Predator escape: an ecologically realistic scenario for the evolutionary origins of multicellularity

Teacher's guide

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Time required: 4 days

Overview. The evolution of multicellularity was one of a few events in the history of life that allowed for increases in biological complexity. The first step in this transition is the evolution of multicellular clusters. Once clusters have evolved, there is a shift in the level of natural selection- from single-cells to whole clusters. Over many generations, cluster-level adaptation results in the evolution of increased multicellular complexity (e.g., cellular division of labor, the evolution of developmental programs, etc). This process is described in the movie entitled 'Video overview of the yeast experiment'.

Here we will examine the very first step of this process- the evolution of cellular clusters. In the lab, scientists have shown that simply selecting for fast settling through liquid media can result in the evolution of cluster-forming 'snowflake' yeast. Gravity is a simple way to select for cluster formation, because clusters of cells fall through liquid media faster than single cells. As a result, if a random mutation arises that results in cluster formation, these will have a *huge* competitive advantage over the wild-type unicellular yeast.

While these experiments are easy to do and give researchers a lot of experimental control, they aren't a very good model for types of selection that unicellular organisms face in nature. After all, there aren't any centrifuges in nature. In this lab, students will use unicellular and snowflake yeast to test a key hypothesis about this transition[1]: that predation by small-mouthed organisms can select for cluster formation.

Goals. Give rotifers (small animals that prey on single-celled organisms) unicellular and multicellular yeast. Observe rotifer predation, and then calculate the relative survival of uni and multicellular yeast during predation. Perform a statistical analysis on this result.

Timeline. This lab is short, taking approximately four 50-minute class periods. There are a number of accessory lessons that can be added, which are included in the appendices. Teachers are encouraged to mix and match these lessons as they see fit. The main document here describes just the core curriculum.

Monday	Tuesday	Wednesday	Thursday	Friday
14 working days prior to Day 1: assess materials and order rotifers				
Day 1: Give pre-test. Read student guide. Intro to rotifers and selection by predators.	Day 2: Examination of rotifer predation	Day 3: Quantification of rotifer predation	Day 4: Discussion and review of lab reports. Give post-test	

At least two weeks before start of lab, place an order for rotifers (and optionally *Paramecium aurelia* if this lab-add on will be used), ask to have delivered the day before you plan to do the lab. All other supplies that are needed should be ordered at this time to ensure timely delivery.

Materials. The following materials are necessary, and with the exception of an autoclave (not strictly required) and microcentrifuge (not strictly required), are relatively simple and inexpensive to obtain. Many of these materials may already be in your lab such as scales, microscopes, micropipetters, depression slides and coverslips. Cheaper or more available alternatives for common items are also provided below. Supplies listed are for one class of 20 students.

Included in this kit:
Saccharomyces cerevisiae unicellular yeast spores (strain Y55)
Saccharomyces cerevisiae snowflake yeast spores, evolved after 3 weeks of settling selection (C1W3)
YPD growth medium , contains agar (6.5 g)
Culture dishes
Sterile water
1% (w/v) aq Congo red cell stain (warning: toxic! Do not use without first reading the MSDS. **Wear gloves and protective eyewear while handling.**)
1 % (w/v) aq methylene blue cell stain (warning: toxic! Do not use without first reading the MSDS. **Wear gloves and protective eyewear while handling.)**
Saccharomyces cerevisiae unicellular yeast (strain Y55) *fixed and stained with 1% Congo red.* **Wear gloves and protective eyewear while handling.**
Saccharomyces cerevisiae snowflake yeast (C1W3) *fixed and stained with 1% methylene blue.* **Wear gloves and protective eyewear while handling.**
Microscope depression slides (also called concavity slides)

Must be purchased separately:
Mixed rotifers (1 culture)
1.5 mL microcentrifuge tubes (100 tubes)
Bottled water (1 L, spring water is best for rotifer health)
Micropipettes (capable of dispensing 5 and 100 μl)
Micropipette tips
Standard microscope slides (1 box)
Microscope coverslips (1 box)
Compound microscope (1-5)

Instructor preparation
Step 1: Thoroughly read student guide, make copies for each student.
Step 2: One day before lab, organize student workstations.

Student work station checklists
Student work stations
The list below is a list of materials that should be present at each student work station prior to the beginning of the lab.

Common work station

The list below is a list of materials, supplies, and equipment that should be present at a common location and accessible by all. It is up to the discretion of the instructor as to whether or not students should access and mix staining solutions and plate, suspend, or isolate yeast, or whether the teacher should aliquot solutions in microtubes and include at student work stations.

Student work stations	Number required	☑
Beaker for waste	1 per group	☐
Permanent marker	1 per group	☐
Compound microscope	1 per group	☐
Pictures of rotifers with food in their stomach	1 per group	☐
Latex gloves	1 box per group	☐
Eye protection	1 pair per student	☐

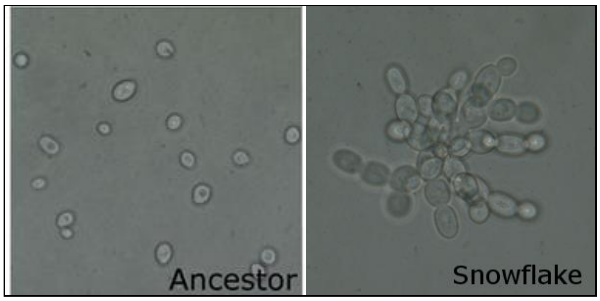
Common work station	Number required	☑
Mixed rotifers	1 stock container	☐
Stock solution of fixed red-stained unicellular yeast	1	☐
Stock solution of fixed blue-stained multicellular yeast	1	☐
0.1-10 µl micropipette	1	☐
100 µl micropipette	1	☐
Micropipette tips	2 boxes	☐
22 mm x 22 mm glass coverslips	1 box	☐
Glass slides (depression)	1 box	☐

Lesson 2A: Observing Rotifer Predation

Introduction

In this lab, students will observe the effect of rotifer predation on the survival of unicellular and multicellular yeast. Scientists have found that when selecting for rapid settling through liquid media (a way of selecting for large size), unicellular yeast evolved into simple multicellular organisms in as little as seven days. Genetically, this resulted from a single mutation that knocked out a gene required for mother-daughter cell separation after mitosis. This experiment was important because it showed that simple multicellularity can evolve rapidly, but it does not use a very ecologically realistic selective agent. Clearly, there were no centrifuges in nature a billion years ago.

Here we will examine the ability of another form of selection against small size, predation, to favor multicellularity. Rotifers (Figure 1) are small multicellular organisms that prey on single celled algae, bacteria, and ciliates. We will give hungry rotifers uni and multicellular yeast, then examine their ability to eat each growth form.



Methods

This experiment utilizes two yeast strains: strain Y55 was isolated from a vineyard in France, and is a regular, unicellular yeast. Strain C1W3 was derived from Y55 after three weeks of selecting for rapid settling through liquid media. This is a

'snowflake' yeast.

Mounting live rotifers for microscopic examination

Materials

·Yeast (both strains Y55 and C1W3) fixed and stained with Congo red and methylene blue (supplied in kit)
· (2) Glass depression slide (alternative: plastic depression slide)
· (2) 22mm x 22mm coverslips
·Micropipetter capable of pipetting 100 µL of liquid
·Micropipetter capable of pipetting 1 mL of liquid (alternative: plastic pipettes)
·Corresponding micropipette tips
·Rotifers

Procedure

1. Add 100 µL of predator to depression slide.
   Hint: Get rotifers from the bottom of the container
2. Add 5 µL of blue stained C1W3 multicellular yeast. **Mix tube by inversion before removing yeast.**
3. Add 5 µL of red stained Y55 unicellular yeast. **Mix tube by inversion before removing yeast.**
4. Add coverslip and immediately view on microscope

Observations

You must observe at least 25 rotifers (a larger sample size is encouraged if time permits) and make a determination on which yeast is the predominant yeast in the stomach of a given rotifer (Figure 2). Note the behaviors of the rotifers. How do they eat? Can you observe any yeast being consumed? How long does it take them to fill their stomach?

In the space below (or on another sheet of paper), draw a picture of a rotifer eating yeast. Use arrows to indicate the movement of water around the rotifer head.

Figure 1- Rotifer. The mouth is at the top, and the stomach is visible through the transparent body. This rotifer has been eating red yeast.

Lesson 2B: Quantifying Rotifer Predation

Introduction

In this experiment, students will quantify the number of each type of yeast cell in rotifer stomachs. In comparison to the previous exercise, here students will actually quantify predatory selection, and will analyze their results statistically. This approach is more rigorous- it will not only allow us to calculate the relative fitness of multi:unicellular yeast, but it will also allow us to determine if this result is statistically robust.

Methods

If the school has a microscope with a camera: students can take images of flattened rotifers (e.g., Figure 2) for counting the number of red and blue yeast inside their stomachs. To do this, follow the protocol above, but let the yeast and rotifer mix stand for ~3 minutes prior to pipetting onto a microscope slide. Rather than using the concavity slide, transfer 10 µL of the yeast-rotifer mixture onto a standard slide and flatten by placing a coverslip on top. Otherwise, use the images provided with the lab.

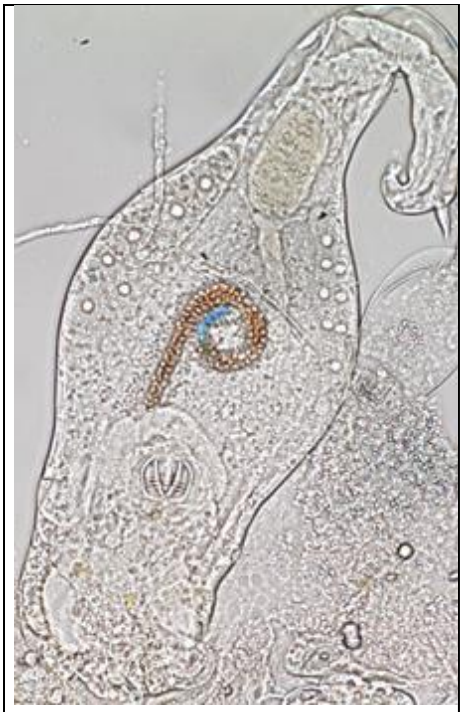


Figure 2- Both red and blue yeast are visible in the stomach of this rotifer.

Each group member will receive a picture of a flattened rotifer. Each student will record the number of yeast of each color in their rotifer and then the number in the rotifers of their group members. Each circle in the stomach of a rotifer is one yeast cell (Figure 3).

Data Collection

In the table below, count the number of red unicellular and blue multicellular yeast found in your rotifer stomach. Include the number of each yeast strain your group-mates find in their rotifers. Finally, sum the total number of uni and multicellular yeast your group found across all of your rotifers, and put this in the 'total' box.

	Rotifer 1	**Rotifer 2**	**Rotifer 3**	**Rotifer 4**	**Rotifer 5**	**Total**
Number of red unicellular yeast						
Number of blue multicellular yeast						

Relative survival during predation

Now we will calculate the relative survival of multi to unicellular yeast during rotifer predation. This is a key element in their Darwinian fitness, because yeast that are eaten by predators are killed and cannot pass their genes on to future generations. First, calculate the proportion of killed yeast that are multicellular:

$$\text{Proportion multicellular consumed} = \frac{\#\ blue\ multicellular\ yeast}{\#\ blue\ multicellular\ yeast + \ \#\ red\ unicellular\ yeast}$$

Statistical analysis

To determine if the above difference is significant, we will perform a statistical analysis. In essence, this analysis determines the probability that the difference in predation between uni and multicellular yeast would have been observed by chance. For example, if you flip a coin 100 times and you get 53 heads and

47 tails, this difference isn't large enough that we're could say with much confidence that the coin was biased towards heads. As the results get more divergent from our expectation of 5 0:50, the chance that the coin really is fair goes down. We're going to use the same principles here to determine if the differences we see in yeast death by rotifers is significant.

We will use a chi-square test, which compares the observed frequencies of uni and multi cells to expected frequencies. To generate the expected frequency of red vs. blue cells, assume that both uni and multicellular yeast stock solutions were at the same cell density (cells / mL). Assuming there was no rotifer preference for either yeast strain, we expect that half the total number of yeast counted should be multicellular, and half should be unicellular. Therefore, to calculate the 'expected' number of multis and unis (for use below), divide the total number of counted cells by two.

Figure 3-Each of the dark circles above is a yeast cell in the stomach of a rotifer. These are all red unis.

The chi squared statistic (denoted χ^2 because χ is the Greek letter 'chi') is calculated by summing the squared difference between the observed and expected number of multicellular yeast in the rotifer stomachs, and the unicellular yeast in rotifer stomachs.

$$\chi^2 = \sum \frac{(\#\ Obs - \#\ Exp)^2}{\#\ Exp}$$

For example, say I counted 200 yeast cells in total, so I expect there to be 100 multi and 100 uni cells in the rotifer stomach. But, when we counted them, I found there were 50 multi cells and 150 uni cells. The χ^2 statistic is calculated as:

$\chi^2 = \frac{(50-100)^2}{100}\ [this\ is\ the\ multi\ expectation] + \frac{(150-100)^2}{100}\ [the\ uni\ expectation]$ = 50

Fill out the following table with the information necessary to conduct a chi-square analysis.

Number of observed multis consumed (# Obs)	Number of expected multis consumed (# Exp)	$\frac{(\#\ Obs - \#\ Exp)^2}{\#\ Exp}$ for multis	Number of observed unis consumed (# Obs)	Number of expected unis consumed (# Exp)	$\frac{(\#\ Obs - \#\ Exp)^2}{\#\ Exp}$ for unis

What is your chi squared statistic? Make sure to show your work (either here or in the boxes above).

Finally, we need to use the chi squared statistic to determine the probability that we got the difference between uni and multi predation simply by chance if rotifers really have no preference. As you can see on the distribution below, if your χ^2 statistic is greater than 3.9, then there is a less than 5% chance that your results were caused by chance alone. At that point, we're pretty confident that the rotifers really do have a preference. If your χ^2 statistic is greater than 3.9, the difference in predation you observed is statistically significant at a level generally accepted by scientists to be robust. If this was your result- congratulations!

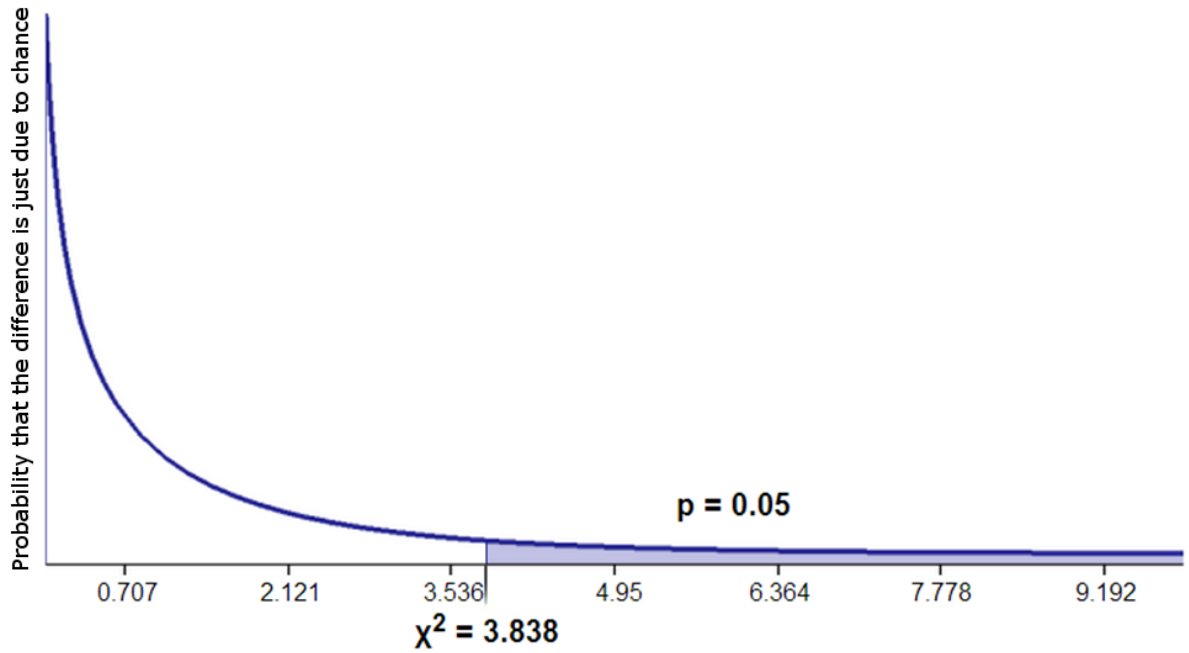


Figure 4- Chi squared distribution. When the chi squared test statistic is above 3.84, then the chance that the difference in rotifer predation was due just to chance is below 5%. At that threshold, we are pretty sure the rotifers have a preference!

Discussion

Depending on teacher preference, students will answer discussion questions in their lab notebooks or discuss these questions as a class. At the culmination of this lab, you will be asked to incorporate your thoughts and write up a full lab report.

References

1. Grosberg, Richard K., and Richard R. Strathmann. "The evolution of multicellularity: a minor major transition?" Annu. Rev. Ecol. Evol. Syst. 38 (2007): 621-654.

Appendix 1. Additional exercises

a. *Paramecium* predation

Overview. Astute students may ask about our choice of predator. After all, rotifers are multicellular animals, so clearly rotifers aren't representative of the unicellular predators that may have led to the first origin of multicellularity. This is true, but rotifers are so easy to work with that we used them anyway. However, the same lab above can be conducted with single-celled predators.

Goal. Examine the preference of single-celled predators for uni vs. multicellular yeast.

Timeline. This lab can be done in conjunction with the above lab- it probably will take about as much time to do as the rotifer experiments.

Methods. Repeat the above experiment, but use *Paramecium aurelia* instead of rotifers as the predator. Note: in contrast to the rotifers, *Paramecium* tend to aggregate at the top of their growth chamber, so get your *Paramecium* from the air-water interface.

Unlike rotifers, which add yeast to a linear stomach, the *Paramecium* engulf yeast in circular vacuoles. Flattened *Paramecium* (Figure 5) yield readily countable yeast in which red and blue colors are clearly visible. This cell was flattened by placing the *Paramecium* on a standard slide under a standard coverslip.

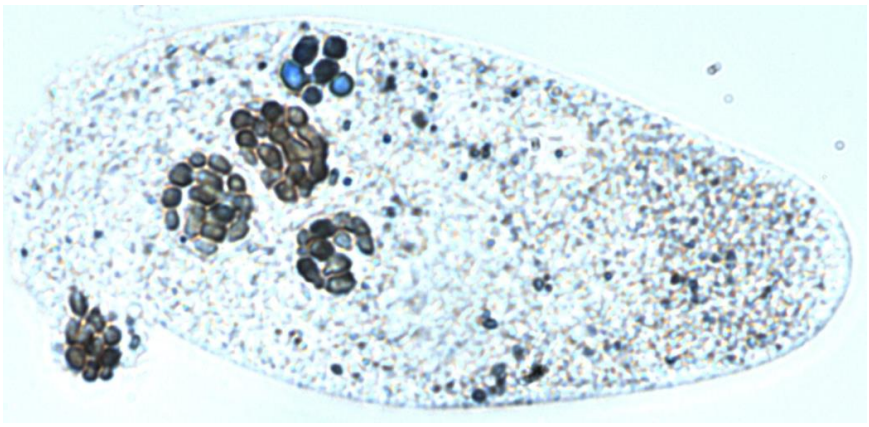


Figure 5- A flattened *Paramecium aurelium* with both red and blue yeast visible.

Additional discussion questions

What are the differences between rotifers and *Paramecium aurelium* on the relative survival of multicellular yeast? Why do you think you saw these differences? How do these predators differ from one another? What is each predator's mode of feeding? Why might this affect the size of prey they can consume?

b. Growing and staining your own yeast

Students can grow and stain their own yeast, rather than use the pre-stained yeast included in the kit. The methods for this are described below.

Streaking our yeast onto agar plates

Notes:
·The incubation of the yeast will take about 3-4 days in an incubator set to 30°C, 1 week without.
·Enclosed in your reagent bag are dried spores of the yeast *Saccharomyces cerevisiae*, strain Y55 (unicellular) and strain C1W3 (multicellular). The latter is a 'snowflake' yeast, derived from Y55 with 3 weeks of settling selection.
·The yeast spores are dried onto filter paper. Use a different agar plate for each strain of yeast.

Materials
(2) 1.5mL microcentrifuge tube
    Alternative: covered/capped test tube
(2) Sterile loop/stick
    Alternative: pipette tip or sterilized toothpicks
Sterile water
Sterile forceps
Flame
Saccharomyces cerevisiae unicellular yeast spores (strain Y55)
Saccharomyces cerevisiae multicellular yeast spores (strain C1W3)

Procedure
1. Carefully open the foil package labeled Y55.
2. Using flame-sterilized forceps, remove paper from foil package.
3. Unfold and place the filter paper onto a culture plate of YPD medium with sterile forceps. Label plate.
4. Moisten with 50 µL or so of sterile water or liquid YPD. Streak the paper around on the agar surface using sterile forceps. Leave the filter paper on the culture plate.
5. Incubate at 30°C. You should see colonies in a few days.
6. Repeat process with C1W3.

*When plating more than one yeast culture, be sure to flame sterilize forceps between each plate.

As an alternative, transfer the filter paper directly into a 10 mL tube of liquid Yeast Peptone Dextrose (YPD) medium with sterile forceps. Incubate these tubes at 30°C with vigorous shaking (requires shaking incubator). Within 2-3 days there should be plenty of growth. One can then extract 100 µL from these stock tubes for initial tube inoculation.

Suspending yeast into liquid *(in preparation for staining with Congo red and methylene blue)*
Procedure
1. Fill 1.5 mL microcentrifuge tube with 1 mL sterile water
2. Using an inoculation loop, toothpick, or pipette tip, scrape off as much Y55 yeast as you can.

3. Insert sterile loop into microcentrifuge tube containing the water and twirl to release yeast. Label tube *Y55 stock.*
4. Shake tube to suspend yeast.
5. Repeat steps 1-3 with C1W3 yeast. Label tube *C1W3 stock.*

Staining Yeast

Stain Y55 (unicellular yeast) with Congo red 1% (w/v) aqueous stain and stain C1W3 (multicellular yeast) with methylene blue 1% (w/v) aqueous stain. **Warning: these stains are toxic. If instructors choose to have students complete this step it should be done so under close supervision. Alternatively, use the pre-stained yeast. Make sure to wear gloves and protective eyewear.**

Materials

(2) 1.5 mL microcentrifuge tube
2 mL 70% ethanol (alternative: isopropyl alcohol)
2 mL deionized or distilled water (tap water will work in a pinch)
Microcentrifuge
100 μL 1% Congo red stain
100 μL 1% Methylene blue stain
Micropipetter capable of pipetting 100 μL of liquid
Micropipetter capable of pipetting 1 mL of liquid (alternative: plastic pipettes)
Corresponding micropipette tips
Y55 yeast in 1 mL liquid in 1.5 mL centrifuge tube
C1W3 yeast in 1 mL liquid in 1.5 mL centrifuge tube

Procedure

1. Centrifuge 1mL aqueous yeast for 30 seconds @ 5,000 g.
2. Remove supernatant with micropipetter or equivalent.
3. Re-suspend yeast pellet with 1mL ethanol (70% or 95%) (Isopropyl alcohol will work too).
4. Wait 5 minutes.
5. Centrifuge yeast/ethanol solution for 30 seconds @ 5,000 g. Remove supernatant.
6. Re-suspend yeast pellet with 100uL of either Congo red 1% (w/v) aqueous stain or methylene blue 1% (w/v) aqueous stain.
7. Wait 5 minutes.
8. Centrifuge yeast/stain solution for 30 seconds @ 5,000 g.
9. Remove supernatant
10. Re-suspend yeast pellet with 1mL deionized water or distilled water.
11. Centrifuge yeast solution for 30 seconds @ 5,000 g.
12. Remove supernatant.
13. Repeat steps 10-12.
14. Re-suspend yeast pellet in 1mL deionized water or distilled water.
15. Refrigerate until use (4 degrees C).

Appendix 2. Questions for students

Insert the questions that apply most to your course into lab handouts for your students, or use these to lead in-class discussion.

Pre-lab Questions:

Give a brief description of rotifers. What do rotifers typically eat? Where do they live?
What are the critical steps in the evolution of multicellularity (make sure to show them the introductory video on this topic)?
What are the differences between the unicellular yeast, strain Y55, and the multicellular strain evolved from it, C1W3? How were C1W3 yeast evolved?

Introduction Questions

What does 'settling selection' actually select for?
In Dr. Ratcliff's experiment, how did yeast respond to selection for fast settling?
What does this experiment show?
Some scientists criticized this research because 'centrifuges aren't found in nature'. How does this experiment address that criticism?
Why do we use both unicellular and multicellular yeast in this experiment?
Which yeast do you hypothesize will be eaten the most by rotifers? Why?

Methodology Questions

Why do we stain the yeast strains different colors?
What is meant by the term 'statistically significant'?
Why do we suspend the yeast in ethanol for five minutes during the staining protocol?
Why do we suspend and centrifuge the yeast sample in water twice after removing the ethanol?
Why do we use distilled, deionized or bottled water instead of tap water?

Discussion questions

What yeast phenotypes are favored during settling selection?
Over time, how would you expect the population of unicellular yeast to adapt and change?
Let's say that in our experiment rotifers eat all unicellular yeast, driving them to extinction. How do you think the evolution of multicellular yeast will affect selection on rotifers? (Hint: rotifers vary in their own size and mouth size).
Clearly multicellularity can evolve rapidly. Why aren't all yeast multicellular?
Were there any sources of error in this experiment? How could those sources of error be diminished?
What would you like to do next if you were going to do more research on this topic?