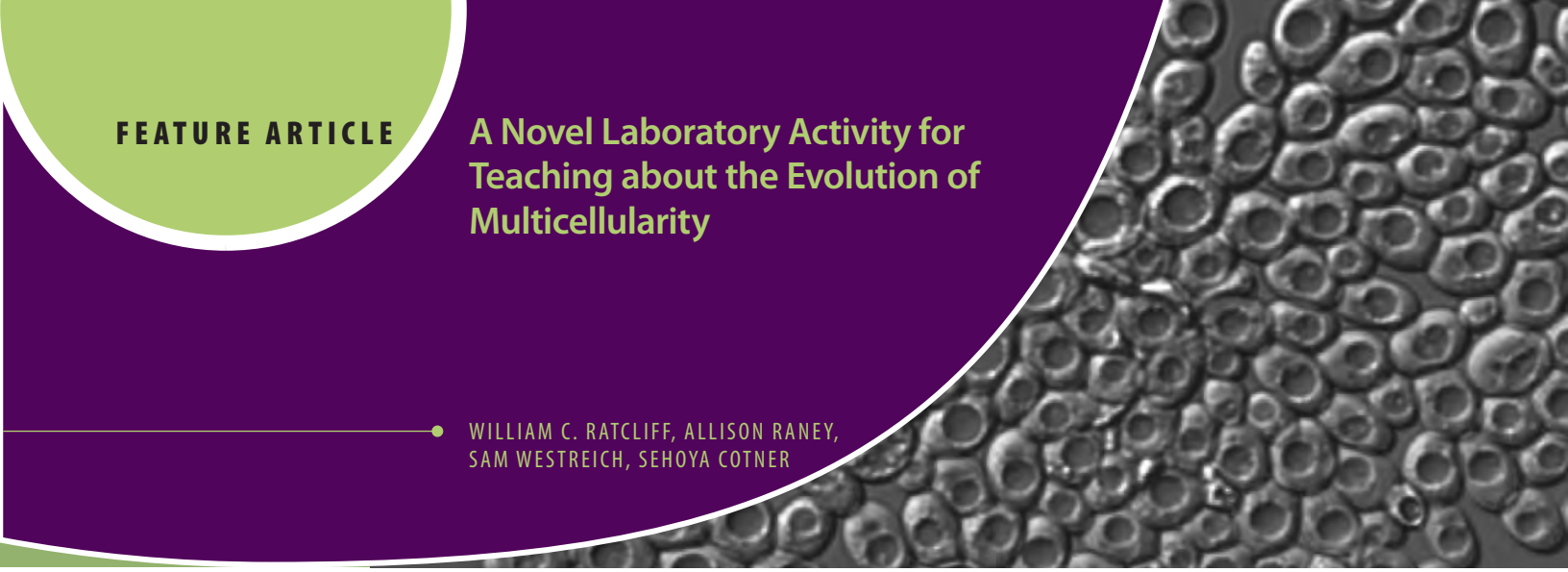
FEATURE ARTICLE

A Novel Laboratory Activity for Teaching about the Evolution of Multicellularity



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Abstract

The evolution of complexity remains one of the most challenging topics in biology to teach effectively. We present a novel laboratory activity, modeled on a recent experimental breakthrough, in which students experimentally evolve simple multicellularity using single-celled yeast (Saccharomyces cerevisiae)*. By simply selecting for faster settling through liquid media, yeast evolve to form snowflake-shaped multicelled clusters that continue to evolve as multicellular individuals. We present core experimental and curriculum tools, including discussion topics and assessment instruments, and provide suggestions for teacher customization. Prelab and postlab assessments demonstrate that this lab effectively teaches fundamental concepts about the transition to multicellularity. Yeast strains, the student lab manual, and an introductory presentation are available free of charge.*

Key Words: *Multicellularity; evolution; complexity; authentic; experimental evolution; curriculum.*

Introduction

A growing body of literature documents a range of strategies for teaching about evolution (see Wei et al., 2012, and references therein), including direct experimentation with live organisms (e.g., Delpech, 2009; Plunkett & Yampolsky, 2010; Green et al., 2011), role playing (e.g., Riechert et al., 2011), and computer simulation (e.g., Codella, 2002; Bromham & Oprandi, 2006; Abraham et al., 2012). Financial and temporal constraints typically limit hands-on experimentation to a few generations, suitable only for microevolutionary questions. As a result, large-scale phenotypic change (macroevolution) is taught only via lecture, computer simulations, or other abstract representation. The origin of multicellular forms from unicellular ancestors was a major transition in evolution (Maynard Smith & Szathmáry, 1995). Despite its fundamental importance in the evolution of large, complex organisms, the evolution of multicellularity has not (to our knowledge) been incorporated into student labs or active-learning exercises. This omission is unfortunate, because copious work (Armbruster et al., 2009; Bailey et al., 2012; Simurda, 2012; for review, see Bransford, 1999) has demonstrated the value of using hands-on methodology to promote deeper understanding and internalization.

The origin of multicellular forms from unicellular ancestors was a major transition in evolution.

Ratcliff et al. (2012) carried out a novel experiment to evolve simple multicellularity in the lab, starting with single-celled microbes. The authors created an environment that favored strains that evolve to form clusters of cells (the first step in the transition to multicellularity) by subjecting baker's yeast (*Saccharomyces cerevisiae*) to daily selection for fast settling through liquid media. Within just a few weeks, yeast that formed snowflake-shaped clusters of cells evolved and displaced their single-celled ancestors. "Snowflake" yeast display several hallmarks of multicellularity, including juvenile and adult life stages, determinate growth, and a rudimentary cellular division of labor utilizing programmed cell death.

Here we describe a new laboratory exercise, modeled on the experiment of Ratcliff et al. (2012). Using simple procedures, nonhazardous microbes and reagents, and inexpensive materials, we have developed and tested a 3-week lab that involves students in cutting-edge research and encourages them to think critically about the evolution of multicellularity.

Methods

In spring 2012, over 300 university students who were enrolled in either Animal Diversity or General Zoology – organismal biology courses with an introductory-biology prerequisite – participated in an "Evolution of Multicellularity" laboratory exercise. Students were divided into 16 sections of ~18 students. Undergraduate teaching assistants oversaw the practical aspects of the lab activities.

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The Laboratory Exercise

Complete lab materials (student and instructor lab manuals, pretest and posttests, video protocols, and other materials) are available at http://www.snowflakeyeastlab.com.

The following materials are necessary and, with the exception of an autoclave and shaking incubator, relatively simple and inexpensive to obtain:

- Unicellular yeast (strain Y55)*
- Snowflake yeast, evolved after 3 weeks of selection (strain Y55_wk3)*
- YPD media (see recipe in "Protocols" [Box 1])
- Test tubes for cell culture (25 × 150 mm)
- Test tube racks
- 1.5-mL microcentrifuge tubes
- 100-µL micropipettors
- Micropipette tips
- Serological pipettes (5 mL)
- Serological pipettes (25 mL)
- Bulbs for the serological pipettes
- 22 × 22 mm coverslips
- Microscope slides (plain)
- Microscope slides (concave)
- Autoclave
- Shaking incubator
- Compound microscope
- Rulers

*Yeast strains can be obtained free of charge by contacting W. Ratcliff (will.ratcliff@biology.gatech.edu). Yeast can be shipped internationally.

The lab procedure spans 3 weeks, with daily settling selection and transfer to fresh media. Each day, students perform a round of settling selection and then transfer surviving yeast to fresh media (for an overview, see Figure 1). Students start with either unicellular yeast (strain Y55) or a snowflake strain that previously evolved during 3 weeks of daily transfer (Y55_wk3) using the "faster settling selection" regime (Figure 1A). Using both strains allows the students to examine 6 weeks of evolutionary time in only 3 weeks, or ~200 generations of yeast.

Using unicellular yeast (str. Y55), students will select only for faster settling, favoring strains that evolve to form clusters. Using snowflake yeast (str. Y55_wk3), students will examine the ability of selection to act on differences among clusters, selecting for either faster or slower settling.

Protocols

YPD media preparation

- Ingredients:
  - 20 g dextrose
  - 20 g peptone
  - 10 g yeast extract
- Dissolve the above ingredients into 1 L water.
- Bring the final volume up to 2 L.
- Using the 25-mL serological pipette, aliquot 5 mL YPD into each 25 × 150 mm test tube, and place into test tube racks.
- Cap tubes.
- Autoclave to sterilize.

Initial tube inoculation

- Obtain 3 test tubes containing 5 mL YPD.
- Inoculate 1 tube with 100 µL of the Y55 stock culture, and 2 tubes with 100 µL of Y55_wk3.
- Label each tube with the inoculation date, strain, and selection scheme (i.e., faster or slower settling). Incubate overnight (30°C, shaking).

Settling selection (days 2+). See Figure 1 for illustrations.

- Obtain three test tubes containing 5 mL YPD. Label tubes with transfer number, strain, and selection scheme.
- Obtain a 5-mL serological pipette with bulb.

Selection for faster settling

- Suck up 5 mL of the turbid yeast culture into a 5-mL serological pipette.
- Allow the pipette to stand upright in a glass beaker or pipette rack for 10 minutes. Make sure the media is not leaking out. If the pipette leaks, seal the bottom with a piece of parafilm.
- Transfer the bottom 0.5 mL to sterile YPD.

Selection for slower settling

Same as above, but after 10 minutes of settling, discard the bottom 4.5 mL of media in the pipette, transferring the top 0.5 mL to sterile YPD.

Repeat the settling selection ~15 times, viewing samples under the microscope at weekly intervals (or more frequently if time permits) to track progress. Labs that only meet once or twice a week will require careful planning: daily transfers can be accomplished by allowing separate lab sections to transfer the same populations. Instructors, TAs, and students may be required to perform transfers when no class is scheduled to meet. When daily selection cannot be performed (such as during a weekend), place the selection lines in the refrigerator.

Analyses to Perform on the Last Lab

By the last lab, each selection line should have undergone ~15 transfers, or ~100 generations. As time permits, have the students measure the following traits on both the evolved lines *and* the ancestors used on day 1 (regrown from stock cultures):

1. **Cells per cluster.** Faster settling mainly results from the evolution of larger clusters. To measure cells per cluster, students first need to dilute each population grown in YPD 10-fold into nonsterile water, adding 100 µL culture to 900 µL water in a 1.5-mL microcentrifuge tube. Mix by inversion, then place 5 µL onto a standard slide with coverslip, and view on a compound scope. Have each student or group of students find the largest cluster on a slide and count the number of cells in it.
2. **Cell size.** Larger clusters of Y55_wk3 will sometimes evolve as a consequence of an increase in the size of individual cells. Think of this as building a larger house not by changing the design, but simply by using larger bricks (Figure 1C, D).

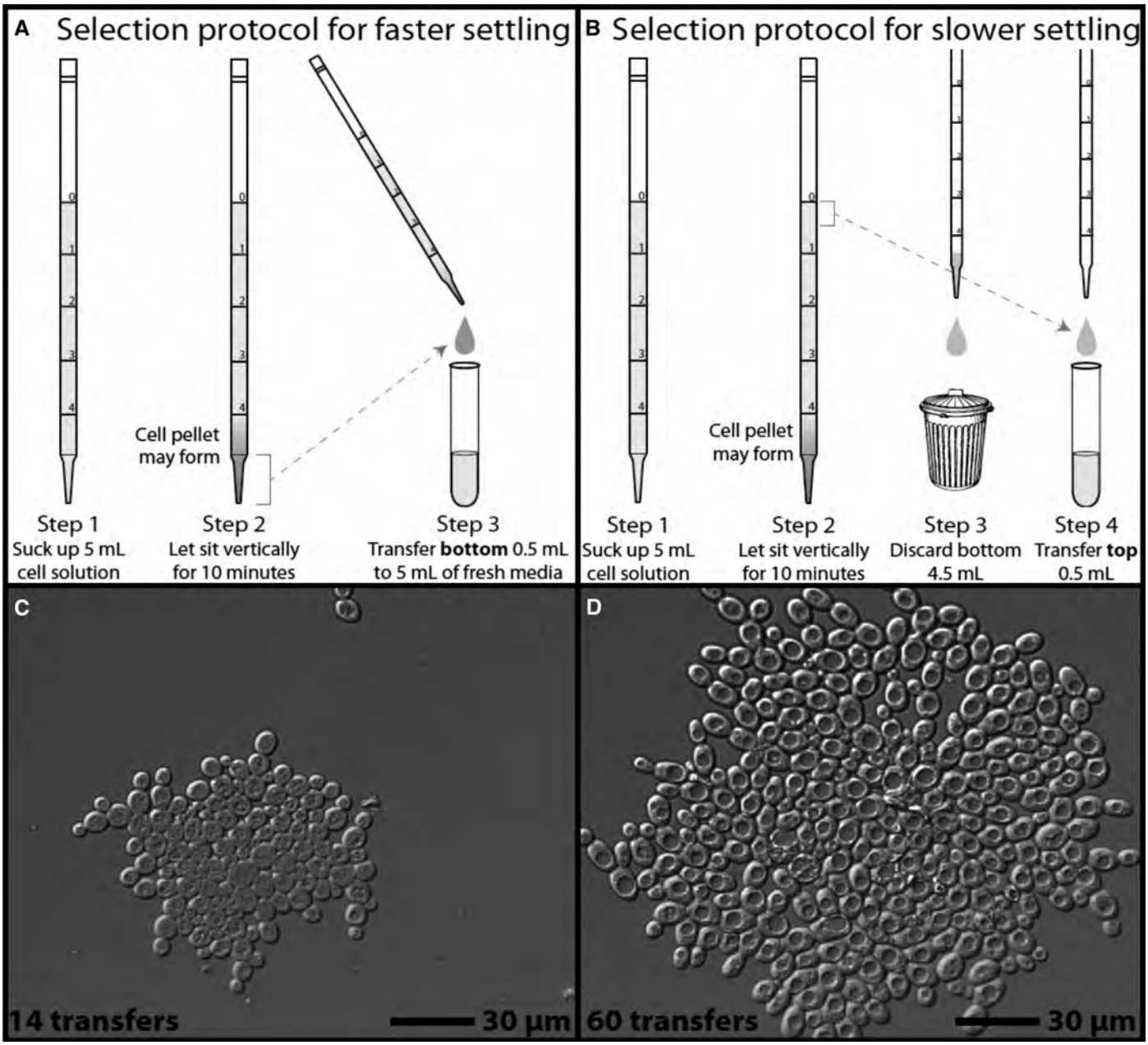

Figure 1. Settling selection protocols illustrated. Selection regime for (A) faster or (B) slower settling. Snowflake yeast evolve faster settling by evolving larger size. (C) An isolate taken from 14 transfers. (D) An isolate taken from 60 transfers; this cluster is larger not only because there are more cells per cluster, but also because the size of individual cells has increased.

Have students note the size of cells in the evolved lines compared with the single-celled ancestor.

3. **Settling speed.** Suck up 5 mL of each of the five populations (two inoculum strains plus three selection lines) into a serological pipette and stand it upright for 5 minutes. Using a ruler, measure the height of the cell pellet that forms at the bottom of the pipette. This measurement is a proxy for settling rate.

Measures 1 and 3 can be reported to the teacher and graphed for the whole class, allowing students to examine the distribution of maximum cluster size and settling speed for their evolved lines, in relation to their starting lines, across multiple independently evolving replicate populations.

Additional Exploration

Expanding on the above selection schemes is possible and encouraged but depends on time, student interests and abilities, and equipment availability. Additional projects could focus on other ecological scenarios that could favor the evolution of cell clusters, such as evasion of small-mouthed predators (e.g., rotifers; see http://www.snowflakeyeastlab.com/predation.htm), protection from UV exposure or desiccation, or increased resistance to antibiotics. Students can investigate various hypotheses about size-related advantages using simple, inexpensive, and readily available supplies (e.g., UV lamps and live zooplankton). Students with access to a fluorescent compound microscope can explore the evolution of programmed

cell death (apoptosis) in snowflake yeast through live/dead staining, following protocols described in Ratcliff et al. (2012).

For further ideas about how to teach this material, especially evolutionary concepts about cooperation, conflict, and biological individuality, see the modules created by Professor Rick Michod and Matthew Herron at the University of Arizona (http://www.eebweb.arizona.edu/faculty/Michod/complexity/). For a summary of recent work on the evolution of multicellularity in the green algae *Volvox* (aimed at advanced high school or undergraduate readers), see Miller (2010).

Possible Discussion Topics

Certain themes may arise during postlab discussions or written reflections, including the following:

- *What is experimental evolution?*

  Experimental evolution involves testing hypotheses about evolutionary processes in controlled laboratory or field settings. By observing evolutionary change in an organism such as yeast over generations, students can view evolution in action in an ecologically relevant context. With organisms that have short generation times, experimental evolution allows students to see morphological change occurring over relatively short time scales.

- *Did multicellularity arise more than once?*

  Multicellularity has evolved at least 25 times independently (Grosberg & Strathmann, 2007), resulting in multicellular organisms as diverse as animals, plants, seaweed, and fungi. The purpose of this exercise is not to suggest that settling selection led to the evolution of multicellularity in any of these groups. Instead, the goal is to demonstrate that simple environmental constraints and relatively few generations could have been sufficient for the evolution of a key step in these transitions.

- *What are benefits and costs of multicellularity?*

  Early multicellular organisms likely benefit from their larger size, resisting things like predation, toxin or UV exposure, or desiccation. More derived multicellular organisms benefit from cooperation among component cells. This division of labor can allow multicellular organisms to perform some tasks more efficiently, and can facilitate the evolution of complex traits (like differentiated tissues) that provide novel multicellular-level functionality.

  Multicellularity is not without its costs, however. Multicellular organisms require more resources and take longer to mature than unicellular organisms. Further, their complexity makes them more vulnerable to disruption. For example, conflicts among cells within an organism, such as cancer, can lead to dissolution and death of the organism; cancer is not a problem for single-celled organisms.

- *Is multicellularity reversible?*

  In principle, all multicellular organisms should be able to evolve back to unicellularity. It may be difficult to imagine complex multicellular organisms like vertebrate animals evolving back to unicellularity, because individual cells are so specialized and integrated into the multicellular whole. However, whether or not increased multicellular complexity makes it more challenging to evolve back to unicellularity remains an open question. The HeLa cell line presents an interesting example of the evolution of unicellularity from a human ancestor. Cervical cancer cells isolated from Henrietta Lacks have been grown in the lab since 1951 and are so widely used that their collective biomass is estimated to be about 50 million tons. HeLa cells are exceptionally adapted to life as a single-celled microbe and have become a major contaminant of laboratory cultures (Skloot, 2011). Their success as a unicellular organism has even led to a formal description as a new species of microbe, named *Helacyton gartleri* (Van Valen, 1991).

- *What is the distinction between a multicellular organism and a multicelled cluster?*

  Scientists have not reached consensus on what constitutes a multicellular organism. Like other prominent concepts in biology (e.g., what counts as alive, what species are, etc.), determining whether an organism is multicellular is trivial at the extremes of the spectrum (e.g., *Escherichia coli* and blue whales), but challenging for intermediate states. A key question is this: When does a cluster of cells stop being a group of single-celled organisms and become one multicellular organism?

  One way to answer this is to determine whether single cells are still "individuals" or if they are "parts" that work together for the benefit of the multicellular individual, like skin cells in animals. Put in evolutionary terms, once the Darwinian fitness (survival and reproduction) of single cells within the cluster is less important than the contribution of single cells to the fitness of the cluster, then the group of cells can be considered a multicellular organism. Ratcliff et al. (2012) showed that single cells in snowflake yeast evolve to commit cellular suicide (apoptosis), to act as "break points" within the multicellular cluster. This reduces the fitness of single cells (they die) but increases the fitness of the cluster, allowing it to regulate the number and size of propagules they produce. So, by this definition, snowflake yeast (at least those that have evolved apoptosis) can be considered simple multicellular organisms.

Assessment

In order to determine the level of background knowledge demonstrated by the students, individual assessments were given to each student before the lab exercise (see Appendix). Nine weeks after the exercise, the students were each given a similar postexercise assessment, designed to test their understanding and retention of the material. Statistics were calculated only for students who completed both prelab and postlab assessments. Matched-pairs t-tests were performed for each question, with error from multiple comparisons controlled with a Bonferroni correction.

○ Results

Prelab and postlab assessments demonstrated that students learned and retained key concepts about the evolution of multicellularity. Substantial improvement was seen for the question in which students identified that "multicellularity arose several times, in several independent lineages" (Figure 2); the prelab average was 66.2% correct and the postlab average was 79.7% correct ($t_{147} = 2.84$, $P < 0.01$). This finding indicates that not only did most of the students understand that multicellularity evolved in multiple lineages, but the exercise was able to emphasize this idea. Students also showed improvement in listing advantages of multicellularity: students were

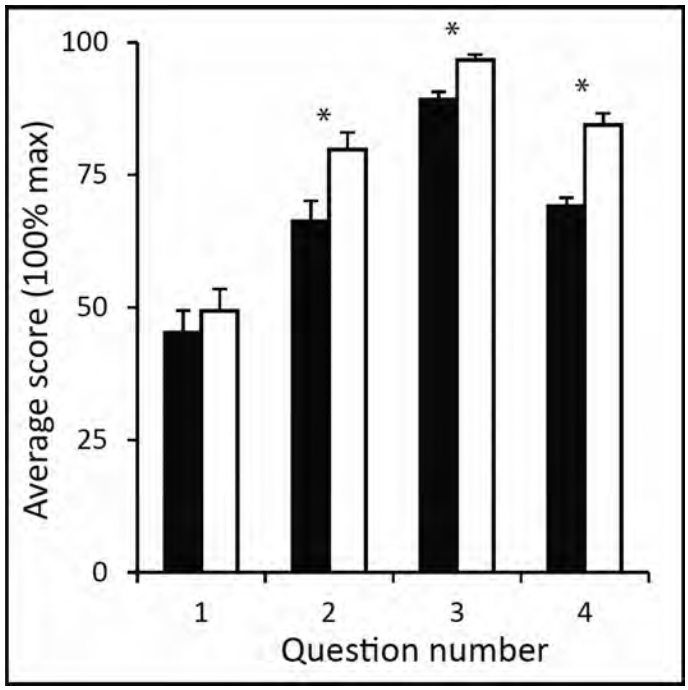

Figure 2. In a prelab and postlab assessment, students showed improvement in their knowledge of the origins of multicellularity (question 2) and could better articulate possible benefits (question 3) and disadvantages (question 4) of multicellularity over single-celled existence. Filled bars represent prelab scores, and open bars are postlab. Error bars indicate SE, and asterisks indicate statistical significance ($P < 0.05$; corrected for multiple comparisons). Students did not show significant improvement in their knowledge of which groups of organisms are multicellular (question 1). See Appendix 1 for a full list of assessment instruments.

able to list an average of 2.68 logical benefits on the prelab quiz, and 2.89 benefits after the exercise and discussion (89.9% and 96.3% of the maximum number of correct answers, respectively; $t_{147} = 4.67$, $P < 0.001$). Similarly, prior to the lab, students could list an average of 2.07 possible disadvantages of multicellularity, compared with a postlab average of 2.53 (69% and 84.3% of the maximum number of correct answers, respectively; $t_{147} = 6.12$, $P < 0.001$).

In the prelab assessment, the most commonly described benefits of multicellularity were physical characteristics of the organism, such as an improved ability to gather food, adapt to novel environments, and survive after the death of individual cells. In the postlab assessment, students were more likely to discuss benefits such as division of labor, specificity of cell tasks, and physical differentiation of cells increasing the overall energy efficiency of the individual. Similarly, students tended to list physical qualities (higher risk of predation, reduced surface-area-to-volume ratio) as disadvantages of multicellularity on the prelab assessment, whereas they favored innate qualities related to the evolution of multicellularity (higher total energy costs, conflicts among cells, greater fragility due to higher complexity) on the postlab exam. This change demonstrates a shift in thinking from merely observing the physical effects of a specific quality to considering the greater implications of evolving into multicellularity, as well as the associated benefits and the obstacles that must be overcome by the organism.

These trends are especially promising, because the postlab assessment was not given to the students immediately after completion of the laboratory exercise, but 9 weeks later. Because no other coursework in this class addressed multicellularity, we conclude that this lab was effective in teaching core concepts about the evolution of multicellularity.

○ Discussion

Few topics in evolutionary biology challenge educators like the origins of complexity. The popularity of nonscientific explanations, such as Intelligent Design and other forms of creationism (Scott & Matzke, 2007), attests to our limited ability to address complexity in a scientifically meaningful way. Hands-on activities that are authentic (i.e., using real organisms and legitimate unexplained phenomena) are ideal for clarifying misconceptions and effecting conceptual change (see Chinsamy & Plagányi, 2008). We are thus especially attracted to this demonstration of a legitimate, intellectually available mechanism for the origins of multicellularity.

The lab described here uses the nonpathogenic and fast-growing unicellular fungus baker's yeast to directly explore the evolutionary origin of multicellularity, recapitulating a recent experimental breakthrough (Ratcliff et al., 2012). In 3 weeks, the students take their yeast through ~100 generations of experimental evolution, examining whole-cluster-level adaptation in cluster-forming "snowflake" yeast. This lab provides a platform for teaching key concepts about the evolution of multicellularity, including (1) how multicellularity arose more than once in different lineages; (2) the ecological conditions under which multicellularity could evolve; (3) how multicellularity incurs costs and is, as predicted, reversible; and (4) the distinction between multicellularity and colonial unicellular organisms. Additionally, lab procedures familiarize students with core life-science materials (e.g., micropipettes and growth media) that are used in advanced teaching and research laboratories.

Recommendations for Customization

We encourage our colleagues to modify this lab activity to suit their curricular needs. However, we can make a few recommendations based on our experiences. Brief introductory materials, discussing the basics of yeast biology and the advent of multicellularity, were sufficient for engaging students in a discussion of the experiment and associated predictions. Clear directions, tailored from Ratcliff et al.'s (2012) experiment, should be given to both the students and the teachers/staff. These directions are included in the laboratory manuals (available at http://www.snowflakeyeastlab.com), and it is critical that instructors think through the logistics of medium preparation and glassware washing/sterilization prior to the first run of the lab. After the first week, the process – growth, selection, and inoculation of new medium – is repeated.

We recommend sharing a week-by-week layout with the students, outlining the plan for the duration of the exercise. Student lab materials should contain ample writing and drawing space, so that the students can describe what they are seeing on the slides (prepared from small amounts of yeast from both the unicellular to multicellular selection lines, and the divergent selection lines) in words and pictures, and collect appropriate data. Whenever possible, we

recommend that data for the entire section be pooled, allowing the students to examine the evolution of snowflake yeast traits in multiple independently evolving lineages.

Limitations & Workarounds

This lab is suitable for both high school and college biology classes. The two main constraints are time (it takes several weeks to evolve the yeast) and logistics. The number of evolving yeast populations scales to the number of students; in large classes this can create a substantial burden on staff and/or students to prepare media, clean test tubes, etc. This workload can be reduced if students work in groups. We have also found that errors during transfer (e.g., mislabeling and contamination) are greatly reduced when students are responsible for transferring the same populations every day. This is easily achieved in high school, where classes meet daily, but may not be possible in college classes where lab sections meet weekly. For the latter, it is essential to establish clear labeling schemes and carefully explain the transfer process to students before they start. Finally, this lab requires little in the way of specialized or expensive equipment, other than an autoclave and shaking incubator. If this equipment is not available at the reader's high school, we recommend the reader contact biology faculty at a local college or university and inquire about borrowing outdated/unused equipment or, better yet, propose a collaboration. Research professors funded by federal grants are encouraged to conduct community outreach (in National Science Foundation terms, fulfill "Broader Impacts" criteria), and thus are incentivized to lend time and/or equipment.

Feedback

The purpose of this lab is to provide core experimental curricula; we highly encourage teachers to expand and modify this lab as they see fit (see Additional Exploration, above, for a few ideas, but teachers should feel free to experiment broadly!). We welcome any comments and questions from teachers about which elements of the lab were the most exciting and challenging in the high school environment.

○ Acknowledgments

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Appendix. Questions used in the assessment.

1. Of the following groups, which are *exclusively* multicellular (circle any/all that apply)?
   (a) Animals
   (b) Land plants
   (c) Fungi
   (d) Bacteria
   (e) Algae
2. Which of the following best characterizes our current understanding of the evolution of multicellularity?
   (a) Multicellularity arose several times, but only in a single lineage
   (b) Multicellularity arose once, and gave rise to all animals
   (c) Multicellularity arose several times, in several independent lineages
   (d) Multicellularity arose once, in an organism like present-day *Volvox*
   (e) Multicellularity arose once, in an organism much like present-day baker's yeast
3. List three possible advantages of multicellularity.*
4. List three possible disadvantages of multicellularity.*

While not included in our first assessments, we recommend these questions for use in your class:

1. What distinguishes a multicellular organism from a clump of cells or a colony of unicellular organisms?
2. Describe a plausible scenario by which multicellular organisms could evolve from unicellular ancestors. Pictures are encouraged!

Finally, the instructor lab manual (available at http://www.snowflakeyeastlab.com) contains 40 discussion questions that cover methodology, evolutionary processes, and even philosophical notions of individuality. Several of these questions can be discussed each day, helping students get their bearings and delve deeply into the subject material. We also include 13 wrap-up discussion questions for use at the end of the lab.

*In the postlab assessment, these questions were changed to "What are some possible advantages [for question 3] or disadvantages [for question 4] of multicellularity (list as many as you can think of)." We scored both prelab and postlab quizzes out of a maximum of three correct responses. Because some students listed more than three advantages/disadvantages in the postlab quiz, this estimate is conservative, likely underestimating improvement.