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

Teacher's guide

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Time required: Two 50-minute class periods

Overview. The origin of multicellularity was one of a few events in the history of life that allowed for increases in biological complexity, allowing for the evolution of the large and complex organisms we see today. The first step in this transition is the evolution of multicellular clusters from single-celled ancestors. Once clusters have evolved, natural selection can act on the properties of whole groups. Over many generations, cluster-level adaptation is thought to result in the evolution of increased multicellular complexity (*e.g.*, cellular division of labor, the evolution of developmental programs, etc.).

In this lab, students will examine the very first step of this process - the ecological conditions under which natural selection favors the evolution of cellular clusters. Scientists have recently shown that, in test tubes, snowflake-shaped multicellular clusters of yeast quickly evolve when they select for fast settling through liquid medium. Making clusters is adaptive in this context, because groups of cells sink faster than single cells, which allows them to survive the selective regime (Ratcliff *et al.*, 2012). As a result, if a random mutation arises that results in cluster formation, these will have a large competitive advantage over the wild-type unicellular yeast. Scientists don't think that selection for fast settling is very common in nature (they use it in the lab mostly because it is convenient) — why might multicellularity evolve outside the lab, in nature?

"…there is always an open niche at the top of the size spectrum; it is the one realm that is ever available to escape competition." John Tyler Bonner, First Signals (2001), p. 51-52.

Increased size has long been thought to play an important role in the evolution of multicellularity (Bonner 1965, 1998). Many benefits of simple multicellularity have been proposed, such as resistance to environmental toxins or UV radiation, increased efficiency of cooperative metabolism, reduced competition with single-celled ancestors, and reduced risk of predation. Out of these, the 'predation hypothesis' is invoked the most frequently. Indeed, it has long been argued that multicellularity may have evolved because it allowed organisms to escape being eaten by small-mouthed predators (Stanley 1973, Bonner 2000). Many living microbes will form multicellular groups when they detect the chemical signature of a predator. For example, the unicellular green algae *Scenedesmus acutus* forms multicellular colonies when grown in water that contained *Daphnia* predators (Van den Hoek *et al*,. 1995). Boraas *et al*. (1998) took an experimental evolution approach to studying this question, co-culturing the unicellular algae *Chorella vulgaris* with a flagellate (small unicellular protist) predator. These algae evolved to form multicellular groups containing eight cells within just 100 generations. Interestingly, these algae continued to form multicellular groups long after the predator was removed, demonstrating that this multicellular state was a heritable, evolved trait. In this lab, students will test the predator escape hypothesis directly, using unicellular and multicellular yeast.

Goals. Feed 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 two 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.

At least two weeks before the start of the lab, place an order for rotifers (and optionally *Paramecium aurelia* if this lab-add on will be used). Ask to have them delivered the day before you plan to do the lab. Rotifers (Carolina Biologal Item #133172, genus *Philodina*) and *Paramecium aurelia* (Carolina Biological Item #131546) can be ordered from Carolina Biological Supply Company (www.carolina.com). Rotifers can survive for up to two weeks in the media and container that they were shipped in, but we recommend having them arrive just a few days before class. Carolina Biological provides a rotifer care sheet describing techniques for their culture and care. The Snowflake Yeast Kit, which includes the yeast strains needed for this lab, can be obtained free of charge by contacting Prof. William Ratcliff (will.ratcliff@biology.gatech.edu) several weeks ahead of time. 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 the Snowflake Yeast 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
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)
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 teacher and student guide, and make copies of the student guide for each student.
Step 2: One day before lab, organize student workstations and give students introductory PowerPoint presentation.

How to use the introductory PowerPoint
Included in the lab is a detailed introductory PowerPoint presentation. This should be used to familiarize the students with the conceptual foundations underpinning the evolution of multicellularity, and introduce the lab activity itself. Specifically, the PowerPoint presentation includes an introduction to the evolutionary transition to multicellularity, background on the experiment performed by Ratcliff *et al.* (2012) that created the snowflake yeast used in this lab, and a short description of the lab activity.

The introductory PowerPoint presentation will take roughly 20-30 minutes, and can be given to the students one day before the lab. We have provided notes (visible below each slide) to serve as a guide for your narration. Feel free to modify this presentation to suit your needs. In addition to the full presentation, we provide a simplified version for use when less detail is preferred (*e.g.*, when time is tight or you feel your class would be confused by the additional detail in the full version).

Optional pre-lab exercise. One customization we suggest is the inclusion of a pre-lab exercise in which students discuss experimental design and expected outcomes. On slide 27 of the full presentation (slide 21 in simplified version), we suggest adding a slide that asks students how they would test the hypothesis that "multicellular yeast may avoid predation because they are too big to eat" experimentally. Once an experimental design has been agreed upon, give students time to write down the expected results of their proposed experiment. This will provide students with the opportunity to think critically about how to design an experiment, rather than simply following a lab's instructions. Note that if you do include this pre-lab option, you should provide the students with their lab manuals after the presentation is over (otherwise they will likely repeat back what they've already read).

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.

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	☐

Exercise 1: Predicting Rotifer Predation

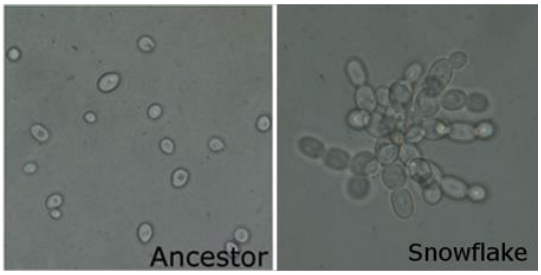

Figure 1. The prey. Snowflake yeast were evolved from the unicellular yeast on the left by selecting for rapid settling through liquid media.

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 (Figure 1). Genetically, this resulted from a single mutation that knocked out a gene (*ACE2*) 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 students will examine the ability of another form of selection against small size, predation, to favor multicellularity. Rotifers (Figure 2) are small multicellular animals that prey on single-celled algae, bacteria, and ciliates. They eat food by creating a vortex with the dense region of cilia on their head, which funnels microbes into their mouth. Their bodies are largely transparent and they move slowly, which makes them ideal for this lab. Students will give hungry rotifers uni- and multicellular yeast, then examine their ability to eat each growth form (Exercise 2). First, they will observe the rotifers and the yeast separately on the microscope and make a prediction about how the yeast will fare when they are given to the hungry rotifers.

Note: Exercise 1 can be skipped if you only have one 50-minute session in which to perform this lab. If so, go straight to Exercise 2, and have the students immediately feed the rotifers uni- and multicellular yeast to make observations.

Figure 2. The predator. *Philodina* rotifers have transparent bodies. Red yeast are visible in this rotifer's stomach.

Methods

This experiment utilizes two yeast strains (Figure 1): 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 the snowflake yeast 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 detritus at the bottom of the container.
2. Add 5 µL of blue stained C1W3 multicellular yeast to a standard microscope slide. **Mix tube by shaking vigorously before removing yeast.**
3. Add 5 µL of red stained Y55 unicellular yeast to the standard microscope slide. **Mix tube by shaking vigorously before removing yeast. You can't hurt the yeast, but they form clumps when sitting for long periods of time and need to be broken up.**
4. Add coverslip to both the depression slide and the standard microscope slide and immediately view on a compound microscope.

Students often confuse predictions with hypotheses. To address this, we have them formulate both (the following text is from the student guide):

Do you think rotifers will preferentially eat either uni- or multicellular yeast, or will eat both equally well? Write down your prediction below. Now, convert this prediction into a hypothesis by including an explanation of why your prediction will be true. For example, I predict that when I drop my pencil, it will fall towards the floor. I hypothesize that gravitational attraction between the pencil and Earth will cause my pencil to move towards to floor at a rate that depends on the distance between the two objects.

Predation prediction:

Predation hypothesis:

Exercise 2: Observing Rotifer Predation

After the students have made their predictions, they can feed the rotifers both yeast strains and make observations about the rotifer predation.

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 shaking vigorously before removing yeast.**
3. Add 5 µL of red stained Y55 unicellular yeast. **Mix tube by shaking vigorously before removing yeast.**
4. Add coverslip and immediately view on a compound microscope.

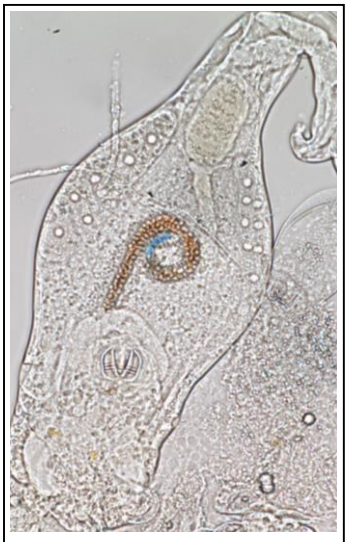

Figure 3. Red and blue yeast can are visible in this rotifer's stomach.

Observations

Students should observe 10 rotifers (a larger sample size is encouraged if time permits) and make a determination on which yeast has been eaten more (see Figure 3 for an example). In the space provided in the student guide, have students note the behaviors of the rotifers and answer questions such as: How do they eat? Can you observe any yeast being consumed? How long does it take them to fill their stomach? Additionally, students will be asked to draw a picture of a rotifer eating yeast, drawing arrows to indicate the movement of water around the rotifers' head.

Exercise 3: Quantifying Rotifer Predation

Introduction

Here, students will gather numerical (quantitative) data that will allow them to formally test their hypothesis about the effect of multicellularity on predation. Students will quantify the number of each type of yeast cell in rotifer stomachs (Figure 4). This approach is more rigorous than the previous exercise: it will not only allow the students to calculate the relative survival of multicellular to unicellular yeast, but it will also allow them to determine if their result is statistically robust (*e.g.*, if the difference in predation between multicellular and unicellular yeast is significantly different). Have students work through this exercise in groups of 4 or 5.

Methods

If the school has a microscope with a camera, students can take images of flattened rotifers (see Figure 3) 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, count red and blue cells in the images provided with the lab (can be obtained under the 'rotifers for quantitation' download link on our website). Each circle in the stomach of a rotifer is one yeast cell (see Figure 4).

Each group member will receive a picture of a different flattened rotifer.

Data Collection

Each group of students will count the number of red unicellular and blue multicellular yeast in five different rotifers' stomachs, filling out the table below (this table is provided in the student guide). Each group member should count the number of blue and red yeast in a different rotifer, allowing students to pool their data within their group. Finally, students will sum the total number of uni- and multicellular yeast their group has found across all of their rotifers, and put this in the 'total' box.

Figure 4. Each of the dark circles above is a yeast cell in the stomach of a rotifer. These are all red unis.

	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

Students will now 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 any differences in the relative survival of multi- to unicellular yeast are statistically significant, students will perform a statistical analysis on their results. In essence, this analysis determines the probability that the difference in predation between uni- and multicellular yeast would have been observed by chance when in fact there are *no actual* differences in susceptibility to predation. For example, if you flip a coin 100 times and you get 53 heads and 47 tails, it's likely that the coin is fair and that this difference is due just to chance (to be precise, p=0.54). But if instead you get 90 heads and 10 tails, the probability that the coin really is fair is pretty tiny ($p \approx 0$). As the results get more divergent from our expectation of 50:50, the chance that the coin really is fair goes down. Students will use the same principle here to determine if the differences they see in yeast death by rotifers is significant.

Students will use a chi-square test to analyze their data, which compares the observed frequencies of uni- and multicellular yeast cells to expected frequencies. The following section is in the student guide to help students through calculating the chi-square statistic to determine if their results are statistically significant:

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). This is a reasonable assumption, because both uni- and multicellular yeast grow until they run out of food, which produces the same number of cells per mL of media in each strain (don't worry, we've checked). When you fed yeast to the rotifers, you provided them with the same number of uni- and multicellular yeast cells. Therefore, the overall ratio of unicellular to multicellular yeast added to the rotifer slide will be very close to 50:50. Assuming there was no rotifer preference for either yeast strain, we expect that half the total number of yeast counted in the rotifers' stomach should be multicellular, and half should be unicellular. To calculate the 'expected' number of multis and unis (for use below), divide the total number of counted cells by two.

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.

$$\chi^2 = \frac{(\#\ Observed_{uni} - \#\ Expected_{uni})^2}{\#\ Expected_{uni}} + \frac{(\#\ Observed_{multi} - \#\ Expected_{multi})^2}{\#\ Expected_{multi}}$$

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:

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

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 calculate what the probability is that there really is no difference in predation between our yeast strains, and that our results are just due to chance. As you can see on the distribution below (Figure 5), if your χ^2 statistic is greater than 3.9, then there is a lower than 5% probability that multicellularity actually has no effect on predation and that your results were caused by chance alone. If your $\chi^2 \geq 3.9$, we're pretty confident that the rotifers really do have a preference. In fact, it is statistically significant at a level generally accepted by scientists to be robust. If this was your result, congratulations!

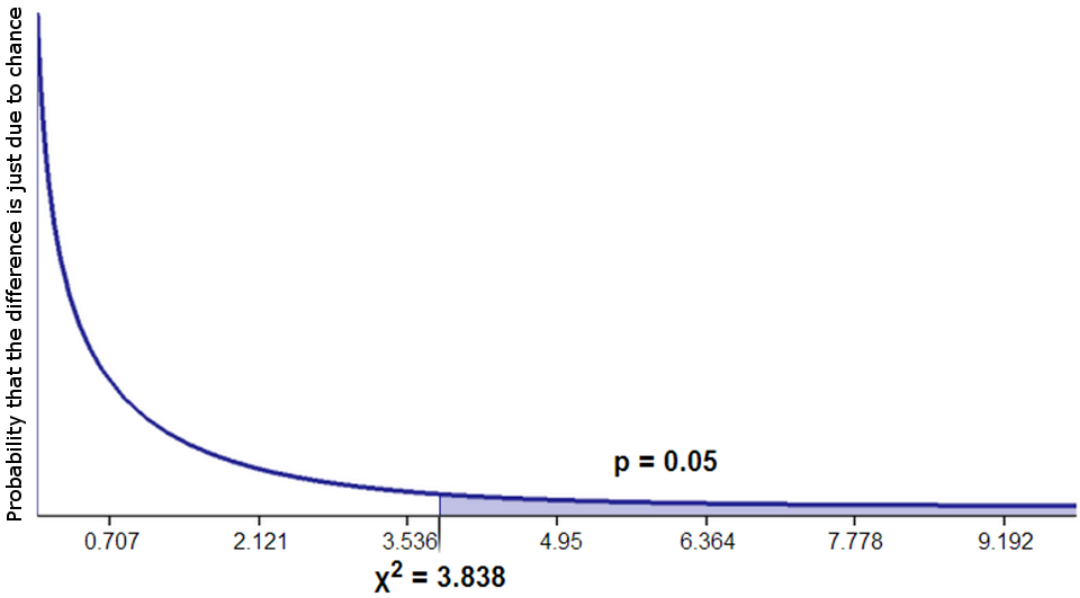

Figure 5. Chi-squared distribution. When the chi squared test statistic is above 3.84, then the chance that the difference in rotifer predation was due just to chance is below 5%. At that threshold, we are pretty sure the rotifers have a preference.

Students will be asked to report their chi-squared statistic and determine if rotifers have statistically significant preference for unicellular yeast over multicellular yeast. Following this lesson, have students answer discussion questions to reflect on the lab in the space provided in the student guide.

Discussion

Discussion questions, as well as pre-lab, introduction, and methodology questions, have been provided in Appendix 2 of this guide. Depending on teacher preference, students will answer questions in their lab notebooks or discuss these questions as a class. At the culmination of this lab, have students incorporate their thoughts and write up a full lab report.

References

1. Ratcliff WC, Denison RF, Borrello M, Travisano M. 2012. Experimental evolution of multicellularity. *Proc. Natl. Acad. Sci. USA* 109:1595-1600
2. Bonner JT. 1965. *Size and Cycle*. Princeton, NJ: Princeton Univ. Press. 219 pp.
3. Bonner JT. 1998. The origins of multicellularity. *Integr. Biol.* 1:28–36

4. Stanley SM. 1973. An ecological theory for the sudden origin of multicellular life in the late Precambrian. *Proc. Natl. Acad. Sci.USA* 70:1486-1489
5. Van den Hoek, E, Mann DG, Jahns HM. 1995. *Algae: An Introduction to Phycology*. Cambridge, UK: Cambridge Univ. Press. 623 pp.
6. Boraas ME, Seale DB, Boxhorn JE. 1998. Phagotrophy of a flagellate selects for colonial prey: a possible origin of multicellularity. *Evol. Ecol*. 12:153-164

Additional reading

1. Grosberg, RK, & Strathmann, RR (2007). The evolution of multicellularity: a minor major transition? *Annual Review of Ecology, Evolution, and Systematics*, 621-654.
   This is our favorite review on the evolutionary history of multicellularity, and it includes an excellent review of the conceptual foundations underpinning the field.
2. Ratcliff, WC, Denison, RF, Borrello, M, & Travisano, M (2012). Experimental evolution of multicellularity. *Proceedings of the National Academy of Sciences, 109*(5), 1595-1600.
   This is the first paper describing the evolution of snowflake yeast. As it was intended primarily for scientists, it is a little technical for high school students.
3. Ratcliff, W. C., & Travisano, M. (2014). Experimental Evolution of Multicellular Complexity in *Saccharomyces cerevisiae*. *BioScience*, 64(5), 383-393.
   This is perhaps the ***best paper for your students to read****. It's written for a non-specialist audience, and covers several of the snowflake yeast papers.*
4. Ratcliff, WC, Raney, A, Westreich, S, & Cotner, S (2014). A Novel Laboratory Activity for Teaching about the Evolution of Multicellularity. *The American Biology Teacher 76(2)*, 81-87.
   This paper covers a different teaching exercise: evolving your own snowflake yeast from unicellular ancestors.
5. Szathmay, E., Maynard-Smith, J. (1995). The major transitions in evolution. *Nature 374*, 227-232.
   This is the canonical paper on major transitions in evolution, such as multicellularity. It's short and a really good read.
6. Godfrey-Smith, P. (2013). Darwinian individuals. *From groups to individuals: evolution and emerging individuality. The MIT Press, Cambridge, 17-36.*
   This is an easy to understand paper on the philosophy of biological individuality that would be good to read if students wanted to learn more about this topic.

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 6) 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.

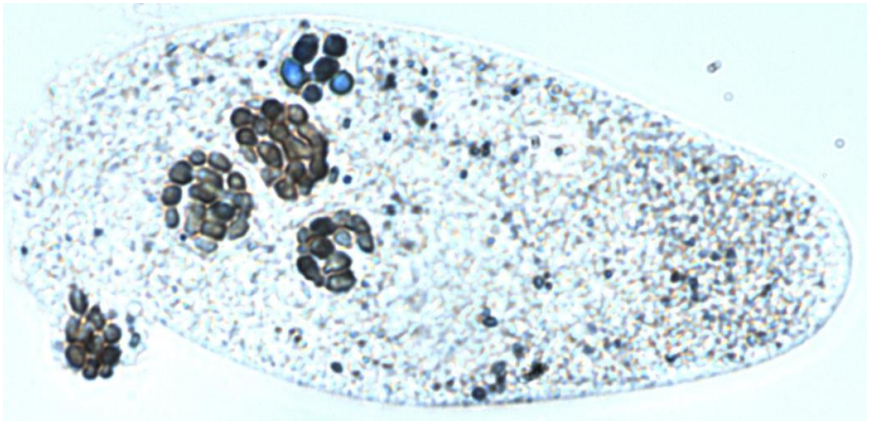

Figure 6. A flattened *Paramecium aurelium* with both red and blue yeast visible.

Additional discussion questions

Are there any differences between predators in how much the discriminate against 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?

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?