Outline for today

- Random vs fixed effects

- Two-factor example

- Why the calculations are different with random effects

- Unbalanced designs with random effects

- Examples of experiments with random effects

- Linear mixed-effects models

- Example: Estimating repeatability of a measurement

- Other designs with random effects, briefly

- Assumptions of linear mixed-effects models

- An example violating an assumption, with a solution

What are fixed effects

Fixed effects are <u>predetermined</u> categories of a variable, of <u>direct interest</u>, and are <u>repeatable</u>. An experiment with the same treatment levels could be repeated.

For example:

- medical treatments in a clinical trial
- predetermined doses of a toxin
- diet or fertilization treatments
- age groups in a population
- habitat
- season

Any conclusions reached in the study about differences among groups can be applied <u>only</u> to the groups included in the study. The results cannot be generalized to other treatments, habitats, etc. not included in the study.

Example of factorial experiment with fixed effects

Field transplant experiment to investigate how herbivores affect the abundance of the red alga, *Mazzaella parksii* in the intertidal habitat of coastal Washington State using (Harley 2004). I analyzed a subset of treatments:

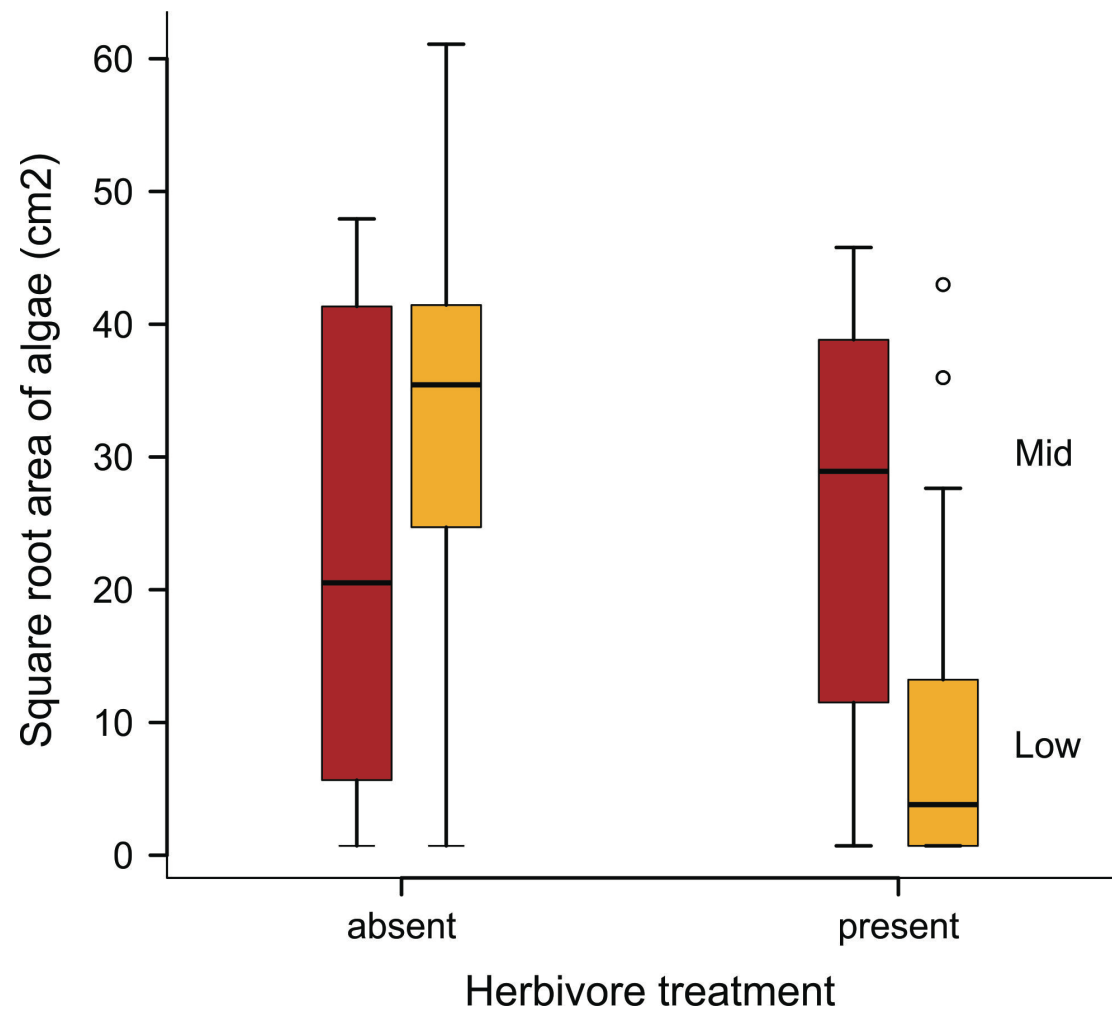|	**herbivory treatment**	
intertidal zone	**present**	**absent**
low	*n* = 16 plots	*n* = 16 plots
mid	*n* = 16 plots	*n* = 16 plots

N = 64 plots in a completely randomized design

Factorial experiment with fixed effects

Response variable: surface area of red alga, *Mazzaella parksii* in plots.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_2 X_1 X_2$$

Linear model for factorial experiment with fixed effects

```
z <- lm(sqrtarea ~ herbivores * zone)
```

The denominator of the F statistic for the treatment effect is $MS_{residual}$

Source	SS	df	MS	F	P
zone	89	1	89	89/238= 0.37	0.54
herbivory	1512	1	1512	1512/238= 6.36	0.01
zone*herbivory	2617	1	2617	2617/238= 11.0	<0.01
residual (error)	14271	60	238		

The residuals represent the sole source of random variation in the analysis.

The residual mean square ($MS_{residual}$) is the appropriate quantity to use as a reference when asking whether treatment mean squares are larger than random variation (chance), i.e., whether F ≫ 1.

What are random effects

Randomly sampled categories of a variable, representing *groups* or *clusters* of measurements or units. An experiment with the same treatment levels could <u>not</u> be repeated. For example:

- randomly sampled families made up of siblings
- randomly sampled subjects measured repeatedly
- randomly placed transects each of multiple quadrats in a sampling survey
- field plots each assigned multiple treatments
- environment chambers each containing many aquariums
- aquariums containing multiple individuals

Groups are assumed to be randomly sampled from a *population of groups*. Therefore, conclusions reached about groups *can* be generalized to this population.

What are random effects

In some cases, the random effects are of no interest themselves – a nuisance.

- field plots each assigned multiple treatments
- environment chambers containing aquariums

In other cases, measuring the <u>variance</u> associated with each level of random groupings is one purpose of the study.

- families of siblings (to estimate heritability, etc)
- subjects measured repeatedly (to estimate repeatability)
- transects with multiple quadrats (to estimate α- and β-diversity)

Repeated measurements made on the same group are not independent.

Modeling random effects properly (using mixed effects models) avoids pseudoreplication.

Factorial experiment when one of the factors is random

Futuyma and Philippi (1987)

Fall cankerworm, *Alsophila pometaria*

http://cfs.nrcan.gc.ca/subsite/glfc-sugarbush/alsophila-pometaria-images

Caterpillars of the fall cankerworm feed on the leaves of hardwood trees. Adult female moths are wingless. Many reproduce clonally, producing only daughters genetically identical to themselves.

Research question: What is the effect of tree species on growth; and how much do clones vary in growth?

(This is a not a question about specific clones but about the population of clones.)

Factorial experiment when one of the factors is random

Design: Sample 9 female moths from a population in NY. Raise larvae from 9 clones on leaves of 4 tree species. Measure individual growth after 15 days.
Two factors: **Tree species** (fixed), *Clone* (random)
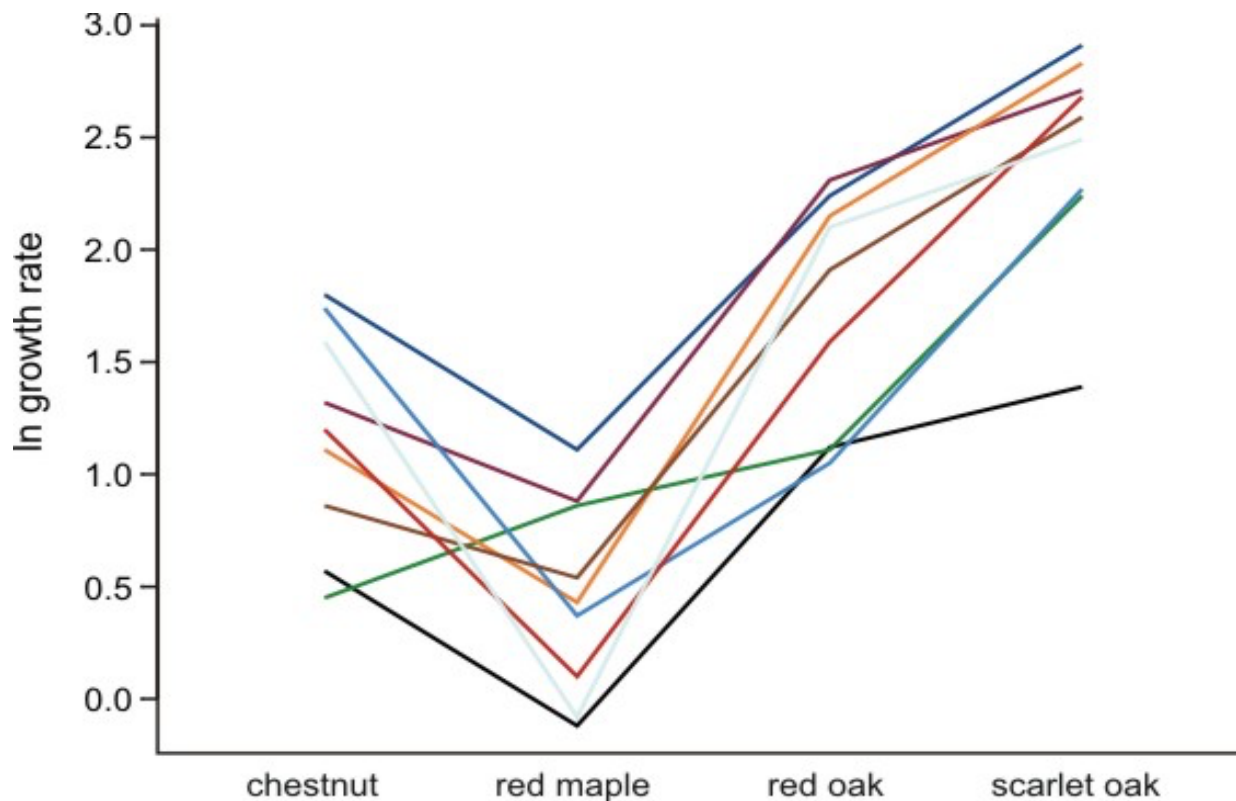
Interaction plot:

Mean growth of caterpillars from 9 families (clones) raised on four tree species.

N = 326 caterpillars total.

Factorial experiment when one of the factors is random

Caterpillars from the same clone are <u>not independent</u>. There are 326 caterpillars but only 9 clones. In this case the residual mean square ($MS_{residual}$) from a linear model fit is no longer the appropriate quantity to use as a reference when asking whether treatment mean squares are larger than expected by chance. To do so would commit pseudoreplication.
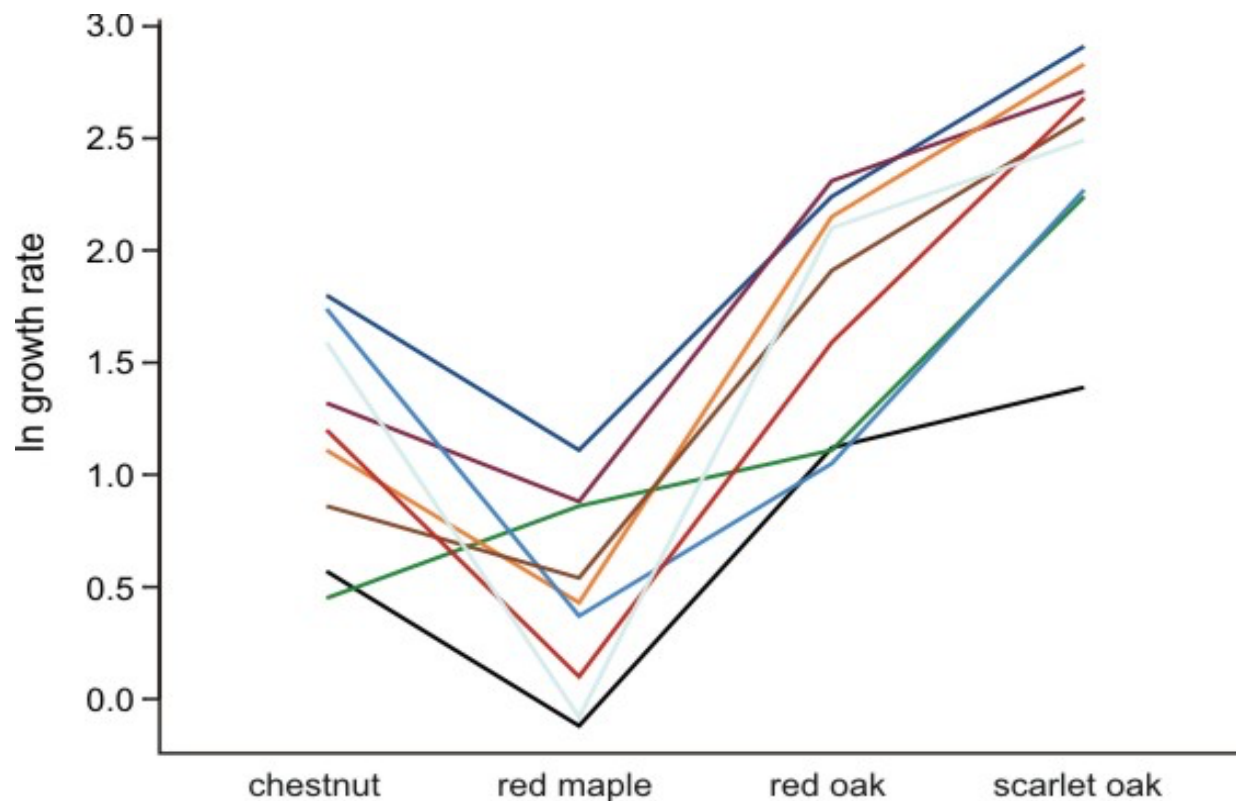

Instead, the variance among clones is the key to testing treatment effects.

(Always report *df* with *F* statistics in your papers to prove you analyzed such data correctly.)

Factorial experiment when one of the factors is random

The presence of a random factor represents an additional source of random variation: the variation among clones.

The mean effect of tree species on caterpillar growth is modeled as the mean of clone means ± variation among clones.
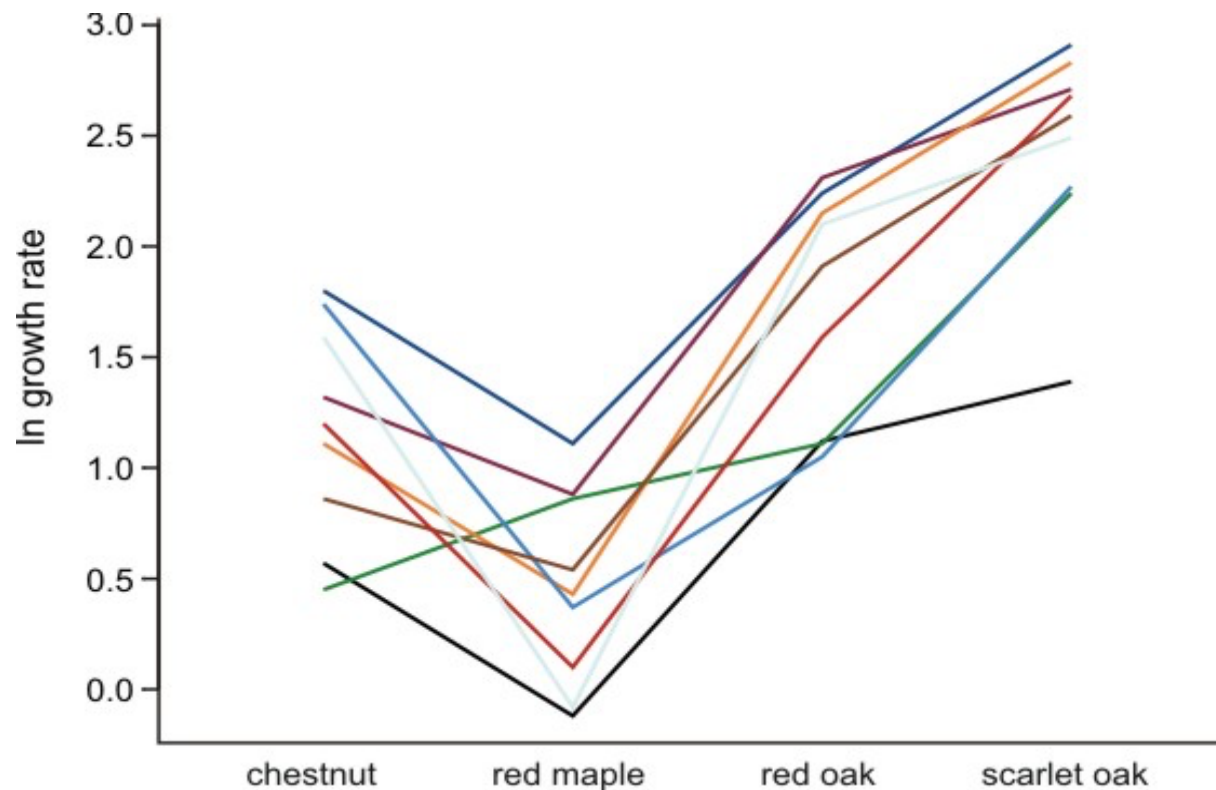
More reasons why analysis is different with random effects

1. Unlike fixed groups, the means of the specific random groups (e.g., clones) are not of direct interest.

2. Interest is focused on the <u>variance among the random groups</u>. A goal of the Futuyma and Philippi experiment was to *estimate* these variances, to measure the amount of genetic variation in the population.

3. When a design including random effects is <u>unbalanced</u>, the standard formulas for *F* statistics (as calculated from ANOVA table of fixed effects) are not *F*-distributed. Standard ANOVA table formulas don't work with unequal sample sizes. Sorry!

4. With unbalanced designs, mixed models calculate approximate *F*-statistics and degrees of freedom for tests of fixed effects. Results are approximate.

How to know when you have random effects in your study

You have random effects:

- whenever your sampling design is nested:
  quadrats within transects; transects within woodlots; woodlots within districts.

- whenever your measurements are clustered spatially or temporally within randomly sampled units i.e., in *blocks*, which are typically analyzed as random effects.

- whenever you divide up plots, or families, clones, ponds, etc, and apply separate fixed treatments to subplots, or siblings, pond-halves, etc.

- whenever you take measurements on related individuals.

- whenever you measure subjects or other sampling units *repeatedly*.

How to analyze random effects

Use <u>linear mixed effects models</u> to analyze random effects.

In R, `lm()` is for fixed effects only.

In R, use `lmer()` in the `lme4/lmerTest` packages or `lme()` in the `nlme` package to analyze models containing random effects. These packages model the variance structure of random effects explicitly.

We'll mostly use `lmer()` because tests of treatment effects using the Kenward-Roger or Satterthwaite approximations for degrees of freedom are most accurate (available in `lmerTest` package).

Attributes of linear mixed-effects models

- They are linear models that include both fixed and random effects.

- They model different error variance for each level of random variation.

- They do not require the assumption of sphericity (a problem of classic repeated measures ANOVA designs when Time is a fixed effect) because they model the correlation structure directly through the random effects.

- Allow for additional correlation structures to model nonindependent errors arising from spatial or temporal autocorrelation.

- Estimation and testing are based on restricted maximum likelihood (REML), which can handle unequal sample size.

- *P*-values for tests of fixed effects are conservative when designs are unbalanced.

- Implemented in the `lme4/lmerTest` and `nlme` packages in R.

# Example 1: Study of measurement repeatability (simple nested design)

The walking stick, *Timema cristinae*, is a wingless herbivorous insect on plants in chaparral habitats of California. Nosil and Crespi (2006) measured individuals using digital photographs. To evaluate measurement repeatability they took two separate photographs of each specimen. After measuring traits on one set of photographs, they repeated the measurements on the second set.
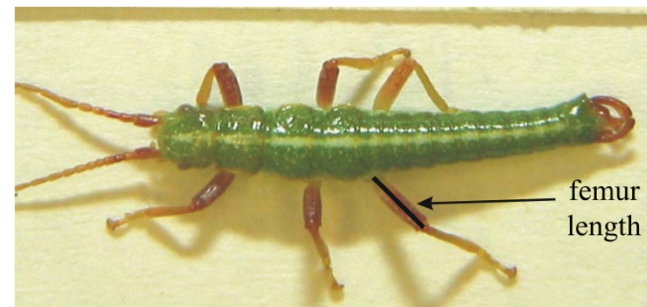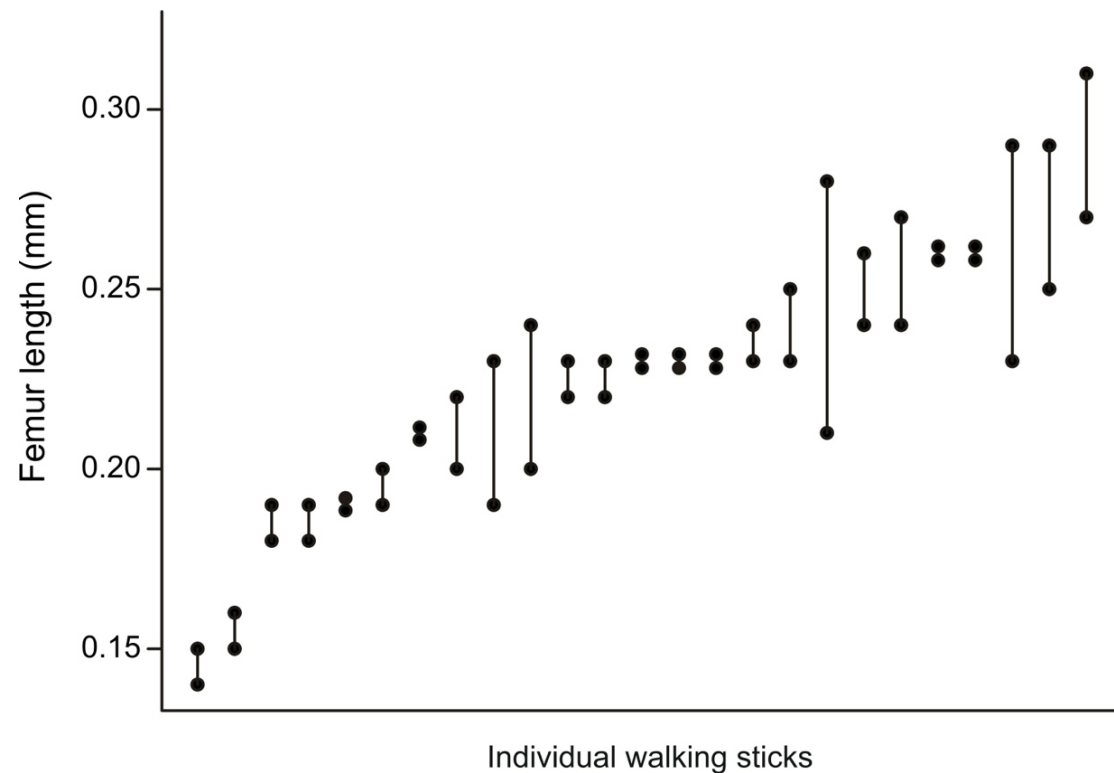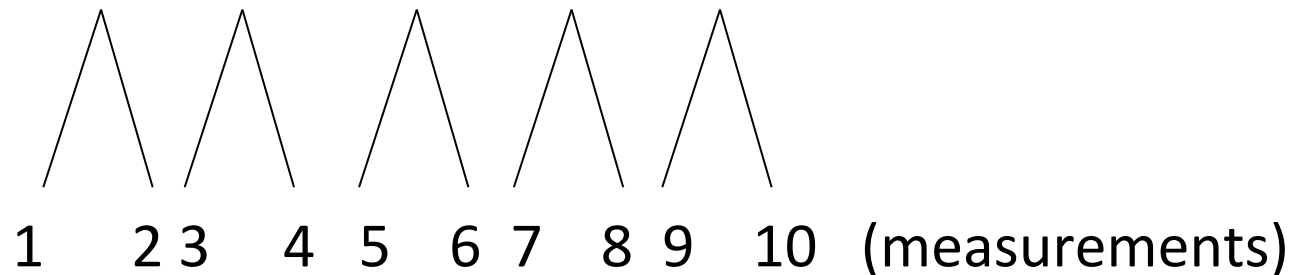
Example 1: Study of measurement repeatability (simple nested design)

Linear mixed model: $Y_i = \beta_0 + u_i + \text{error}$ for individual bugs i
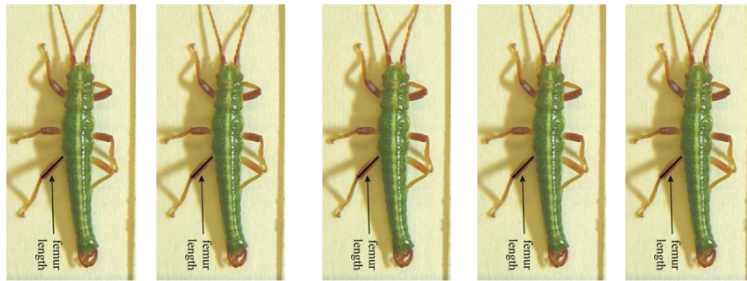
Including a random effect means that the analysis follows design

1   2 3   4 5   6 7   8 9   10 (measurements)

Individual bugs are the random groups in this study, with two repeated measurements per group.

Model has two parts, each with its own source of error variance:

1) Random part: the measurement of individual bug i: $u_i \pm$ random error.

2) Fixed part: the mean of bug means: $\beta_0 \pm$ variation among bugs

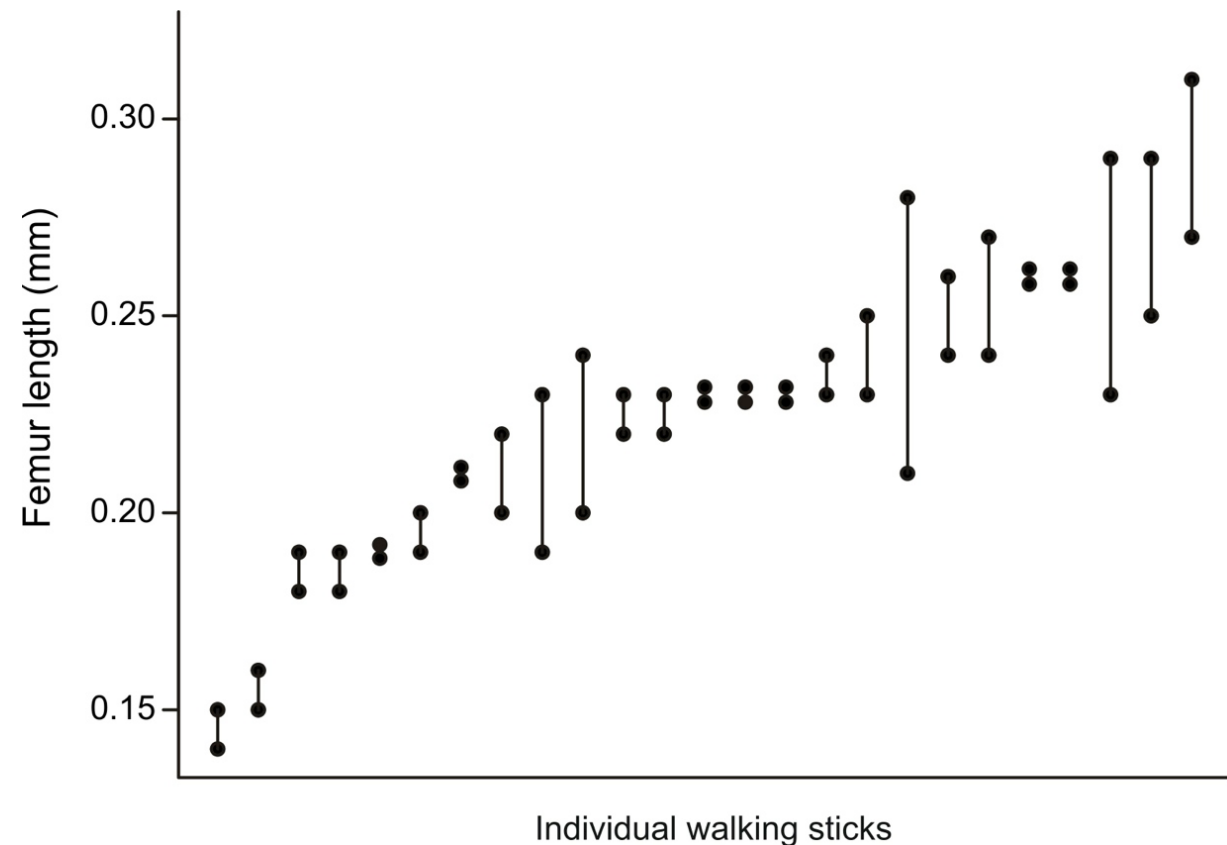# Example 1: Study of measurement repeatability (simple nested design)

```
library(lmerTest)

z <- lmer(femurlength ~ 1 + (1|individual))
```

Fixed part of the formula:

```
femurlength ~ 1
```

Random part of the formula

```
+ (1|individual)
```

Example 1: Study of measurement repeatability (simple nested design)

The **fixed** part of the formula instructs R to fit a fixed constant (intercept) based on the fitted values of the random groups (individual bugs) – i.e., the grand mean.

```
femurlength ~ 1
```

The *random* part of the formula instructs R to fit another constant (an intercept) to the measurements within each individual. This yields a fitted value for each individual walking stick.

```
+ (1|individual)
```


Individual walking sticks
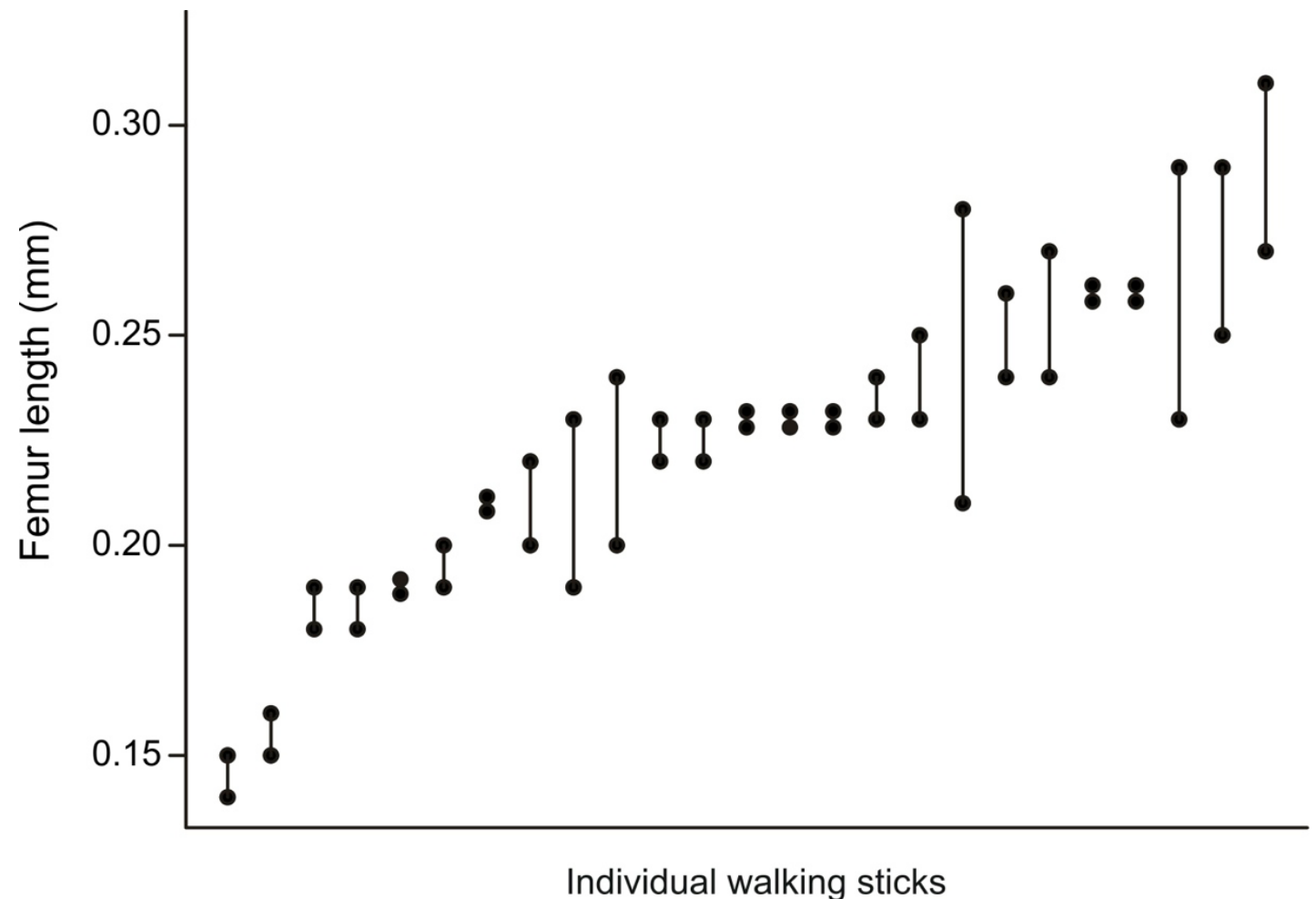
Example 1: Study of measurement repeatability (simple nested design)

```
z <- lmer(femurlength ~ 1 + (1|individual))
```

`fitted(z)` # yields best linear unbiased predictors (BLUPs) shown in plot:

R fits them all together, rather than in two stages, yielding variance components and BLUPs.

The BLUPs are not the means for each insect. They are "shrunk" towards the centre compared with the individual insect means.

Predicted femur length

Individual walking stick
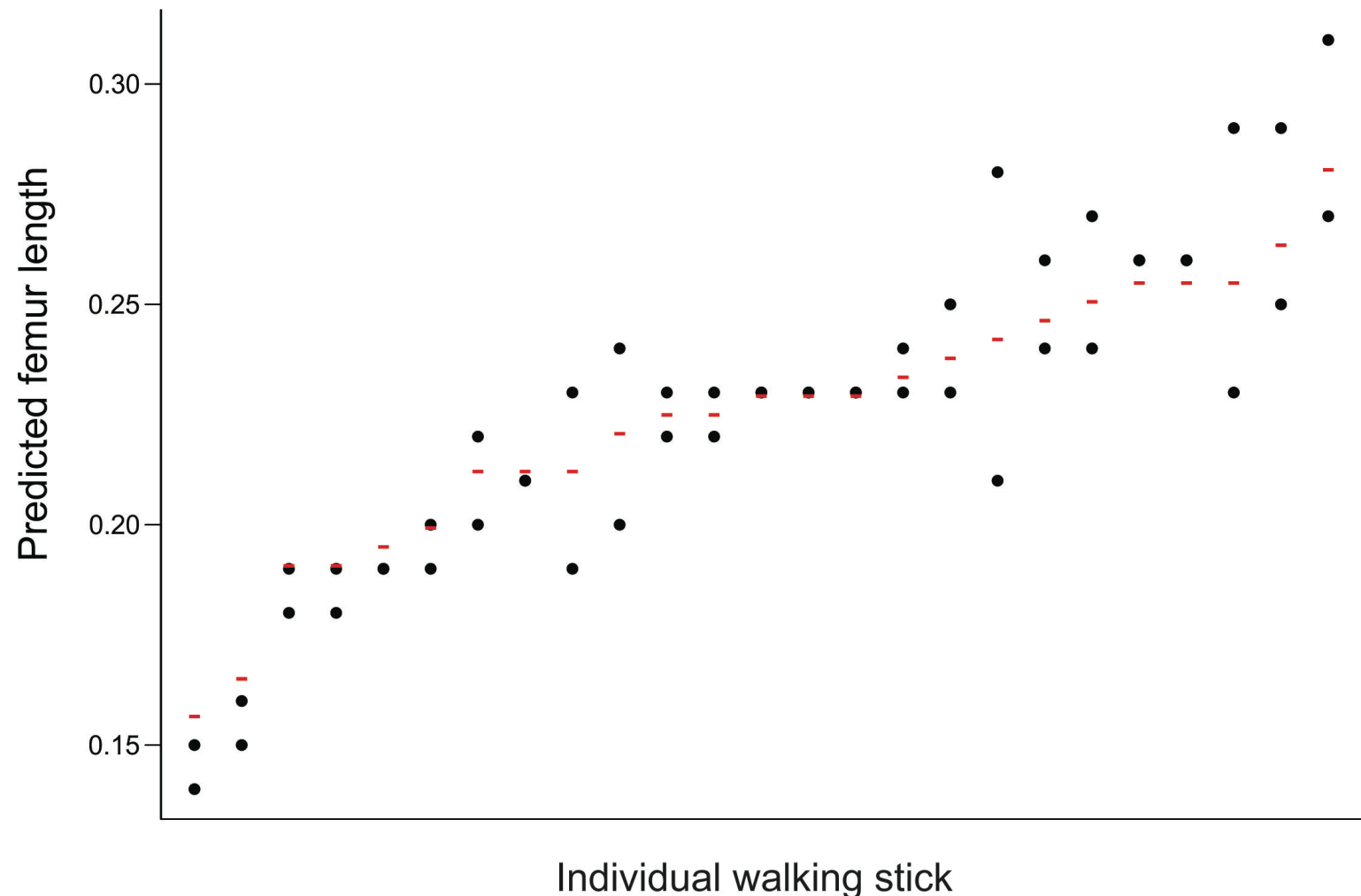
Example 1: Study of measurement repeatability (simple nested design)

`VarCorr(z)` extracts the variance components (square the standard deviations to obtain the variances)

Groups	Name	Std.Dev.	
individual	(Intercept)	0.032464	= variability among random groups (individuals)
Residual		0.018868	= variability within random groups (usual error)

We can use these quantities to calculate the fraction of variation that is among individuals (repeatability):
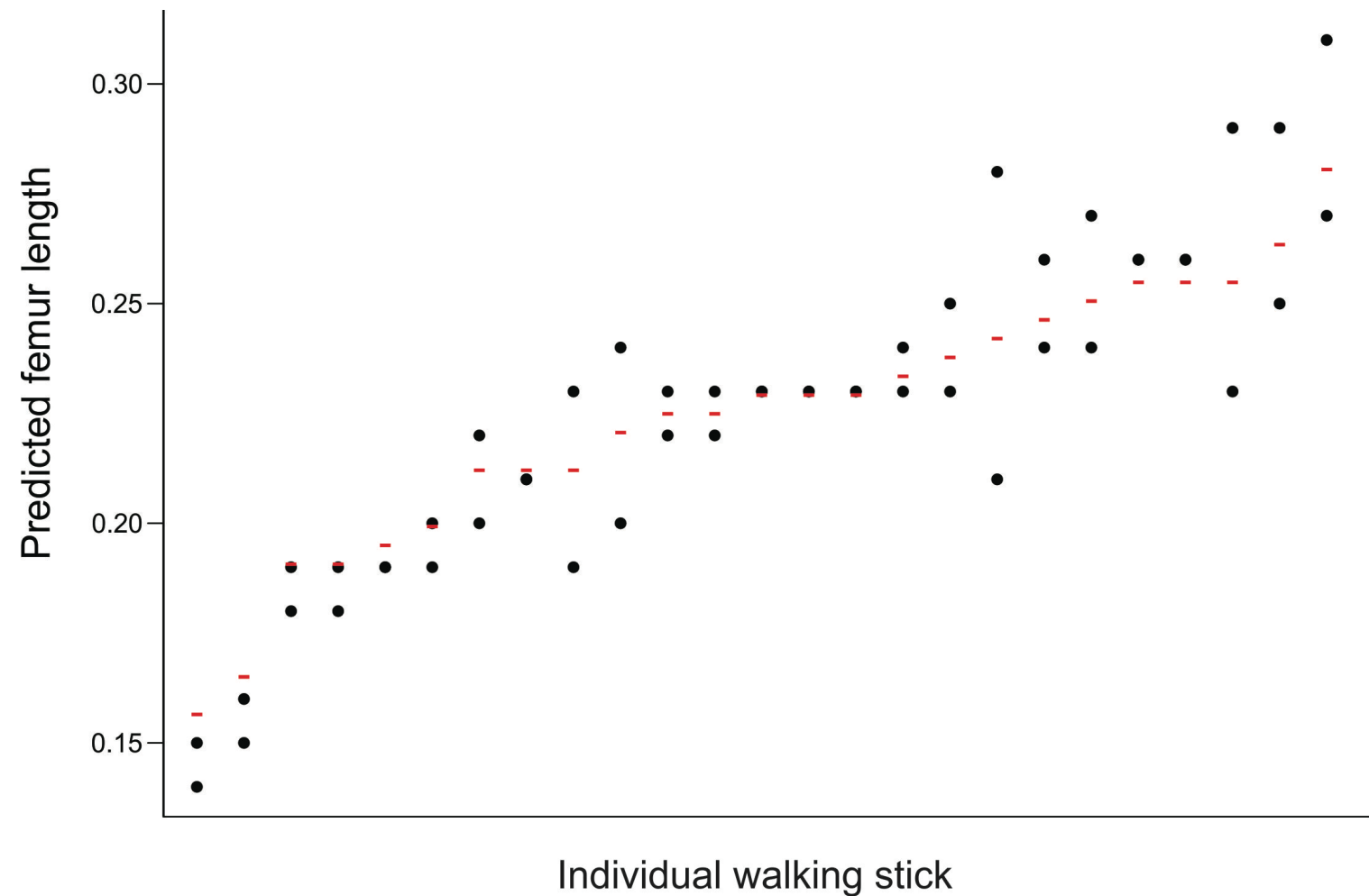
repeatability = σ^2_{among} / (σ^2_{among} + σ^2_{within})

estimate of repeatability = 0.032464^2 / (0.032464^2 + 0.018868^2)

= 0.75

Example 1: Study of measurement repeatability (simple nested design)

Estimate of repeatability = 0.75

That is, an estimated 75% of the variation among measurements is true variation among individual insects in their femur lengths, the rest is measurement error.

Example 2: "Subjects by treatment" repeated measures design

Light sensitivity of 5 goldfish to specific wavelengths of light.

Cronly-Dillon and Muntz (1965) used the optomotor response to measure color vision in the goldfish. Each fish was tested at different wavelength in <u>random order</u>.
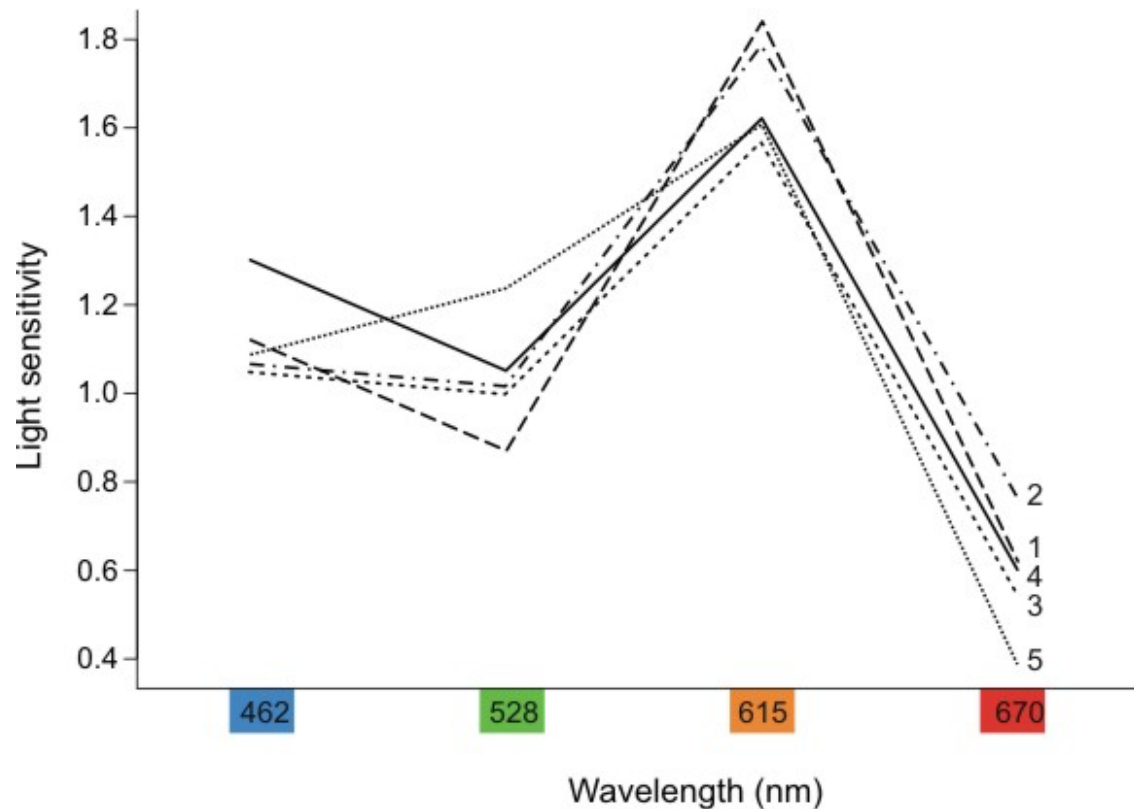
A large value indicates that the fish has high sensitivity --- it can detect a low light intensity.

Factors:

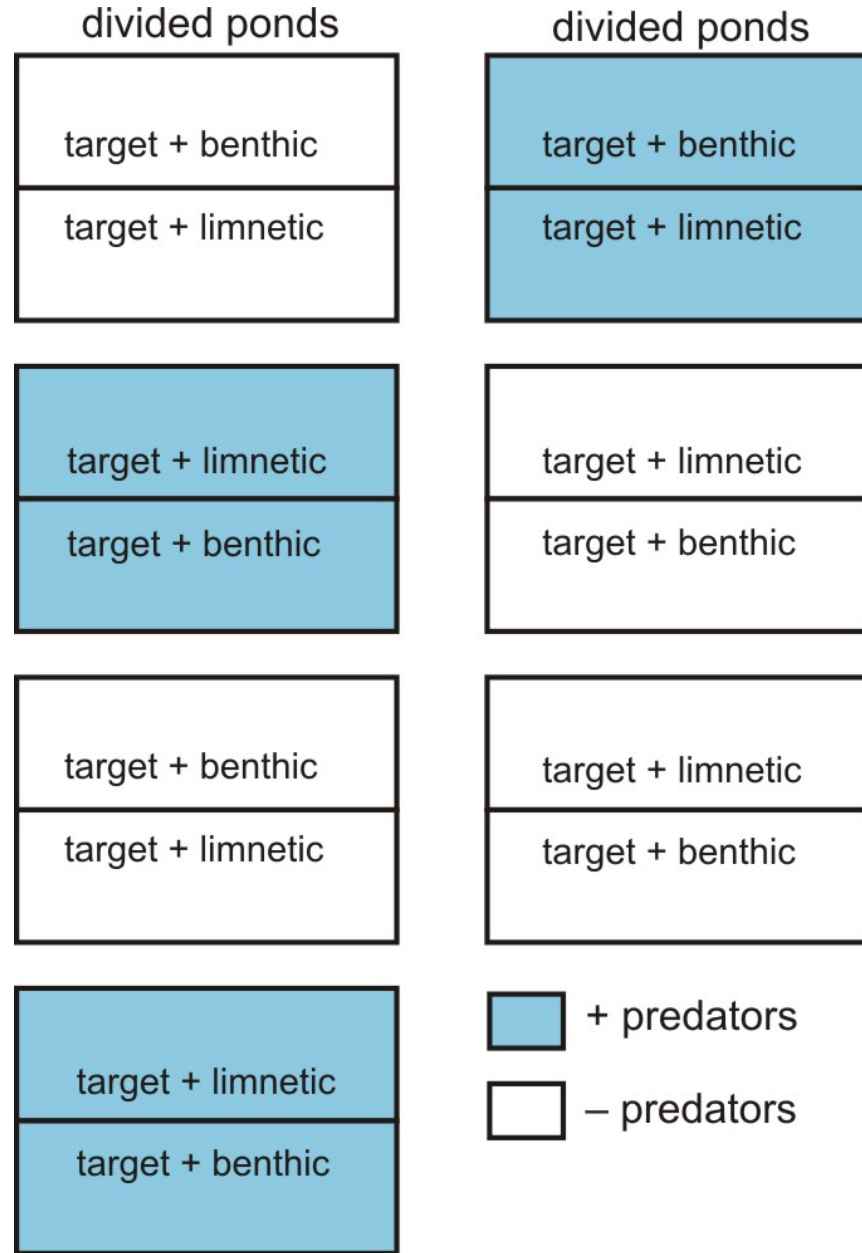Wavelength (fixed, repeated measure)

Fish (random)

Example 3: Split plot design

Factors:

Predation treatment (fixed), whole ponds

Competition treatment (fixed), split ponds

Pond (random)

divided ponds

target + benthic
target + limnetic

target + limnetic
target + benthic

target + benthic
target + limnetic

target + limnetic
target + benthic

divided ponds

target + benthic
target + limnetic

target + limnetic
target + benthic

target + limnetic
target + benthic

+ predators

– predators

Assumptions of linear mixed-effects models

- As with all linear models: Residuals follow a normal distribution with equal variance.

- Groups are randomly sampled from a "population" of groups (i.e., are independent and sampled without bias).

- Group means have a normal distribution.

- Replicates within groups are also randomly sampled (i.e. independent and sampled without bias). Problems can arise when **Time** is a fixed factor – need to model the correlation between adjacent samples, also possible with mixed models.

- No carry-over between repeated measurements on the same subject.

Example 4: When <u>time</u> is a fixed factor

- Analyses of growth curves in time.
- Repeated measures experiments in which the treatment levels are given in the same sequence (i.e., not in random order)

The correlation of a variable with itself across different points in time (or space) is called **autocorrelation**. With time series or longitudinal data, it's the degree to which current values of a variable are related to its past values. This can violate the assumption of independence of errors, leading to incorrect standard errors and unreliable hypothesis tests and confidence intervals.

Autocorrelation can be addressed by incorporating an autoregressive structure, commonly AR(1), into the model. This explicitly models the nonindependence between successive observations.

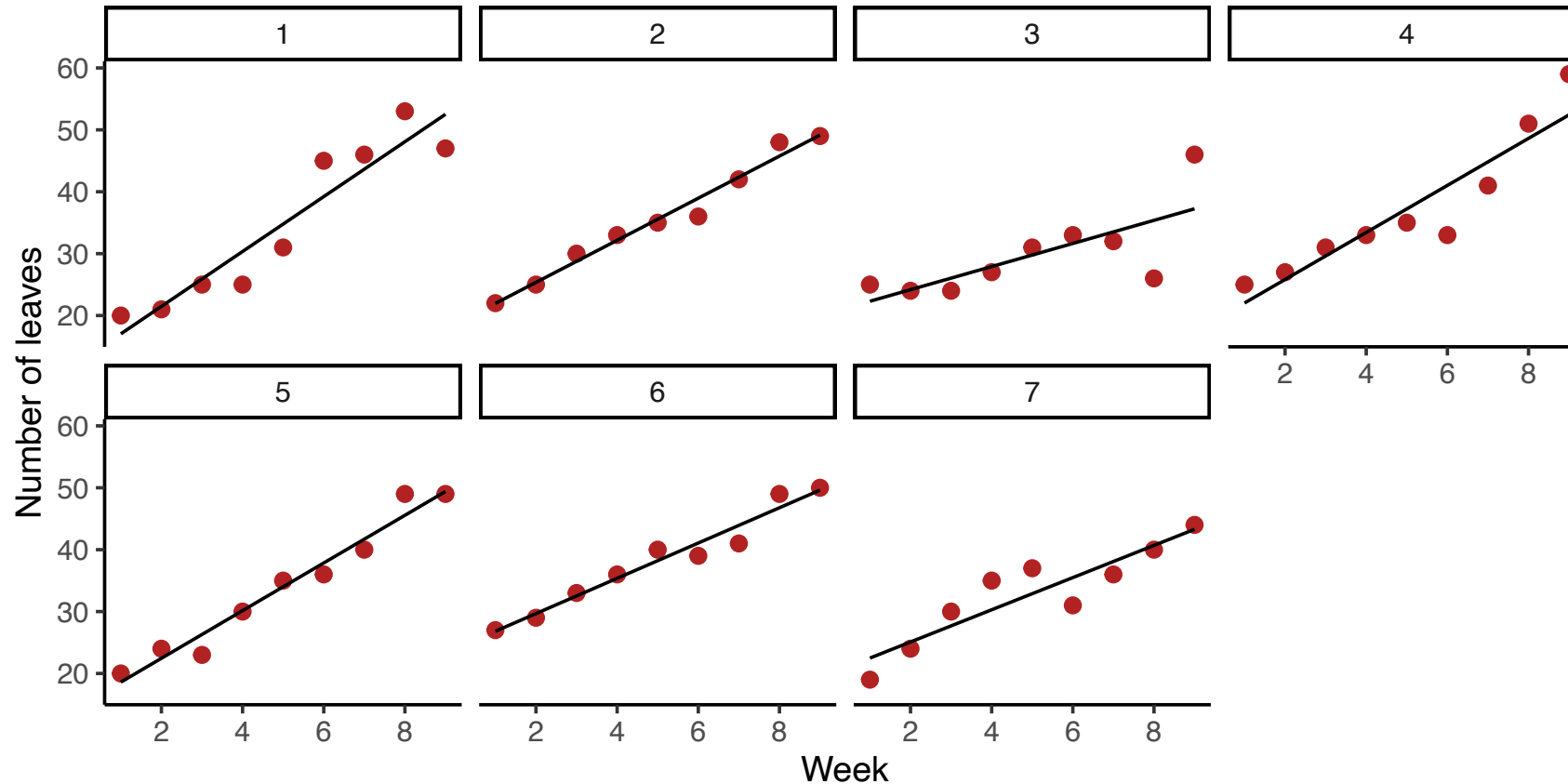**Example 4: When <u>time</u> is a fixed factor**

Growth curves are potentially affected by nonindependent errors. Measurements made in adjacent time periods are likely to have correlated residuals. This correlation should decline between more distant measurements in time.

Example from Quinn & Keough (2024)

Data are number of leaves on each of seven *Lactuca serriola* plants grown in pots over 9 weeks. I'm using the data only for the 75% soil water holding capacity treatment.

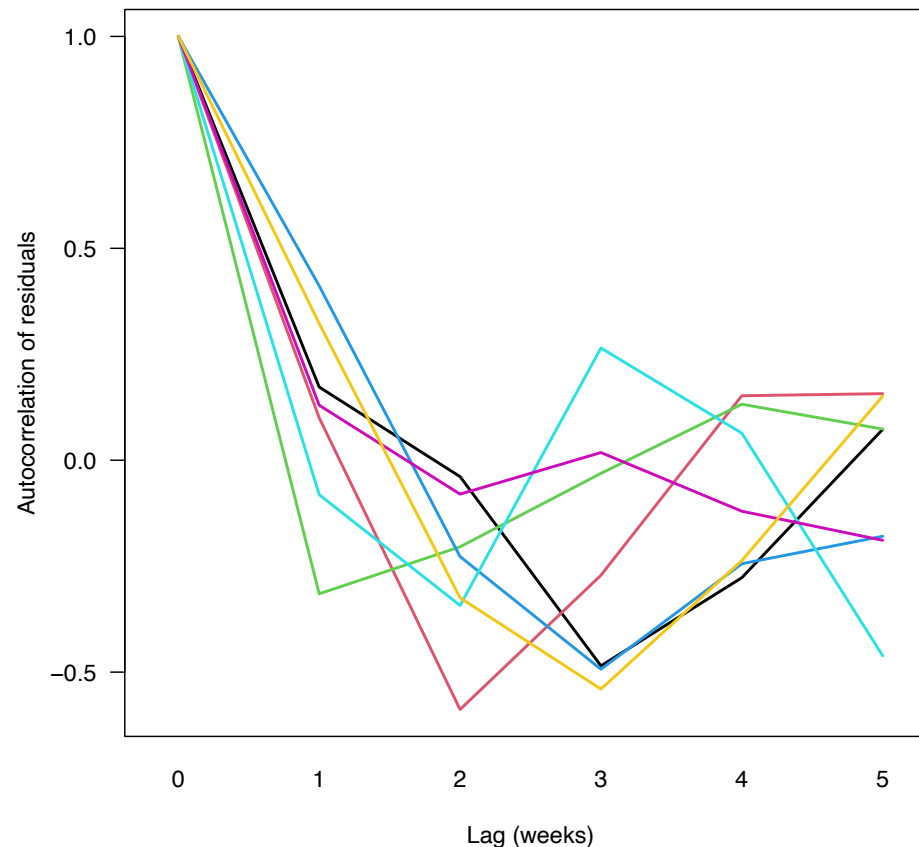# Example 4: When <u>time</u> is a fixed factor

Time in weeks is the within-subjects (repeated measures) fixed factor and individual *plants* are the random subjects. Want to estimate growth rate slope (fixed) and variance among *plants* in growth rate slope and intercept (random).

Example 4: When <u>time</u> is a fixed factor

Mixed model with random intercept and slopes (allows growth curves of different plants to vary in intercept and slope):

```
z <- lmer(nleaves ~ week + (week|plant), data = lactuca)
```

Examine autocorrelation between residuals (one curve per plant). Here the correlation between residuals is relatively week even for adjacent weeks (1 week apart).

Example 4: When <u>time</u> is a fixed factor

Fit mixed model using AR(1) autocorrelation model, which assumes that the correlation between two residuals decays exponentially as the time between observations increases. Needs the `nlme` library because `lme4` doesn't include it.

```
library(nlme)

z <- lme(nleaves ~ week, random = ~week|plant,
      correlation = corAR1(form = ~week|plant), lactuca)
```

The correlation formula is structured as `~time|groups`

```
summary(z)
```

```
Random effects:
            StdDev       Corr
(Intercept) 0.002410178 (Intr)
week        0.357092983 -0.377
Residual    4.262789724

Fixed effects:  nleaves ~ week
            Value Std.Error DF  t-value  p-value
(Intercept) 18.425685 1.5781576 55 11.67544      0
week         3.285107 0.2999463 55 10.95232      0
```

Where to get further advice

I have found Quinn & Keough to be very useful in understanding design and assumptions of mixed-effects models. The 2nd edition has online worked examples in R.

Quinn & Keough 2024. 2nd ed. *Experimental design and data analysis for biologists*.

Online books on mixed-effects models in R (see Books tab at course web site):

Pinheiro and Bates (2000). *Mixed-effects models in S and S-PLUS*.

Zuur et al (2009). *Mixed effects models and extensions in ecology with R*.

Article:

Bates, Douglas, et al. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* 67: 1-48.

Discussion paper for next week:

Murtaugh (2007) Simplicity and complexity in data analysis. Ecology 88: 56–62.

Presenters: Finola & Megan

Moderators: Vivien & _____