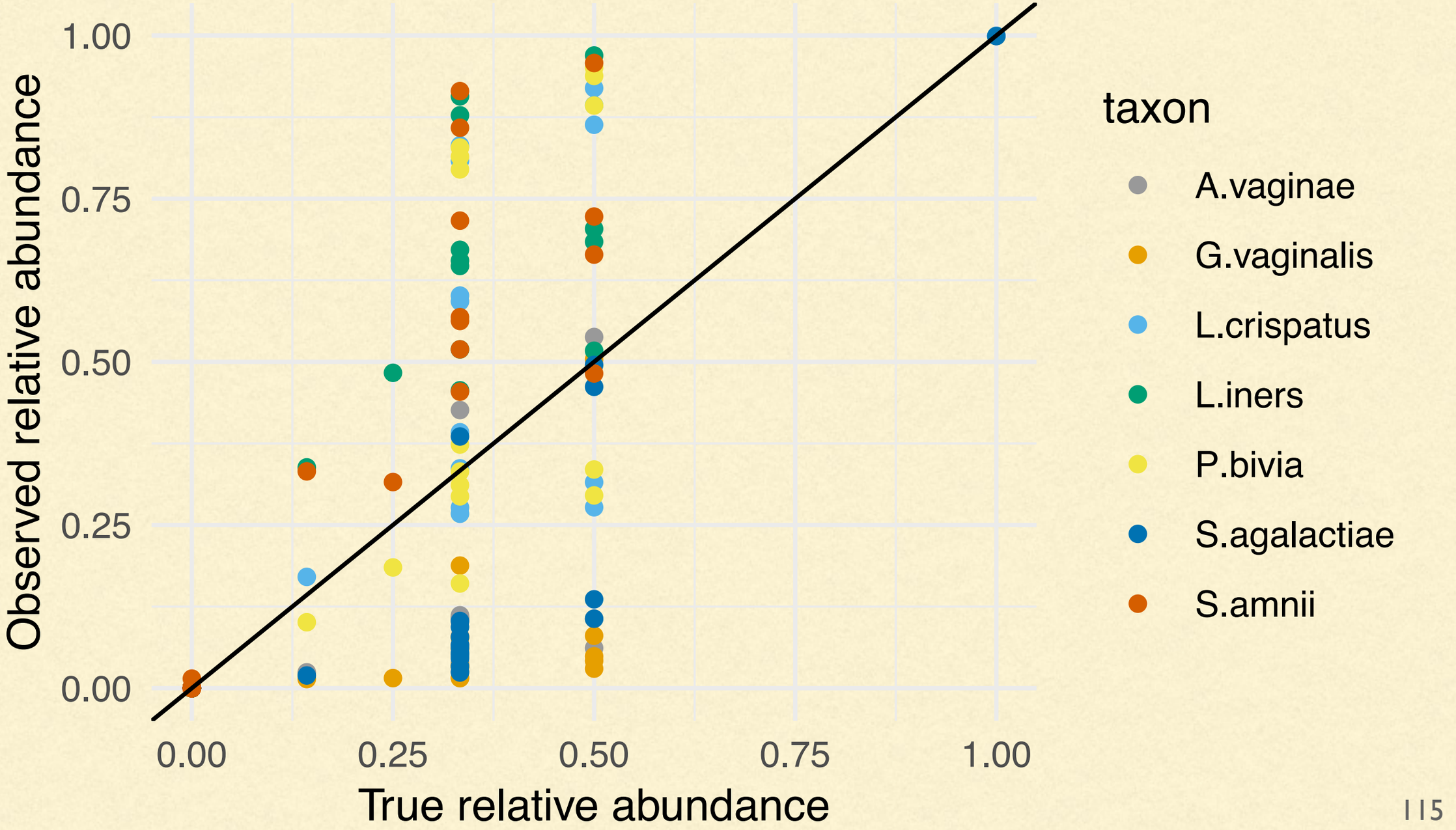
WHAT DO WE OBSERVE?



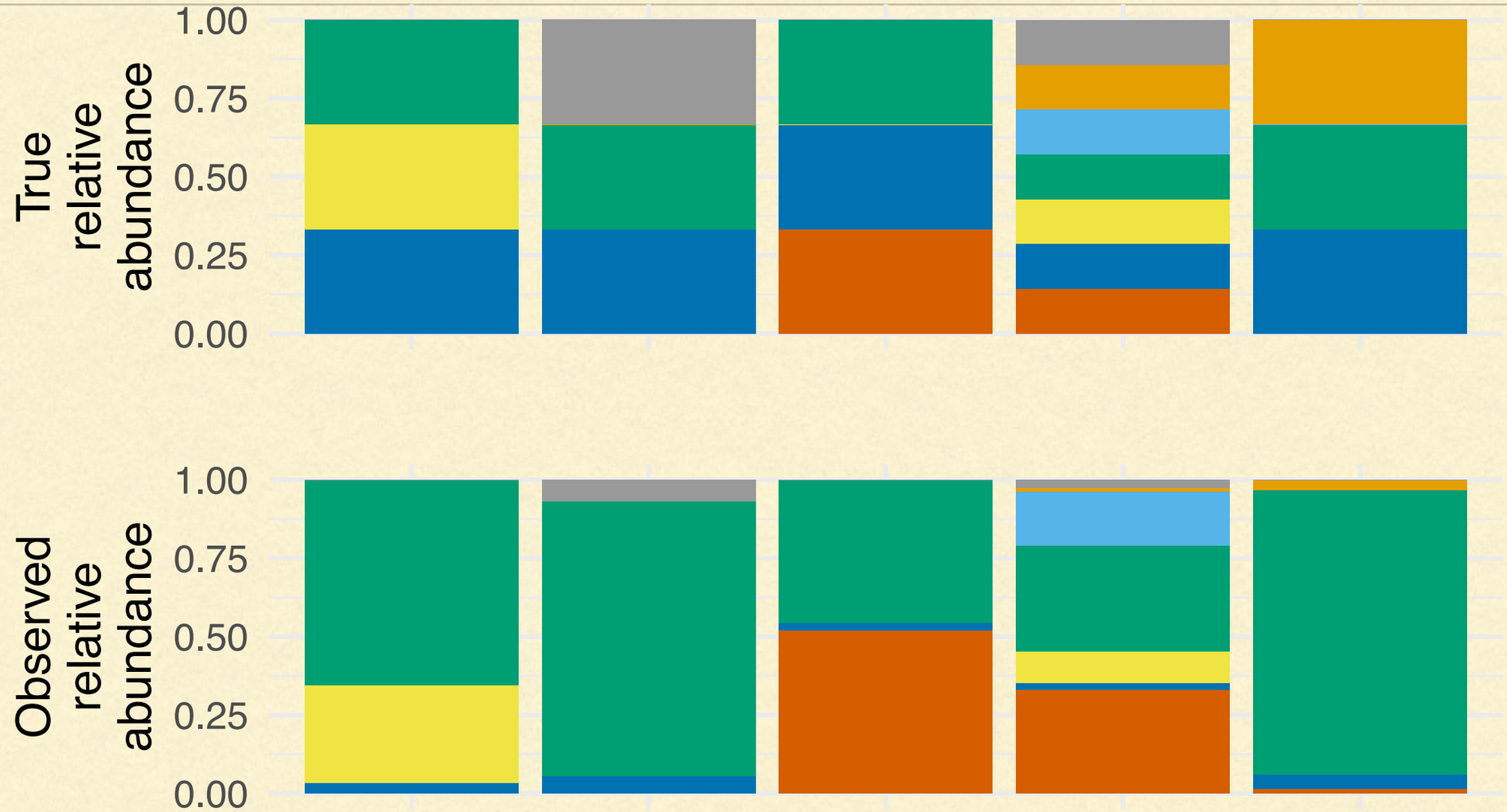
WHAT DO WE OBSERVE?



WHAT PATTERNS CAN WE FIND?

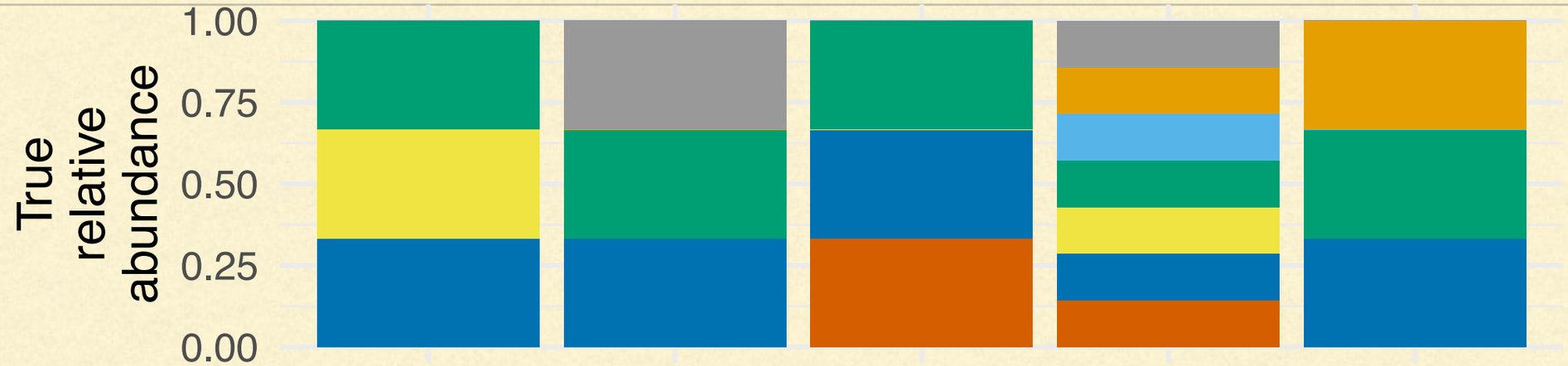


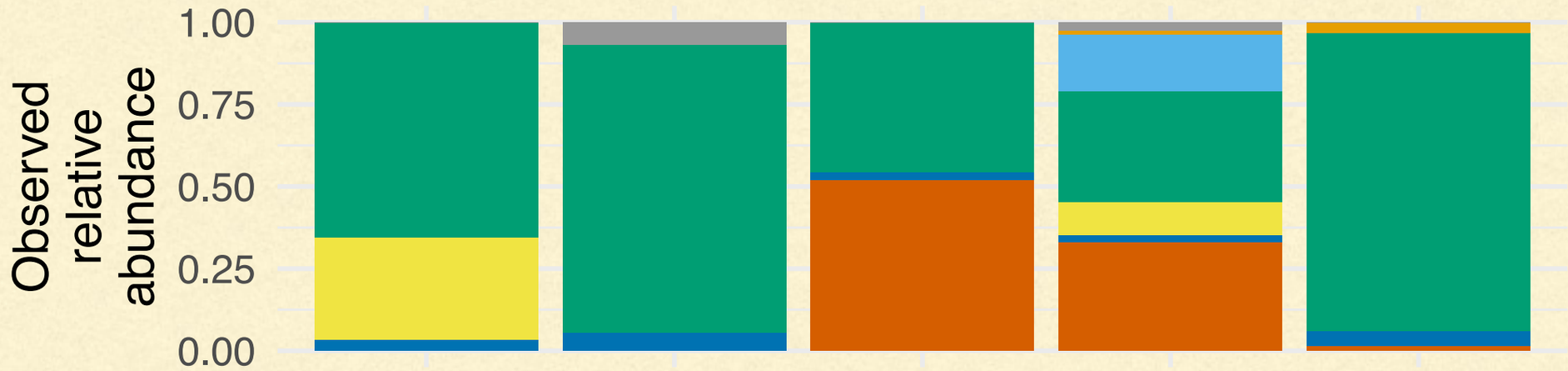
WHAT PATTERNS CAN WE FIND?



Data from Brooks *et al* (2015) BMC Microbiology

WHAT PATTERNS CAN WE FIND?





L.iners/S.agalactiae	19.5	16.1	18.9	17.4	19.9	**18.3 (1.6)**

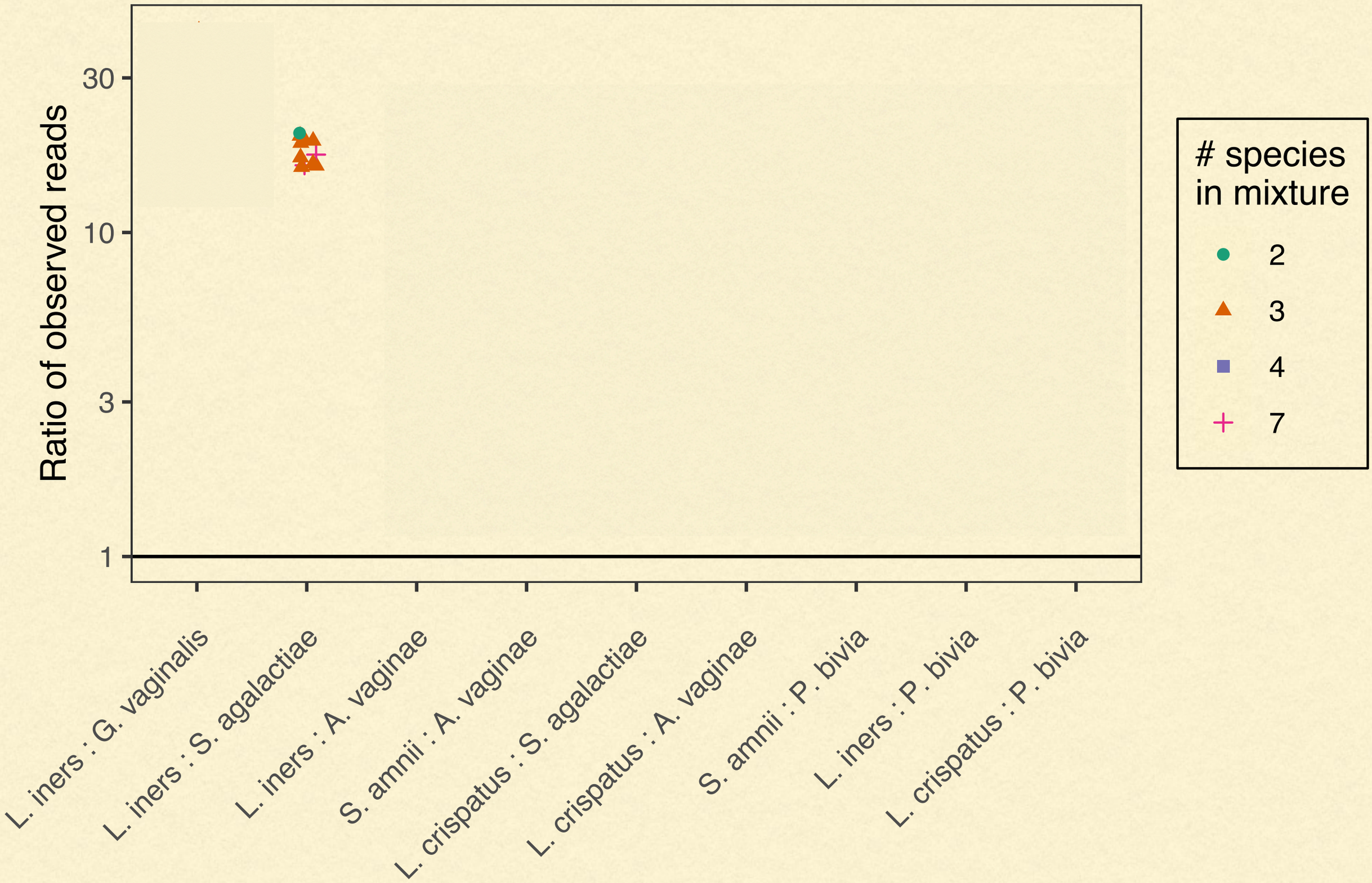


Figure adapted from McLaren, Willis & Callahan, 2019, *eLife*

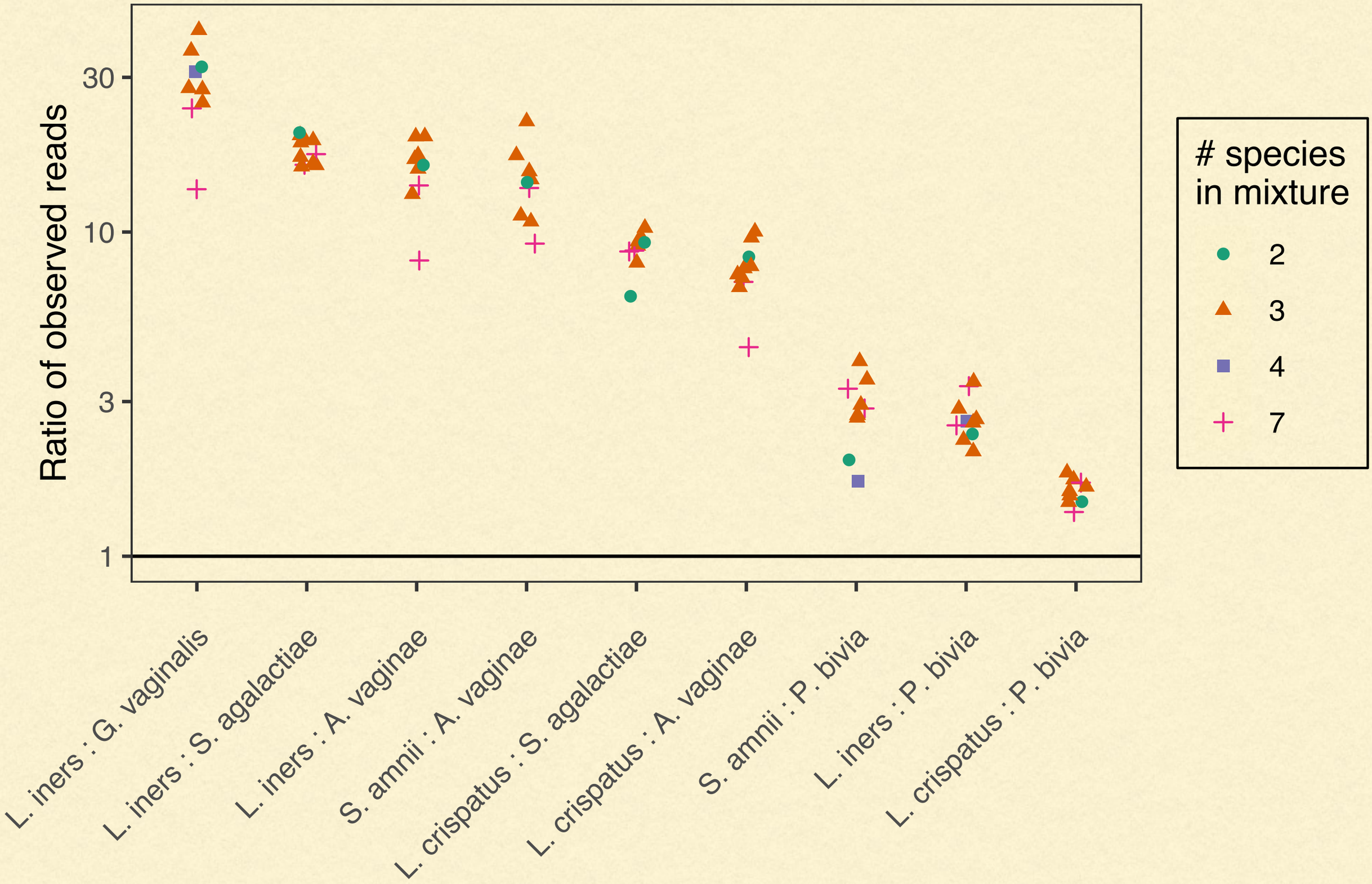


Figure adapted from McLaren, Willis & Callahan, 2019, *eLife*

MODEL SPECIFICATION

- Strong support *against*

$$\text{expected count}_{ij} = \text{true proportion}_{ij} \times \text{sampling intensity}_{i}$$

MODEL SPECIFICATION

- Strong support *against*

$$\text{expected count}_{ij} = \text{true proportion}_{ij} \times \text{sampling intensity}_{i}$$

- Better support for

$$\text{expected count}_{ij} = \text{true proportion}_{ij} \times \text{sampling intensity}_{i} \times \text{detection efficiency}_{j}$$

RESEARCH ARTICLE

Consistent and correctable bias in metagenomic sequencing experiments

Michael R McLaren[1], Amy D Willis[2], Benjamin J Callahan[1,3]*

- Model for bias in observed abundances
  - Validated on 16S and shotgun metagenomic data
- Critical evaluation of model specification

arXiv > stat > arXiv:2204.12733

Statistics > Methodology

Modeling complex measurement error in microbiome experiments

David S Clausen, Amy D Willis

David Clausen (UW)

Ben Callahan (NCSU)

DOI: 10.1111/biom.13503

BIOMETRIC PRACTICE

Biometrics A JOURNAL OF THE INTERNATIONAL BIOMETRIC SOCIETY WILEY

A multiview model for relative and absolute microbial abundances

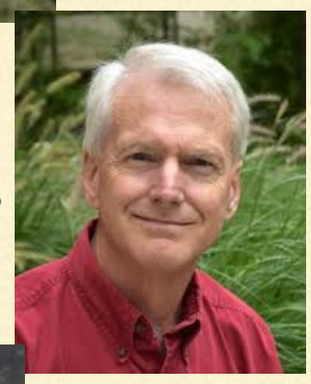
Brian D. Williamson | James P. Hughes | Amy D. Willis

Michael McLaren (MIT)

eLIFE elifesciences.org

Consistent and correctable bias in metagenomic sequencing experiments

Michael R McLaren[1], Amy D Willis[2], Benjamin J Callahan[1,3]*



Jim Hughes (UW)

Evaluating replicability in microbiome data

David S Clausen, Amy D Willis

Biostatistics, kxab048, https://doi.org/10.1093/biostatistics/kxab048

Brian Williamson (Kaiser)

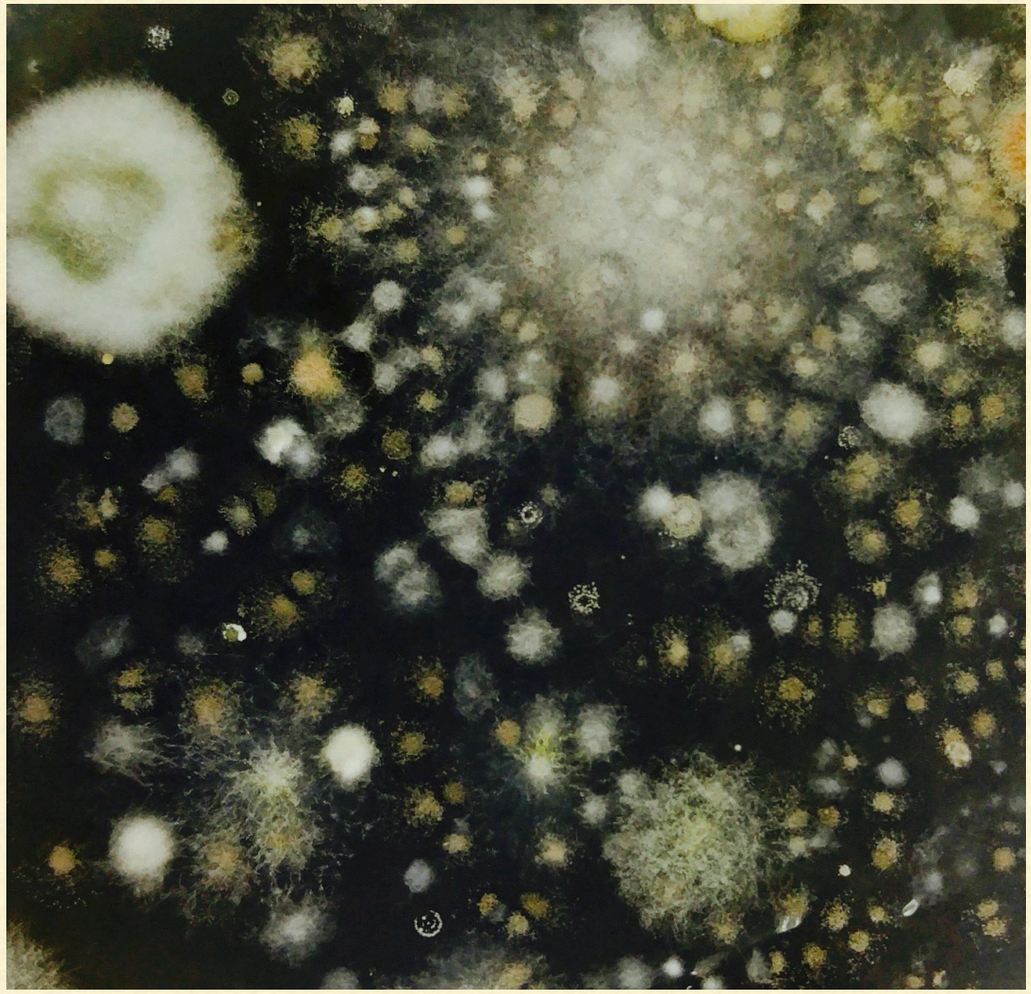
NIH NIGMS

IMPLICATIONS

- Now we know this phenomenon exists, what can we do?
  - Cautious: model relative abundance, but understand limitations
  - Cynical: Only rely on qPCR to discuss abundance
  - Aspirational: Use mock communities for calibration…
  - Aware: model ratios
  - Progressive: radEmu

CLOSING THOUGHTS

- Methods for modeling microbiome data is a fast-moving field, and new methods are constantly emerging
- Talk to lots of people
  - "What's the biggest limitation of this?"
- Stay critical but open-minded



MODELING MICROBIOME DATA

Statistical Diversity Lab @ University of Washington

Amy Willis — @AmyDWillis — Assistant Professor

David Clausen — @davidandacat — PhD Candidate

Sarah Teichman — @sarah_teichman — PhD Candidate

Photo credit: T.D. Berry, Whitman lab, UW Madison

CONSISTENCY OF EFFICIENCIES

STRAIN	GENOME SIZE (MBP)	COPY NUMBER	ESTIMATED EFFICIENCY
L CRISPATUS	2.04	4	**2.03**
L INERS	1.30	1	**6.83**

Data from Brooks et al., 2015; Analysis from McLaren et al., 2019

16S COPY NUMBER IS PREDICTIVE OF PCR BIAS BUT NOT OF TOTAL BIAS

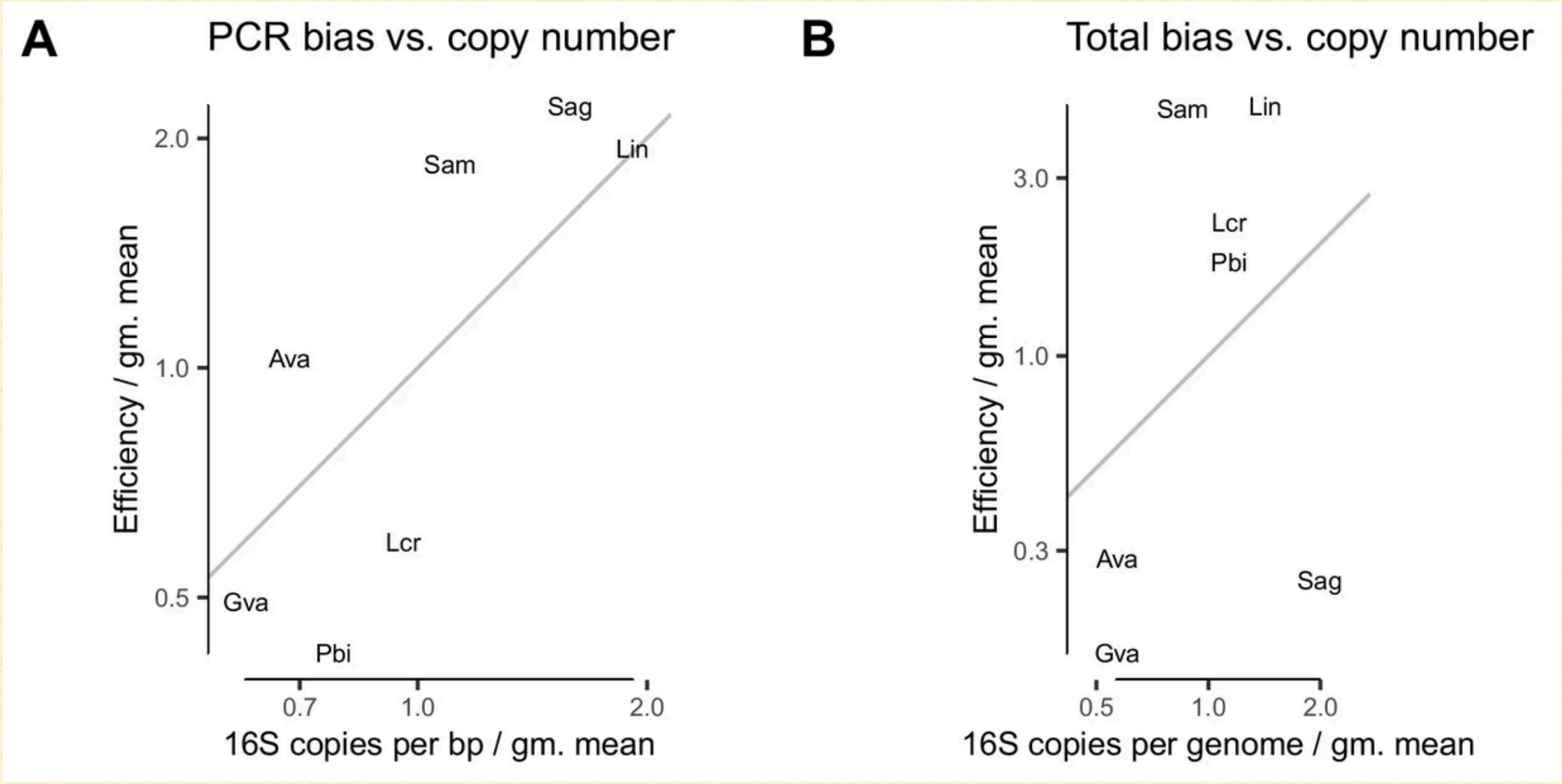


Image from McLaren et al., 2019, *eLife*