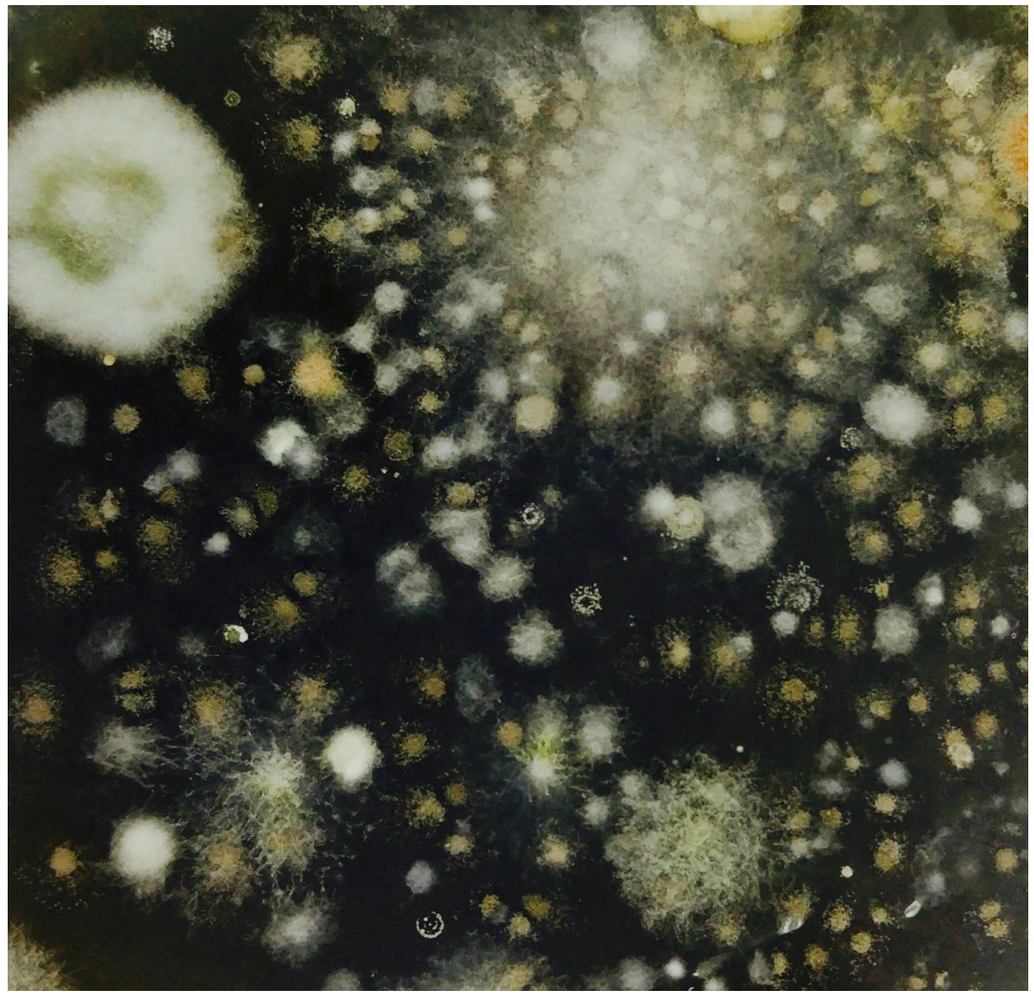
A rigorous & rational approach to

Microbial differential abundance



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Slides: github.com/statdivlab/presentations

Paradigms for microbiome data analysis

- Two statistical paradigms:

1. “How do we *model the data?*”

2. “How do we *learn about biology?*”

Paradigm 1: Model the data

- “Modeling the data” conversations sound like
  - compositional? overdispersed? zero-inflated? Negative binomial? Multinomial-Dirichlet? …
- rarefy?
- best α- and β-diversity metrics?
- best transformation / “normalization”?

Paradigm 2:
Learn about biology

- “Learning about biology” conversations sound like
  - What exists in the environment?
  - What would we like to learn?
  - How does our data reflect that environment?
  - What can we learn from our data? Under what assumptions?

Today: Paradigm 2 for differential abundance

Statistics > Methodology

[Submitted on 7 Feb 2024 (v1), last revised 14 Mar 2025 (this version, v2)]

Estimating Fold Changes from Partially Observed Outcomes with Applications in Microbial Metagenomics

David S Clausen, Sarah Teichman, Amy D Willis

We consider the problem of estimating fold-changes in the expected value of a multivariate outcome observed with unknown sample-specific and category-specific perturbations. This challenge arises in high-throughput sequencing studies of the abundance of microbial taxa because microbes are systematically over- and under-detected relative to their true abundances. Our model admits a partially identifiable estimand, and we establish full identifiability by imposing interpretable parameter constraints. To reduce bias and guarantee

Software: github.com/**statdivlab/radEmu**

What exists in the environment?

- "There is some number of a given biological quantity in every environment"
- "There are 54,601 *S. epidermidis* cells on my index finger"
- "There are 0 transcripts of the gene *Core RC1 subunit PsaA* on this podium”
- "There are 874,455,469 genomes circulating in 100 mL seawater with the 16S variant CGGAGGGTGCA…”

What exists in the environment?

Y_{ij} = true number of genomic unit j in environment i

$X_i \in \mathbb{R}^p$ covariate information
(treatment vs control, age, sex, diet…)

😻 Y_{ij} 💸	1	2	…	J
ENV 1				
ENV 2				
…				
ENV M				
ENV M+1				
…				
ENV N-1				
ENV N				

What would we like to know?

Y_{ij} = true number of unit j in sample i

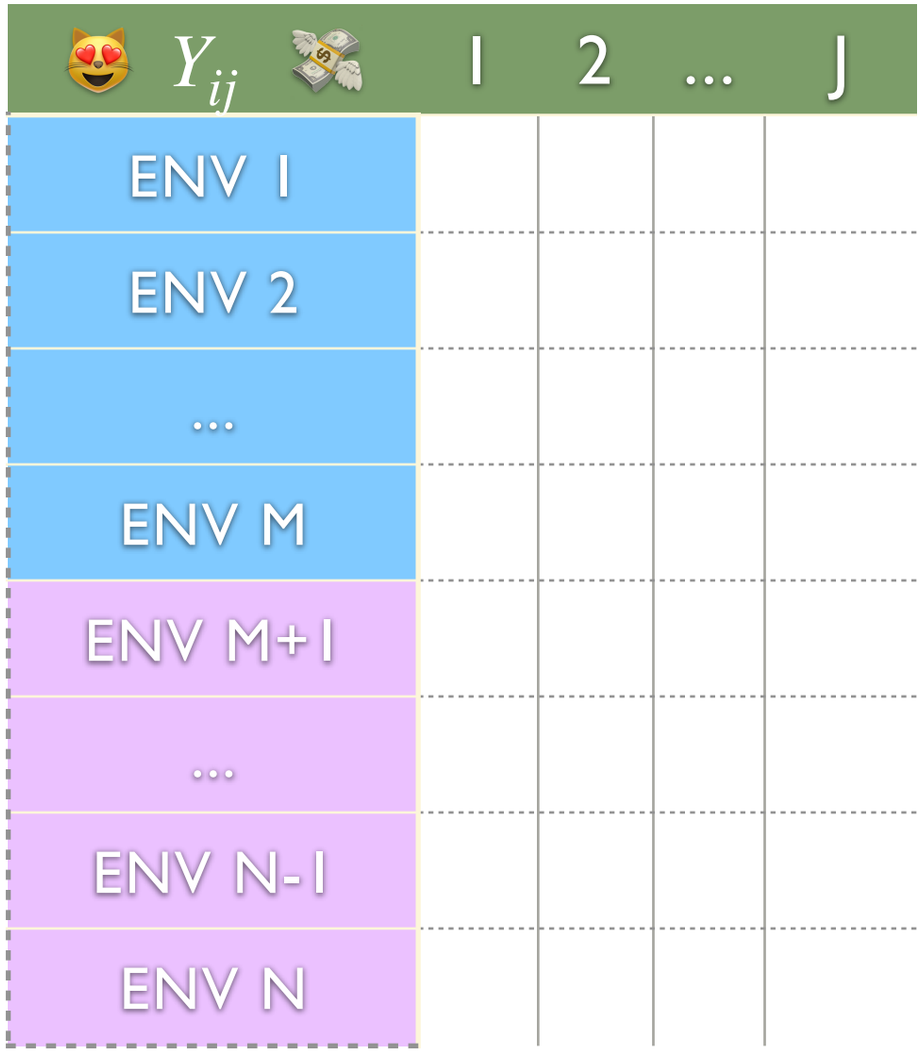
We do **not** observe $\{Y_{ij}\}$…

…but if we did, what would we do?

😻 Y_{ij} 💸	1	2	…	J
ENV 1				
ENV 2				
…				
ENV M				
ENV M+1				
…				
ENV N-1				
ENV N				

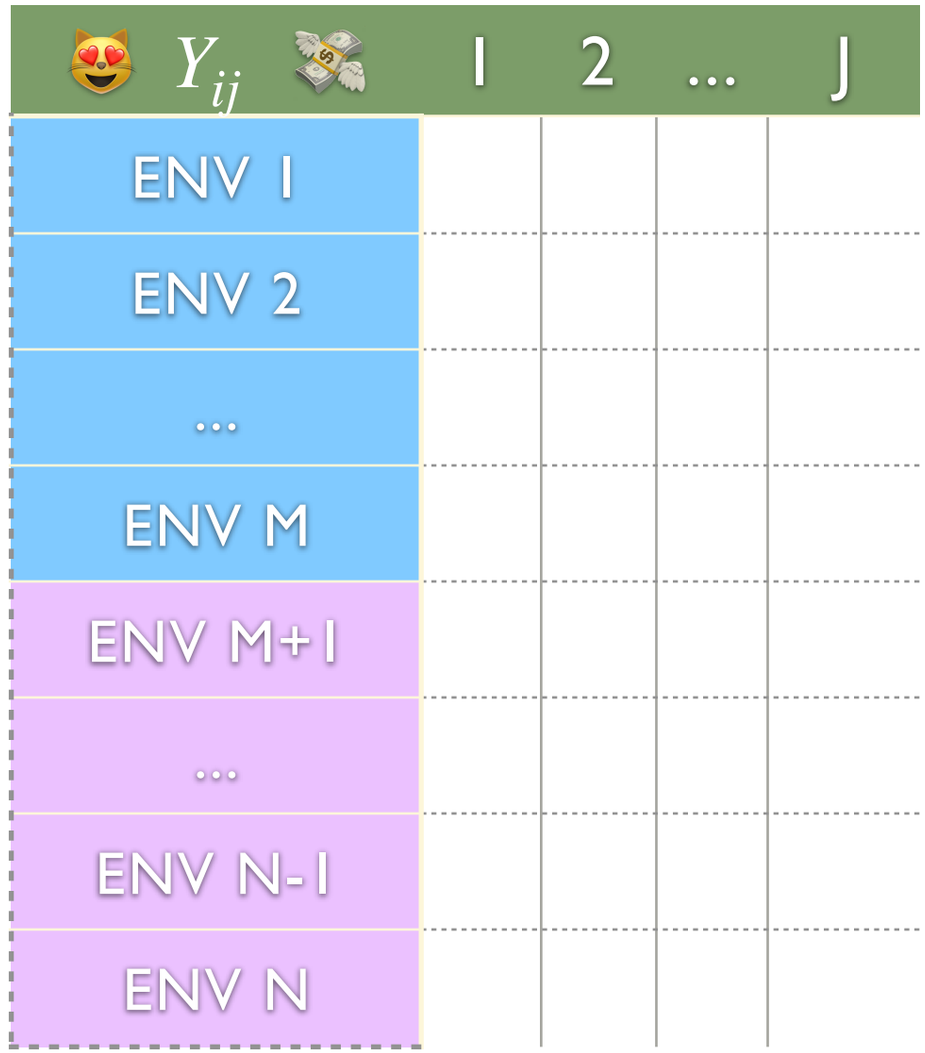
What would we like to know?

- How *abundant* are species?
  - Average of Y_{i4} across environments
- How *present* are species?
  - % of environments in which $Y_{i2} > 0$
- How *diverse* are communities?
  - $\#\{j : Y_{ij} > 0\}$
  - $-\sum_{j=1}^{J} p_{ij} \log p_{ij}$ for $p_{ij} := \frac{Y_{ij}}{\sum_j Y_{ij}}$
- ...



What would we like to know?

- Which species differ in their average abundance?
  - All?
- By how much?
  - 0.1%? 50%? 500%?



What data do we have?

Y_{ij} = true number of unit j in sample i

W_{ij} = number of times unit j observed in sample i from HTS

🌧️ W_{ij} 😿	1	2	…	J
SAMPLE 1				
SAMPLE 2				
…				
SAMPLE M				
SAMPLE M+1				
…				
SAMPLE N-1				
SAMPLE N				

How do we connect the Y_{ij}'s and the W_{ij}'s?

Connecting data to reality

- Traditionally, DA methods assume

$$\mathbb{E}[W_{ij}] = c_i Y_{ij}$$

- Is this reasonable?

Connecting data to reality

- Mock community: An artificially constructed community of known composition

	L.crispatus	L.iners	A.vaginae	S.agalactiae	G.vaginalis	S.amnii	P.bivia
1	0	0	0	1.00	0	0	0
2	0	0	0.5	0	0	0	0.5
3	0.33	0.33	0	0	0	0	0.33
4	0.33	0.33	0	0.33	0	0	0

Connecting data to reality

	L.crispatus	L.iners	A.vaginae	S.agalactiae	G.vaginalis	S.amnii	P.bivia
1	19	4	2	51332	1	14	1
2	0	1	1424	0	0	7	21708
3	4775	11234	0	0	0	1	3249
4	1644	5497	1	4521	0	7	0

	L.crispatus	L.iners	A.vaginae	S.agalactiae	G.vaginalis	S.amnii	P.bivia
1	0	0	0	1.00	0	0	0
2	0	0	0.5	0	0	0	0.5
3	0.33	0.33	0	0	0	0	0.33
4	0.33	0.33	0	0.33	0	0	0

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

Connecting data to reality

1. Despite equal mixing fractions, some taxa are observed many more times

2. Despite being purportedly absent, taxa are observed

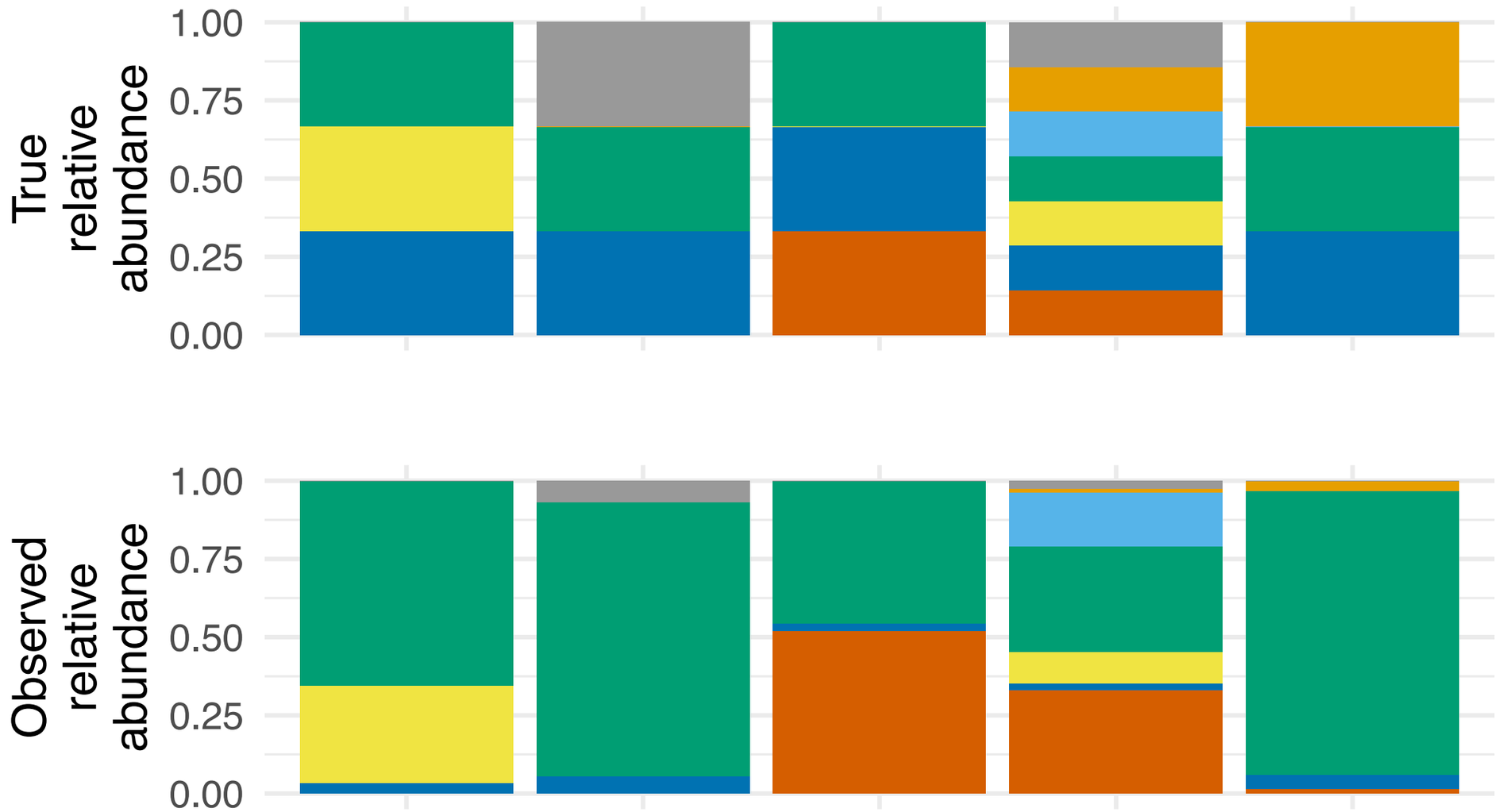
Connecting data to reality

- Despite the common assumption that

$$\mathbb{E}[W_{ij}] = c_i Y_{ij}$$

some taxa are *over-observed* for equal c_i and Y_{ij}

- What model better explains this observation?



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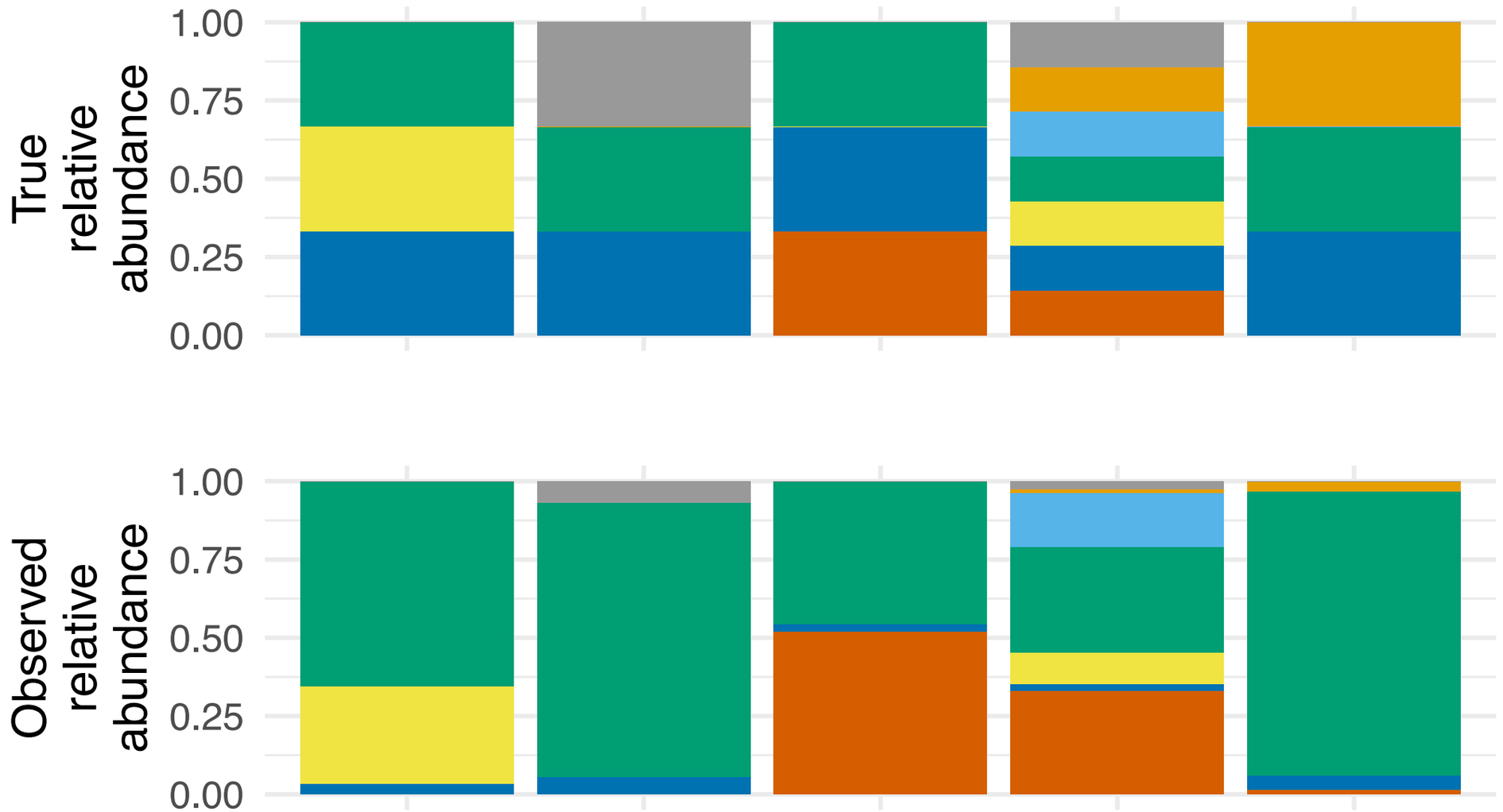


L.iners/S.agalactiae						
	19.5	16.1	18.9	17.4	19.9	**18.3 (1.6)**

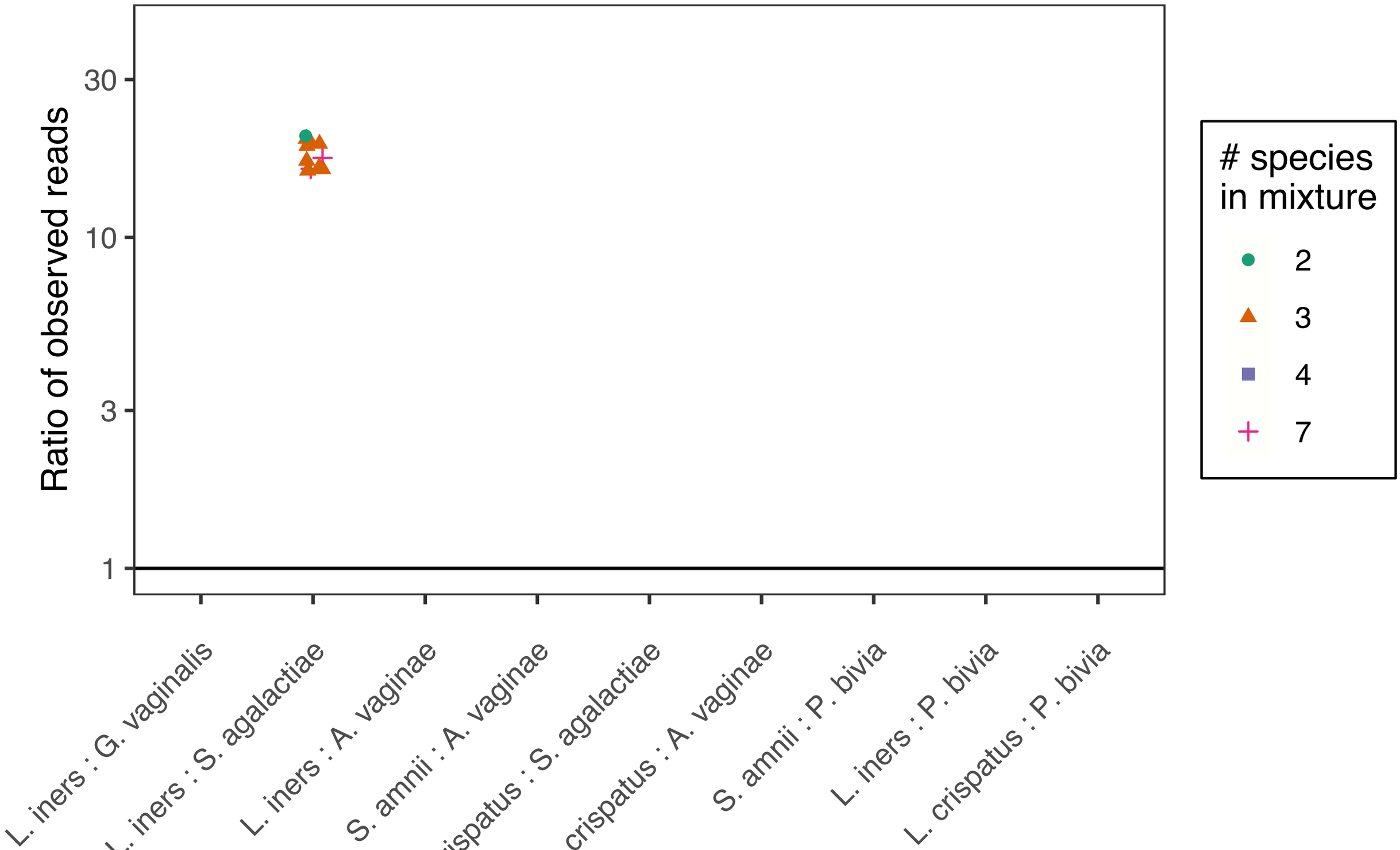


 Figure adapted from McLaren, Willis & Callahan, 2019, *eLife*

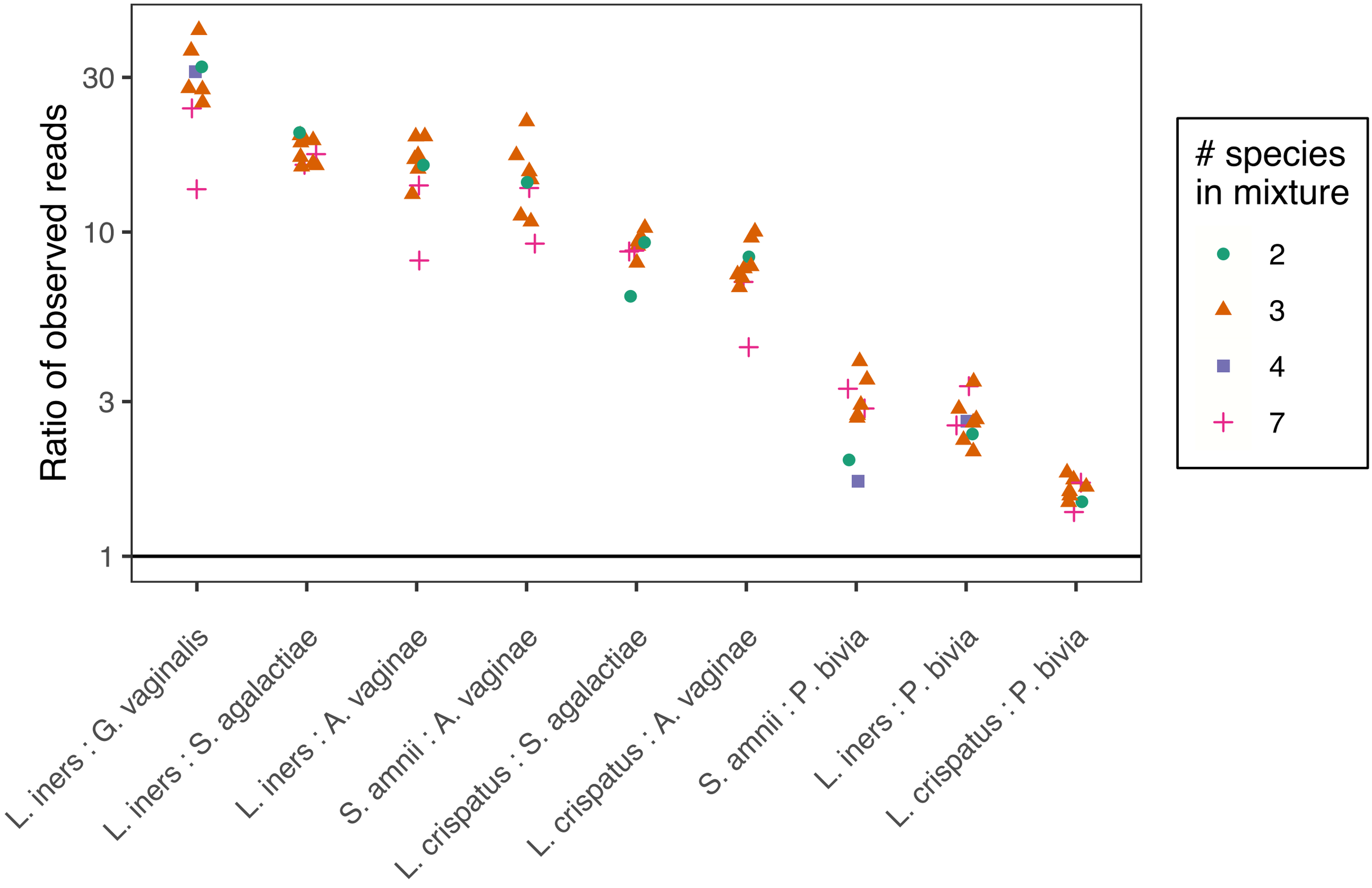


 Figure adapted from McLaren, Willis & Callahan, 2019, *eLife*

Connecting data to reality

- Evidence *against*

$$\mathbb{E}[W_{ij}] = c_i \times Y_{ij}$$

- Better support for

$$\mathbb{E}[W_{ij}] = c_i \times e_j \times Y_{ij}$$

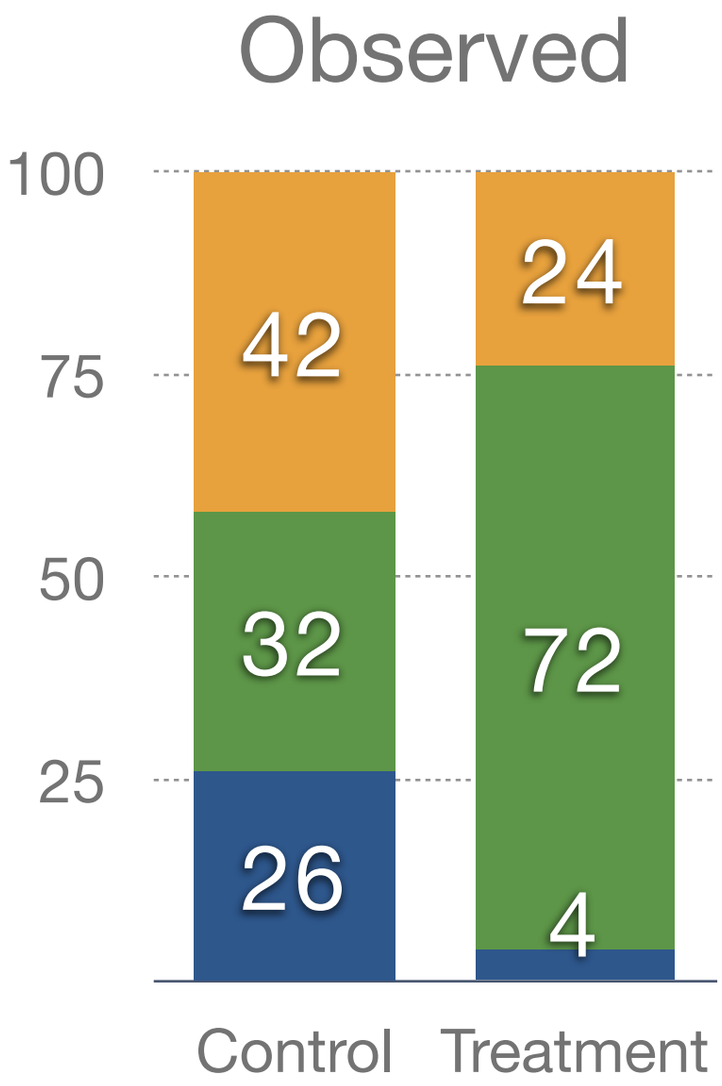
Why is this so important for data analysis?

Connecting data to reality

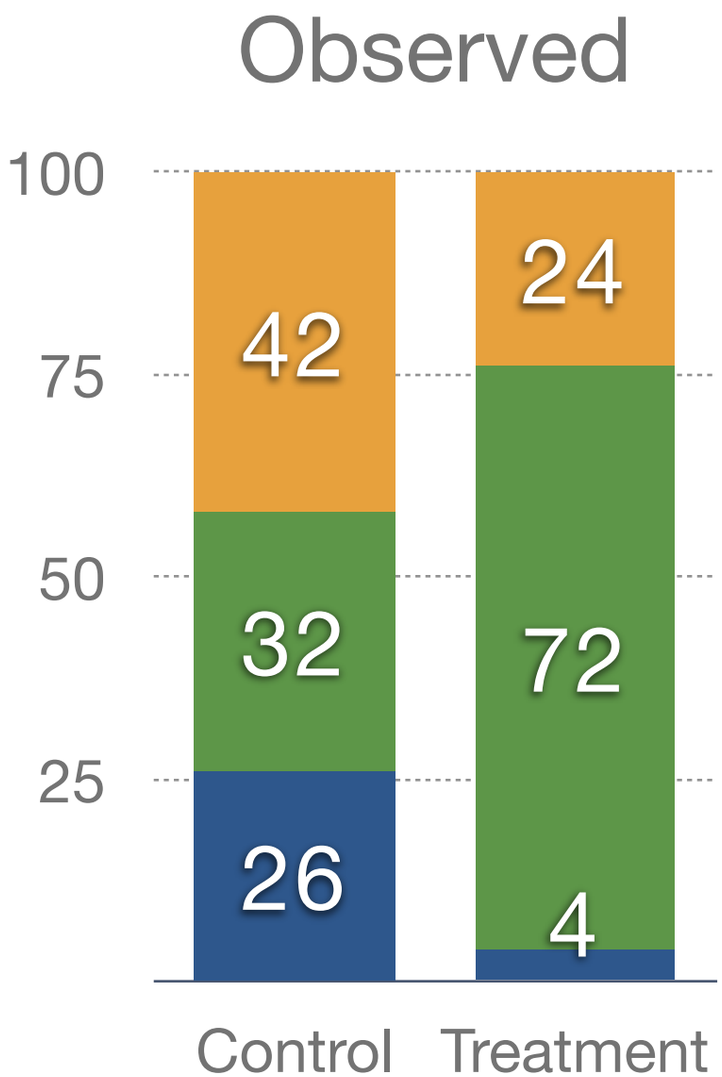
- Stated differently,

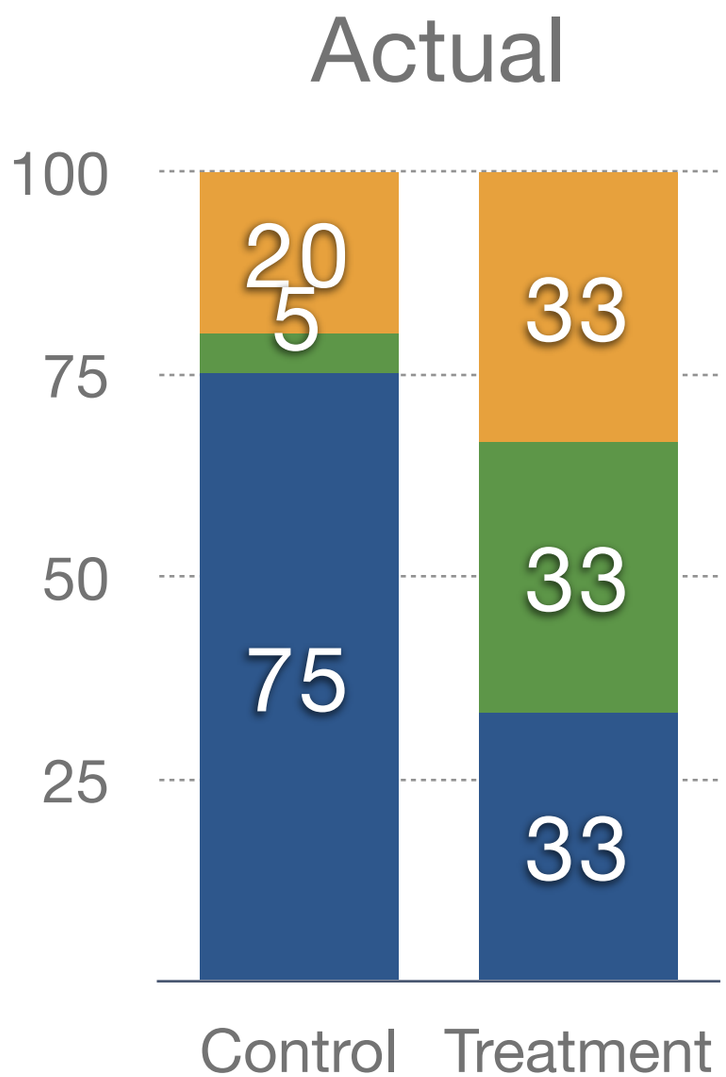
Observed relative abundance $\propto$ True relative abundance $\times$ Taxon-specific efficiencies

Expected value of $\frac{W_{ij}}{\sum_{j'} W_{ij'}} = \frac{p_{ij} e_j}{\sum_{j'} p_{ij'} e_{j'}}$



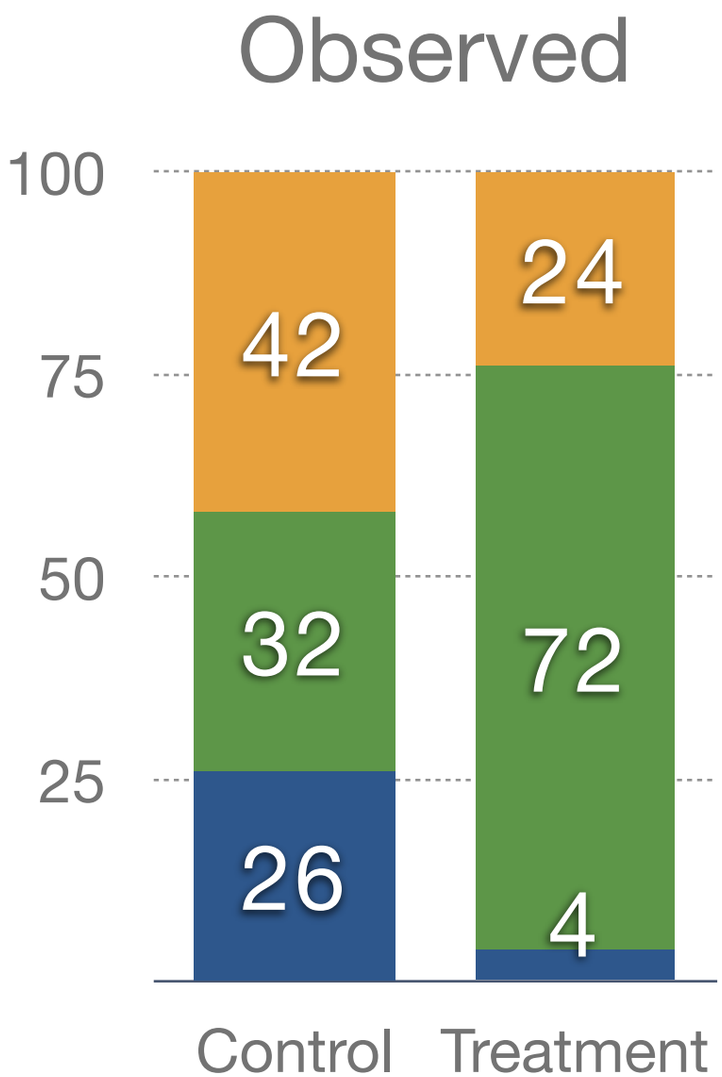
- A tempting conclusion:
  - The relative abundance of **orange** *<u>decreased</u>* in the Treatment sample (right) compared to the Control sample (left)

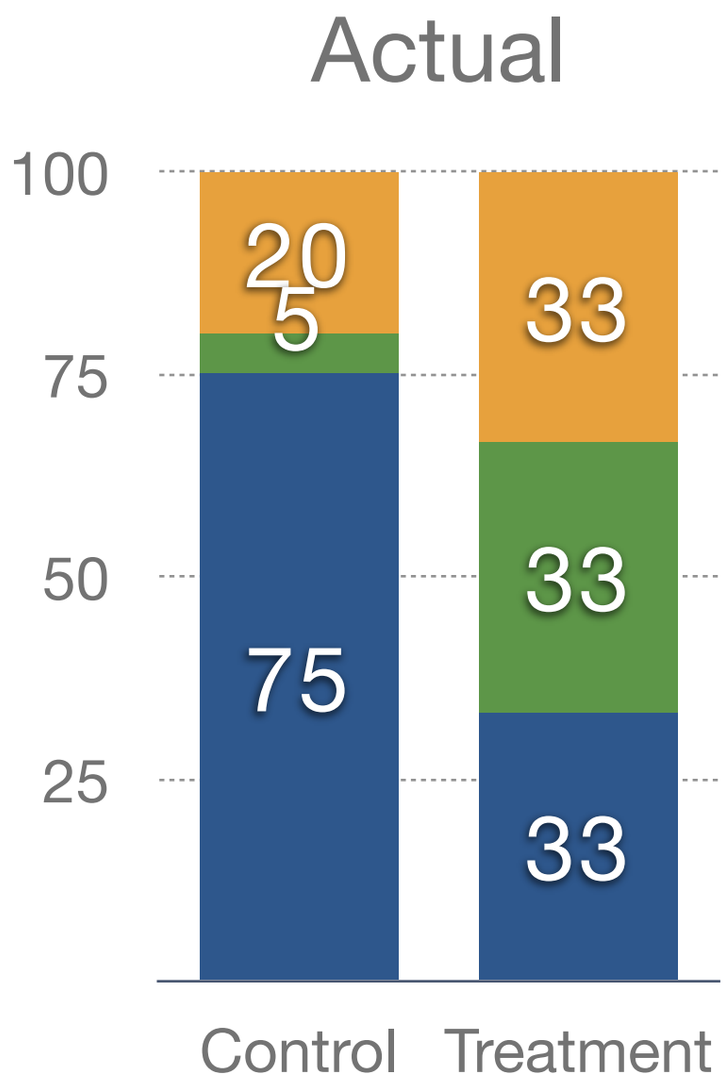


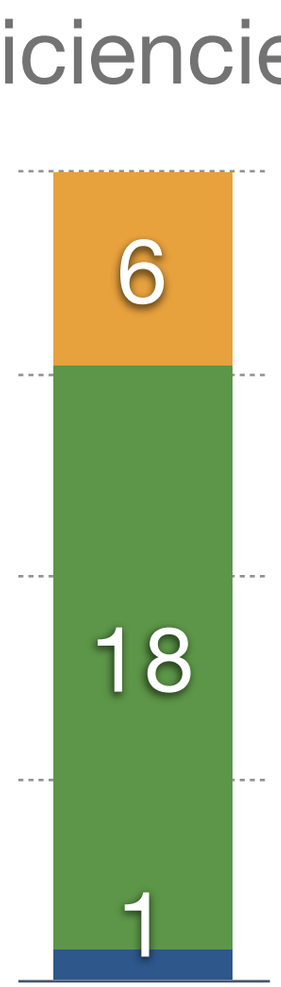


- In fact, the relative abundance of **orange** *increased* in the Treatment sample compared to the Control sample

For more details, see McLaren *et al* (2022+) "Implications of taxonomic bias..."







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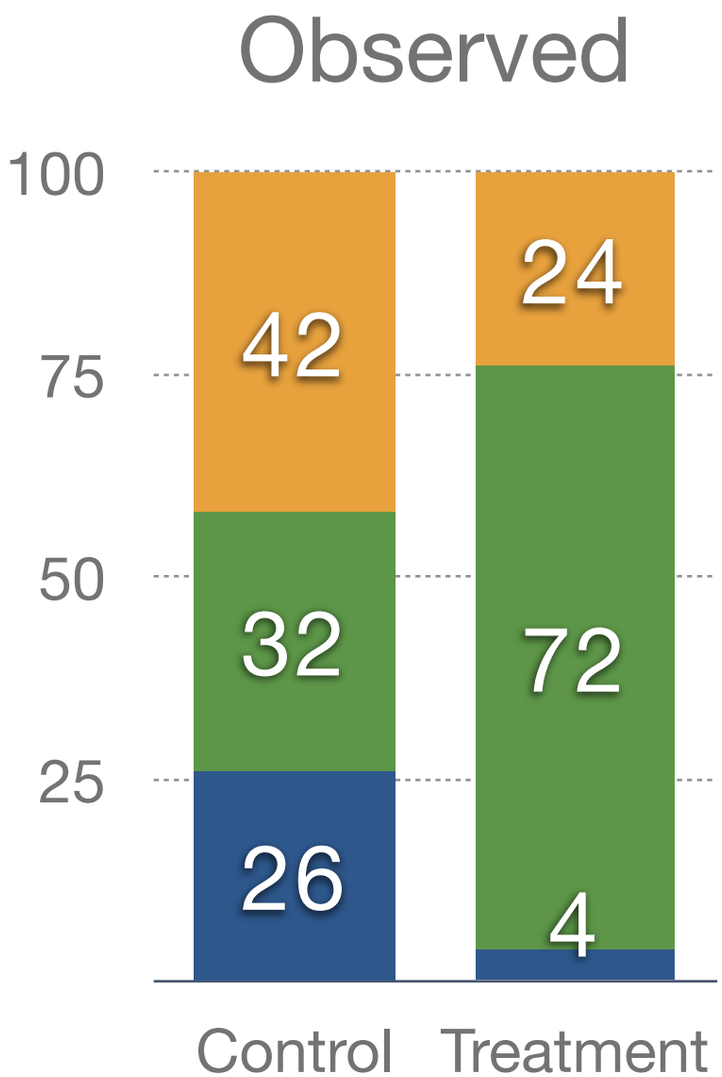
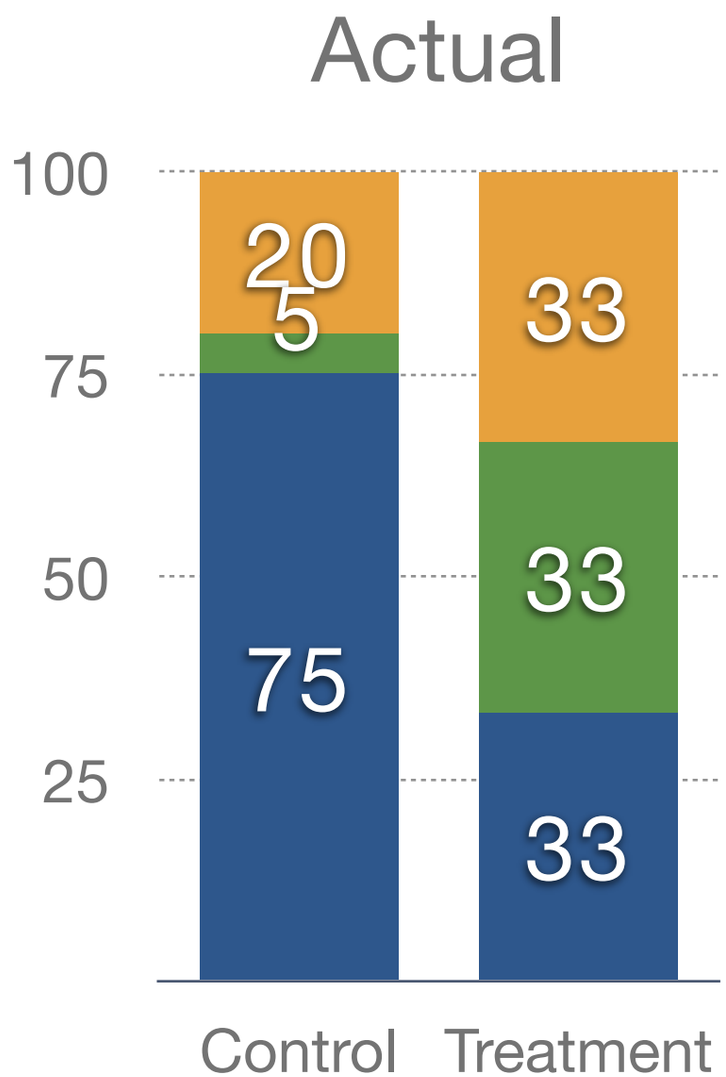
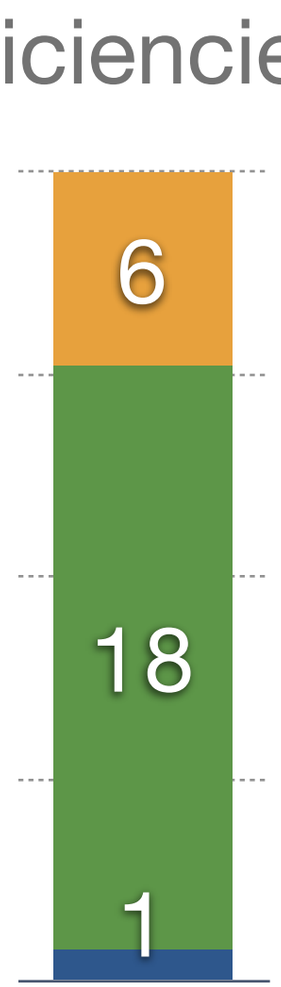






- **Green** is high efficiency; its abundance increased.
  Blue is low efficiency, and its abundance decreased.

- **Orange**'s abundance depends on the abundance of **the other taxa**.

For more details, see McLaren *et al* (2022+) "Implications of taxonomic bias..."

What can't we learn?

- Result: Under the model

$$\mathbb{E}W_{ij} = c_i \times e_j \times Y_{ij}$$

we *cannot* learn about:

- $\mathbb{E}Y_{\{X_i=1\}j} - \mathbb{E}Y_{\{X_i=0\}j}$
- $\mathbb{E}\left[\frac{Y_{\{X_i=1\}j}}{\sum_{j'} Y_{\{X_i=1\}j'}}\right] - \mathbb{E}\left[\frac{Y_{\{X_i=0\}j}}{\sum_{j'} Y_{\{X_i=0\}j'}}\right]$
- $\frac{\mathbb{E}Y_{\{X_i=1\}j}}{\mathbb{E}Y_{\{X_i=0\}j}}$ and $\log\left(\frac{\mathbb{E}Y_{\{X_i=1\}j}}{\mathbb{E}Y_{\{X_i=0\}j}}\right)$

What can we learn?

- Result: Under the model

$$\mathbb{E}W_{ij} = c_i \times e_j \times Y_{ij}$$

we *can* learn about:

- $$\log\left(\frac{\mathbb{E}Y_{\{X_i=1\}j}}{\mathbb{E}Y_{\{X_i=0\}j}}\right) - \log\left(\frac{\mathbb{E}Y_{\{X_i=1\}j'}}{\mathbb{E}Y_{\{X_i=0\}j'}}\right)$$

- $$\log\left(\frac{\mathbb{E}Y_{\{X_i=1\}j}}{\mathbb{E}Y_{\{X_i=0\}j}}\right) - \text{average}_{j'} \log\left(\frac{\mathbb{E}Y_{\{X_i=1\}j'}}{\mathbb{E}Y_{\{X_i=0\}j'}}\right)$$

What can we learn?

- Result: Under the model

$$\mathbb{E}W_{ij} = c_i \times e_j \times Y_{ij}$$

$$\log Y_{ij} = X_i^T \beta_j$$

we *can* learn about

- $\beta_{kj} - \beta_{kj'}$ log ratios-of-ratios
- $\beta_{kj} -$ average$(\beta_{k.})$ log ratios *relative* to average log ratios

What can we learn?

- Res

We can identify
groups (taxa, genes, etc.)
that are
changing the **most**
in abundance
from HTS

we

- p_{kj} — average($p_{k.}$) log ratios relative to average log ratios

🦤 radEmu 🦤

- We propose an estimator of β_{kj} – average($\beta_{k.}$) under the model

$$\mathbb{E}W_{ij} = c_i \times e_j \times Y_{ij}$$

$$\log Y_{ij} = X_i^T \beta_j$$

- Estimator is *consistent* under weak conditions, *efficient* under stronger conditions

🦤 radEmu 🦤

✅ Estimates a ecologically-relevant, model-agnostic, interpretable parameter

> "We estimate that the average abundance of *Oliverpabstia intestinalis* in metagenomes is 11 times greater after commencing dairy work, when compared to the typical fold-differences in the average abundance of taxa across these visits."

> "Under the assumption that most taxa do not differ in average abundance between visits post and prior exposure, we estimate that the abundance of *Oliverpabstia intestinalis* in metagenomes from post exposure visits is 11 times greater than prior exposure visits."

✅ Robust to differential detection

✅ Adjusts for differential sequencing depth

✅ ...

 radEmu 

✅ Model-robust inference controls Type 1 error unlike DESeq2, ANCOM-BC2, t-tests…

✅ Handles zeroes without pseudocounts $\mathbb{E}(\log Y_{ij})$ vs $\log \mathbb{E} Y_{ij}$

✅ Simulation: Smallest estimation error out of methods that control T1E beats ALDEx2

✅ Covariate adjustment; inference under independence & cluster correlation;

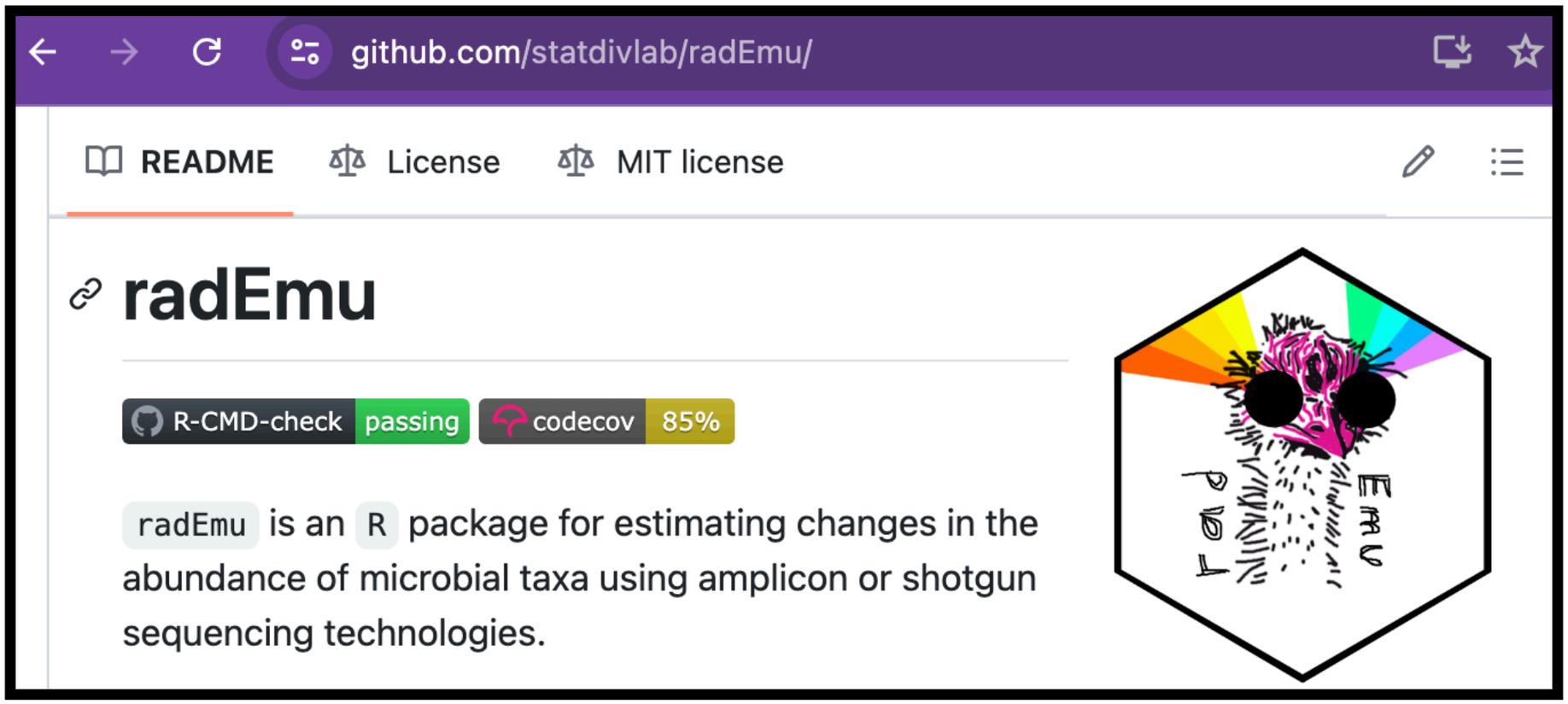
✅ Spike-ins + radEmu = you can interpret fold-differences on the absolute scale

❌ Slower than other methods

Summary

- ~~"How do we *model the data?*"~~
- "How do we *learn about biology?*"
- Want: estimate β in $\log Y_{ij} = X_i^T \beta_j$
- Have: distorted data $W_{ij} \approx c_i \times e_j \times Y_{ij}$
- Result: $\beta_{kj} -$ average$(\beta_{k.})$ is identifiable
- Method: model-robust estimation & inference with 🦤radEmu🦤

Software




```
emuFit(formula = ~ cases + age + sex,
       data = my_metadata,
       Y = my_counts)
```

arXiv > stat > arXiv:2402.05231

Search... Help | Adv

Statistics > Methodology

[Submitted on 7 Feb 2024 (v1), last revised 14 Mar 2025 (this version, v2)]

Estimating Fold Changes from Partially Observed Outcomes with Applications in Microbial Metagenomics

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David Clausen

Sarah Teichman

eLIFE elifesciences.org RESEARCH ARTICLE

Consistent and correctable bias in metagenomic sequencing experiments

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

Michael McLaren (MIT, SecureBio)

NIH NIGMS

Implications of taxonomic bias for microbial differential-abundance analysis

Michael R. McLaren, Jacob T. Nearing, Amy D. Willis, Karen G. Lloyd, Benjamin J. Callahan

doi: https://doi.org/10.1101/2022.08.19.504330

Ben Callahan (NCSU)

R packages: radEmu from github.com/statdivlab

A rigorous & rational approach to

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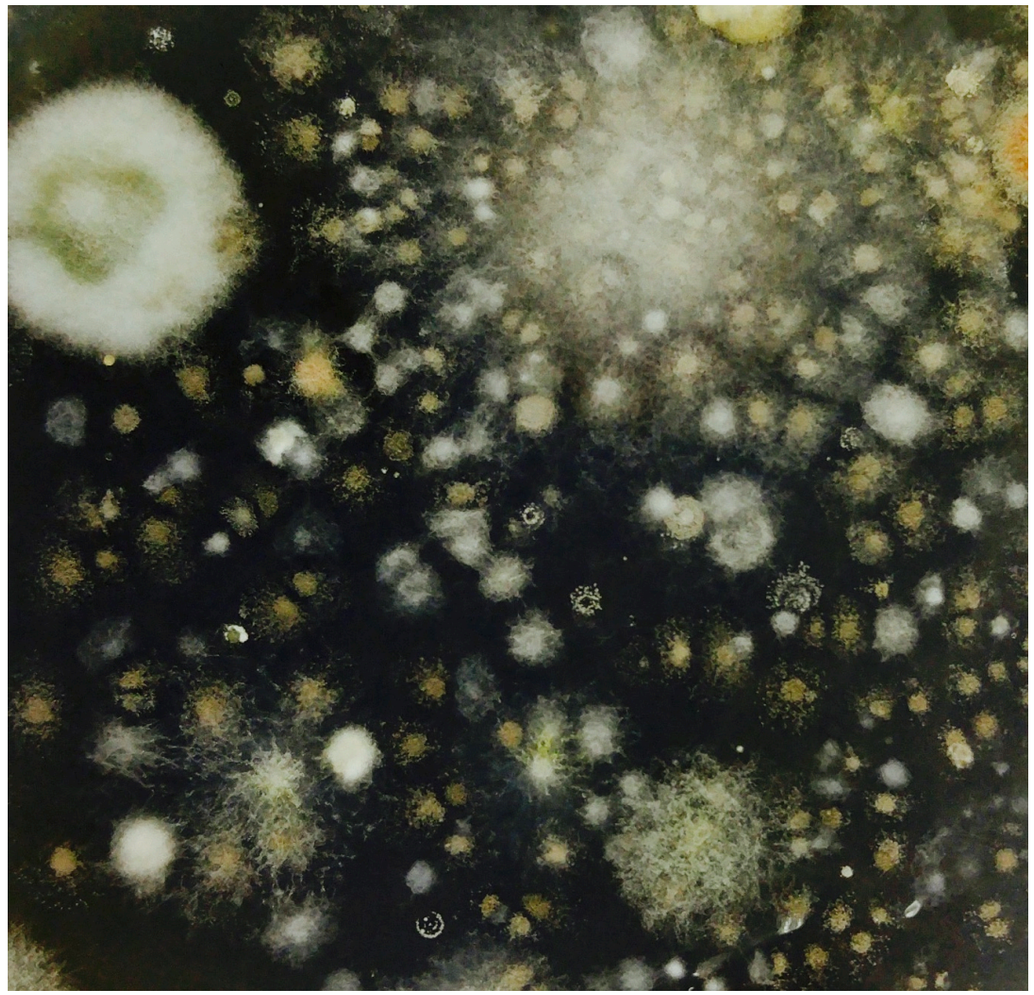

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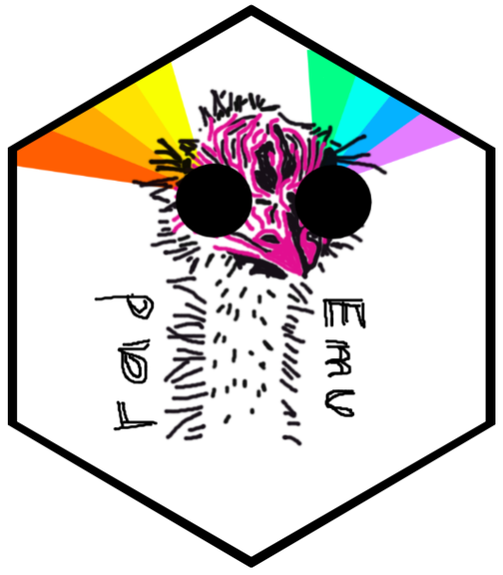
Slides: github.com/statdivlab/presentations

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) BMC Microbiology

Amy's wish list



- You choose a meaningful parameter to estimate
- You choose a sensible way to estimate the parameter
- You choose tests that control Type 1 error

I like radEmu because it meets these criteria!

https://github.com/statdivlab/radEmu/

Comparing radEmu to other methods

- Make W_{ij}'s realistic lots of zeroes, high-variance

- Ask

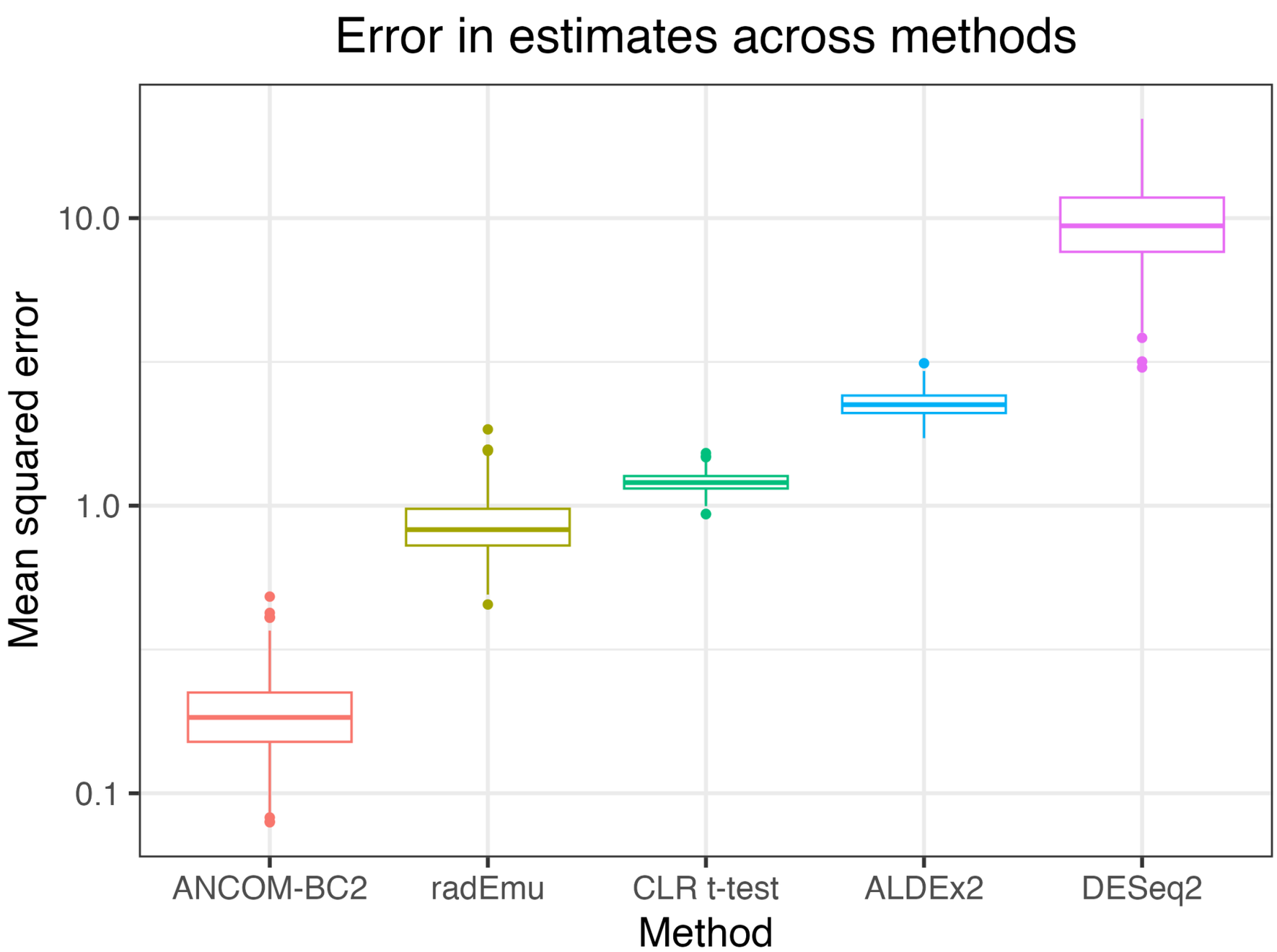
1. "How good are our estimates?"

2. "Do we have error rate control?"

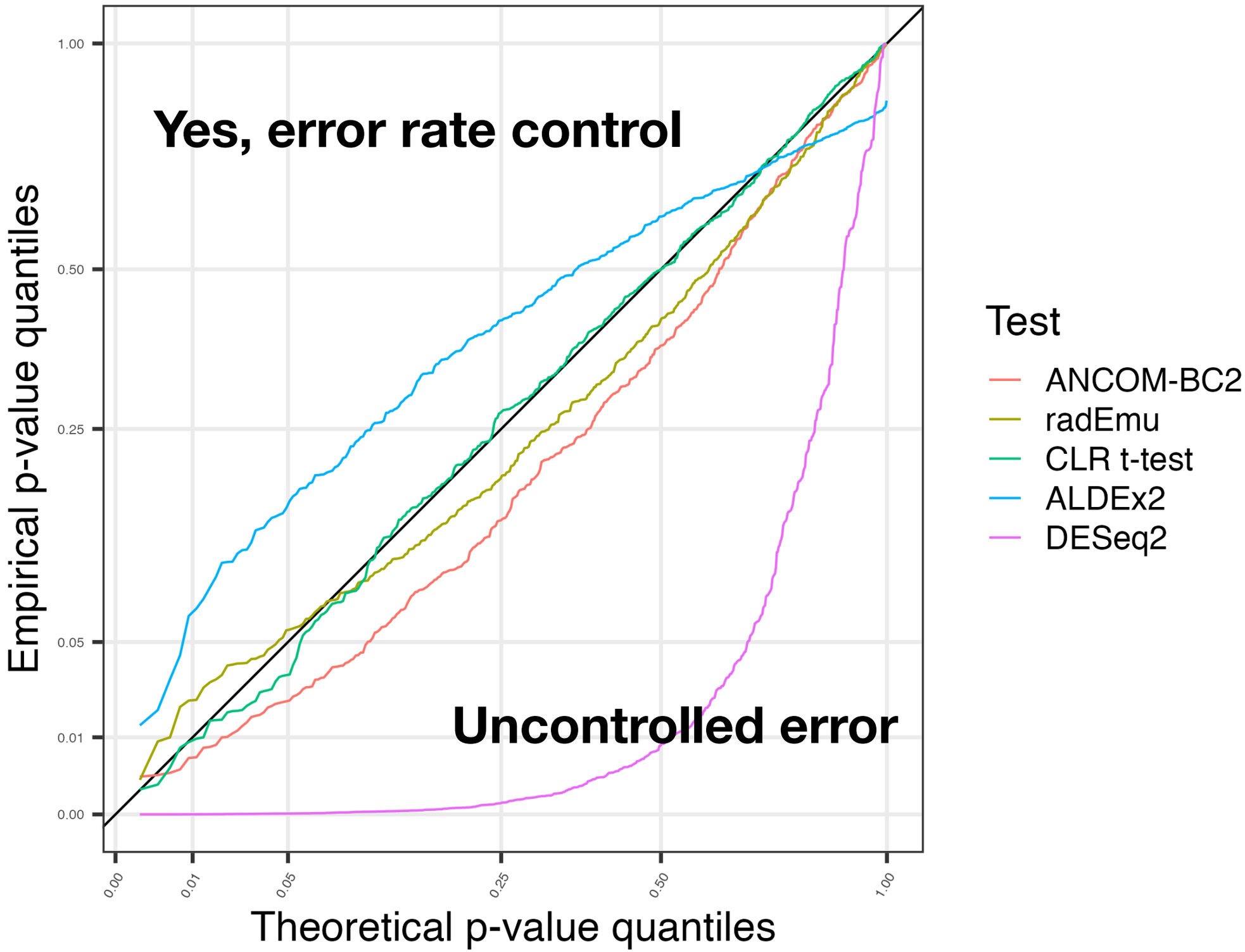
    - Null hypothesis: "Fold difference (cases vs. controls) in *F. praus* is equal to typical fold difference across taxa"

Special thanks to Sarah! 😻

“How good are our estimates?”



“Do we have error rate control?”



Type I error rate control results

Method	1% Type 1 error	5% Type 1 error rate
ALDEx2	0.00	0.01
ANCOM-BC2	0.02	0.11
CLR t-test	0.01	0.06
DESeq2	0.52	0.67
radEmu	0.00	0.04

Simulation takeaways

- TL;DR In a realistically pathological setting,
    - radEmu has the lowest error in estimation out of all methods that control error Type 1 error rate

Identifiability: summary

- Can't estimate *absolute* log-fold differences, but can estimate differences relative to other taxa

  - $\hat{\beta}_{kj}$ is the estimated log-fold difference in the mean abundance μ_{ij} of taxon j across X_k groups

$$\log \mathbb{E} Y_{ij}\left(\mathbf{x}_{x_k+1}\right) - \log \mathbb{E} Y_{ij}(\mathbf{x})$$

  relative to typical differences across taxa

- Challenges current paradigm that we cannot make statements about *absolute abundances* μ_{ij} from *"compositional" data* W_{ij}

Interpretation

- $\beta_{kj} - g(\beta_{k.})$ is the log-fold difference in the mean abundance of taxon j across groups that differ in $X_{.k}$ but are similar in $X_{.\{-k\}}$,

$$\log \mathbb{E} Y_{ij} \left(\mathbf{x}_{x_k+1} \right) - \log \mathbb{E} Y_{ij} (\mathbf{x}),$$

relative to typical differences across taxa