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

Student guide

William C. Ratcliff, Nicholas Beerman, Tami Limberg, and Jennifer Pentz

Introduction. 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 (natural selection favoring mutations that make clusters slightly better than their competitors) is thought to result in the evolution of more complex organisms.

In this lab, you 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, you 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.

The actors

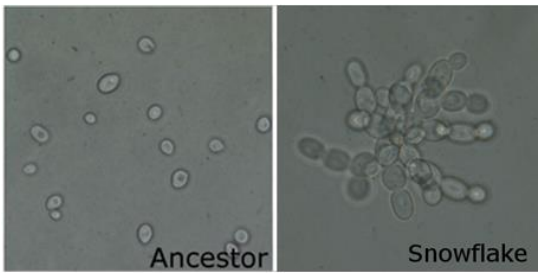

Figure 1. The prey. Snowflake yeast were evolved from the unicellular yeast on the left by selecting for rapid settling through liquid media.

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

Snowflake yeast evolved from single-celled ancestors after three weeks of 'settling selection': artificial selection for faster settling through liquid media (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. After all, there were no centrifuges in nature a billion years ago.

For a long time, scientists have hypothesized that predation could provide a similar selective environment to settling through liquid - namely that predators would be capable of eating (and killing) small, single-celled organisms, resulting in selection for multicellular clusters too large to be eaten. Rotifers are microscopic animals that prey upon single-celled organisms like algae and bacteria (Figure 2). Rotifers live in aquatic environments, like ponds, marshes, and wet moss. They eat food by creating a vortex with the dense region 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. Here, you will give hungry rotifers uni- and multicellular yeast, then examine their ability to eat each growth form.

Exercise 1: Predicting Rotifer Predation

This experiment utilizes two yeast strains: strain Y55 was isolated from a vineyard in France, and is a regular, unicellular yeast. Multicellular strain C1W3 was derived from Y55 after three weeks of selecting for rapid settling through liquid media (Figure 1). We have labeled the unicellular yeast (strain Y55) red, and the multicellular yeast (strain C1W3) blue. Ask your instructor if you are interested in how this was done. In this lesson, you will observe the rotifers and the yeast separately on the microscope and make a prediction on how the yeast will fare once you feed them to the hungry rotifers.

Materials

· Yeast (both strains Y55 and C1W3) fixed and stained with Congo red and methylene blue (supplied in the snowflake yeast kit). **Be sure to wear gloves and protective eye glasses. These stains are toxic.**
· (2) Glass depression slide (alternative: plastic depression slide)
· (2) 22mm x 22mm coverslips
· Micropipette capable of pipetting 100 µL of liquid
· Micropipette 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 depression slide containing rotifers and standard slide containing yeast and immediately view on a compound microscope.

Do you think that 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

You will now gather data to test your hypothesis by feeding the rotifers both uni- and multicellular yeast.

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.

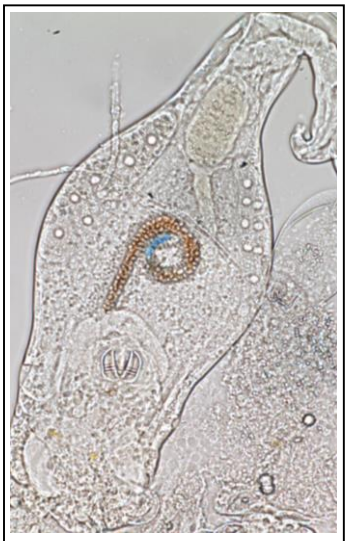

Figure 3. Red and blue yeast can are visible in this rotifer's stomach.

Observations

Observe at least 10 rotifers (more if time permits) and determine which strain of yeast has been eaten more. Note the behaviors of the rotifers. Answer the following questions below: How do they eat? Can you observe any yeast being consumed? How long does it take them to fill their stomach? Draw a picture of a rotifer eating yeast in the box below. Use arrows to indicate the movement of water around the rotifer's head.

Answers to above questions

Rotifer picture

Exercise 3: Quantifying Rotifer Predation

Here, we gather numerical (quantitative) data that will allow you to formally test your hypothesis about the effect of multicellularity on predation. Using a microscope, you will quantify the number of each type of yeast cell in rotifer stomachs (see Figure 4). This approach is more rigorous than the previous exercise: it will not only allow you to calculate the relative survival of multicellular to unicellular yeast, but will also allow you to test if your results are statistically significant (*e.g.*, if the difference in predation between multicellular and unicellular yeast is significantly different). Work through this exercise in groups of 4 or 5 students.

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

Methods

Imaging flattened rotifers. If your microscope has a digital camera, 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. You should see flattened rotifers with yeast cells in their stomach like in Figure 3. Count the number of red and blue cells in the stomach of your rotifer, and record in the table below. Each circle in the stomach of a rotifer is one yeast cell (Figure 4). Fill out the rest of the table with counts provided by your group members.

If the microscope in your lab does not have a digital camera, your instructor will provide you with electronic or printed images of flattened rotifers. Each group member will receive a picture of a different flattened rotifer.

Data Collection

Your group will count the number of red unicellular and blue multicellular yeast in five different rotifers' stomachs, filling out the table below. First, count the number of red unicellular and blue multicellular yeast found in your rotifer stomach, then record the number of each yeast strain your group members 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

You 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, you will perform a statistical analysis on your results. This test will determine 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. You will use the same principle here to determine if the differences we see in yeast death by rotifers is significant.

You will use a chi-square test to analyze your data, which compares the observed frequencies of uni- and multicellular yeast 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). 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 about the same number of unicellular and multicellular yeast cells. 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 uni- and multicellular yeast (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:

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

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!

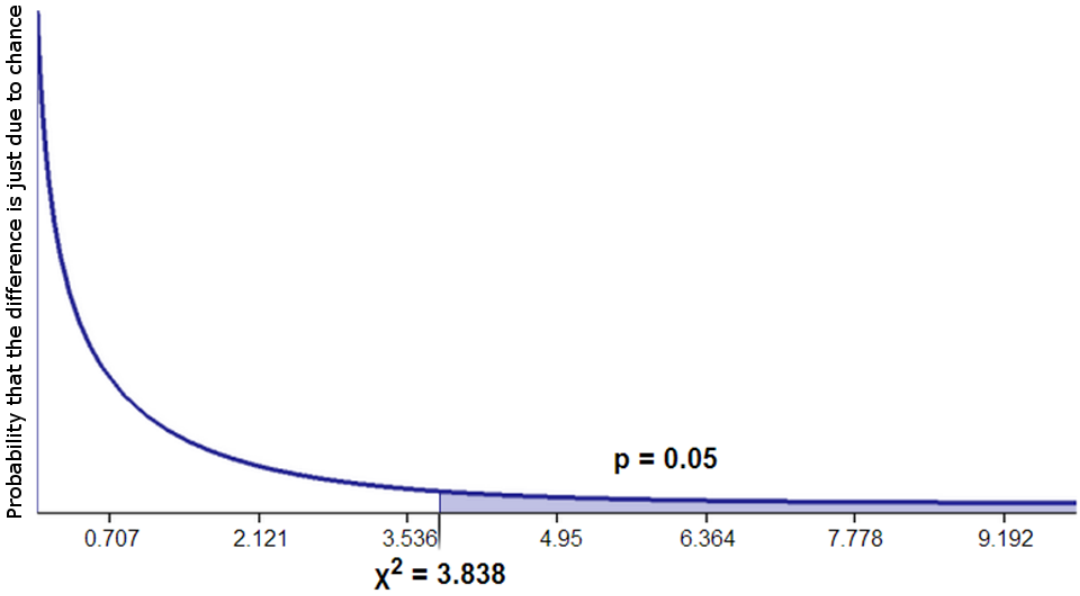

Figure 5. Chi-squared distribution. When the chi-squared test statistic is above 3.84, then the chance that the difference in rotifer predation was due just to chance is below 5%. At that threshold, we are pretty sure the rotifers have a preference.

Discussion

Depending on your teacher's preference, you will either answer discussion questions on page 8, or will have a class discussion.

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 a great review on the evolutionary history of multicellularity, and it includes an excellent review of the conceptual foundations underpinning this 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.
   Interested in the evolution of snowflake yeast? This is the first paper published describing the experiment.
3. Ratcliff, W. C., & Travisano, M. (2014). Experimental Evolution of Multicellular Complexity in *Saccharomyces cerevisiae*. *BioScience*, 64(5), 383-393.
   If you're going to read just one paper on snowflake yeast, this should be it. It's written for a nontechnical audience, and covers several of Ratcliff et al.'s *prior 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 you wanted to learn more about this topic.

Space for answering discussion questions