Fitness competition in caffeine

TIME ESTIMATE

When growing yeast in an incubator, this takes a week, with active time on Day 1 and Day 4, and results available on Day 7 or later if plates are stored. This does not include the 2-3 days needed to have the strains growing on plates from which students pick colonies on Day 1, as detailed in "before the lab."

GOALS

1. Demonstrate that independent evolved strains may differ in their fitness in the evolved environment
2. Demonstrate that independent evolved strains may differ in their fitness between environments (evolved environment vs. original environment)

OVERVIEW

We have been carrying out experimental evolutions with lab strains of *S. cerevisiae* that express vibrant pigments and thus each have a distinct color. Because of these colors, relative abundance of each strain in a mixed culture can be determined by counting colony forming units (CFUs) and calculating the ratio of colors (**Figure 1**). This approach can be used to determine whether yeast with different colors are better adapted to a particular environment.

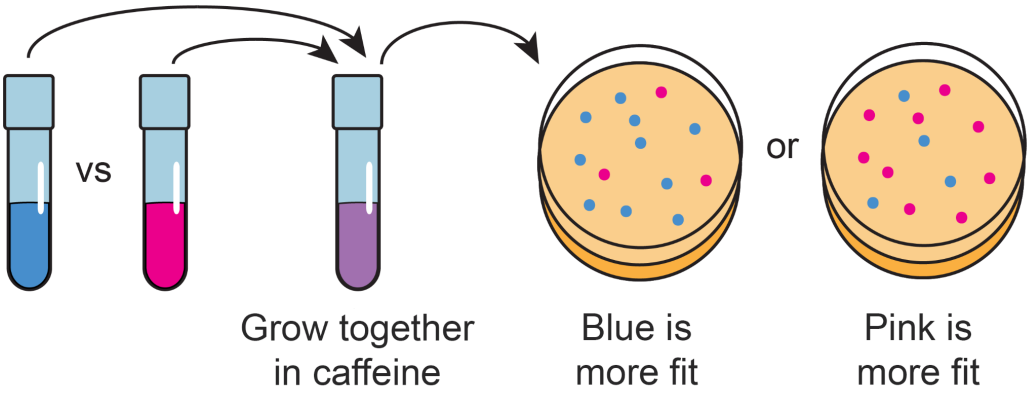

Figure 1: Competition experiment overview. Ratio of CFU colors on agar media indicates the relative abundance of each strain in a mixed culture.

We will use these colors in a competition experiment, which will allow us to determine which yeast from your experiments are best-adapted to caffeine. To do this, you will first grow yeast from the evolution experiment and with different colors in a medium that does not contain caffeine. You will then mix these strains in media containing varying concentrations of caffeine so that they will compete for resources. After the mixed cultures grow, you will plate them onto

agar media on plates. After a few days of growth, you can count the ratio of colors on each plate to determine which strain "won" the competition in each concentration (**Figure 2**).

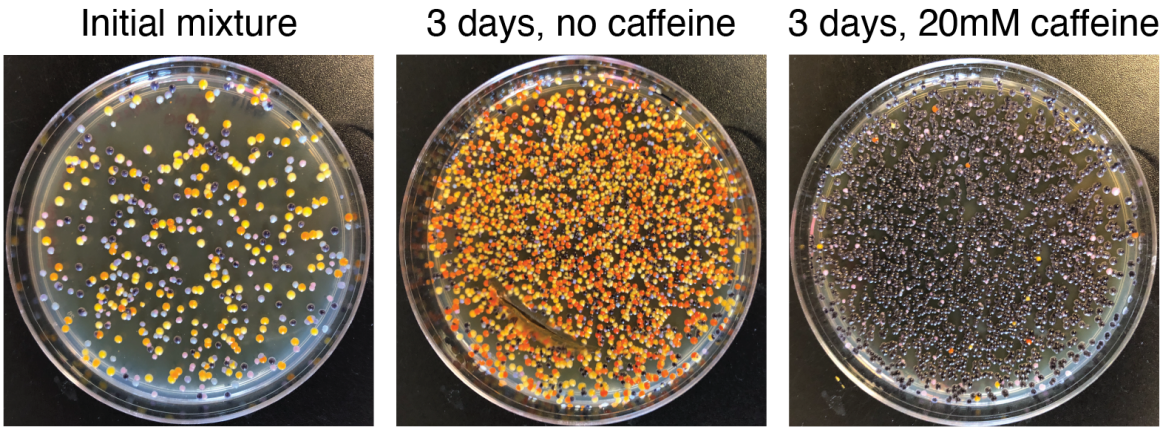

Figure 2: Example of competition experiment outcome. Six strains of different colors evolved in the presence of caffeine were co-cultured in YPD + G418 media containing 0mM or 20mM caffeine for 3 days. A 1:10,000 dilution of each culture was plated onto YPD + G418 agar media, except for 20mM a 1:2,000 dilution . In 20mM caffeine, the black strain outcompetes the others, indicating that it has higher fitness than the ancestral strain in the presence of caffeine, but in the absence of caffeine the yellow and orange strains have higher fitness and outcompete the others.

GLOSSARY

- CFU: Colony Forming Unit; a cell that is capable of growing into a colony of cells when transferred from liquid to solid medium. CFUs are commonly used as a proxy for the number of viable cells in a liquid culture.
- Caffeine: A compound produced by plants found in some products used for foods, including coffee and cocoa beans. Industrial yeast are exposed to caffeine when used for fermenting coffee and chocolate.
- Fitness: A measure of an individual's reproductive success. Note that in this experiment you will not be measuring fitness directly but will instead observe the outcome of competition between individuals with differing fitness in a given environment. Success in this competition is determined by the relative fitness between these individuals.
- G418: Geneticin, an antibiotic commonly used in laboratory experiments. Yeast utilized in this protocol are resistant to G418 due to a plasmid they carry, which also gives them their distinctive color thanks to additional genes on the plasmid that encode pigment production pathways. G418 is necessary for maintenance of the plasmid and additionally helps to prevent contamination by killing other microbes that are not G418-resistant.
- Selection pressure: An environmental condition that favors some genotypes in a population over others.
- YPD: A standard rich yeast medium named for its three ingredients: Yeast extract, Peptone, and Dextrose. Also referred to as YEPD.

MATERIALS AND EQUIPMENT

Yeast strains

- *S. cerevisiae* strains evolved in caffeine carrying different pigment expression plasmids

Equipment

- Pipettes: volume needs will vary based on implementation. You will likely need a P2-20ul, a P20-200ul, and a P200-1000ul or equivalent, and a 5ml serological pipette.
- Culture tubes
- Glass beads or plate spreader

Consumables

- YPD + G418 liquid media (21mL per classroom of 6 groups; 84mL for four classrooms and multi-class final competition)
- YPD + G418 + 10mM caffeine media (3mL per experiment, made from mixing three parts no caffeine and one part 40mM caffeine medias)
    - For four classrooms and one multi-class competition, mix 15mL YPD + G418 with 5mL YPD + G418 + 40mM caffeine
- YPD + G418 + 20mM caffeine media (3mL per experiment, made from mixing equal parts no caffeine and 40mM caffeine medias)
    - For four classrooms and one multi-class competition, mix 10mL YPD + G418 with 10mL YPD + G418 + 40mM caffeine
- YPD + G418 agar plates (8 per experiment, 40 total)
- 1.5mL tubes (12 per experiment, 60 total)
- Sterile swabs, sterile inoculating loops, or sterile inoculating sticks (2 per experiment)

Optional

- *30°C incubator*
- *Test tube roller drum or shaking platform*
- *Vortex machine*

SAFETY

The colored yeast strains are genetically modified and thus considered biohazardous waste. Dispose of inoculating sticks in a biohazard bin, or decontaminate by placing in 10% bleach for 20 minutes before throwing away. Liquid waste containing yeast should be collected and decontaminated using bleach at a final concentration of 10% for 20 minutes before pouring down a drain.

BEFORE THE LAB

1. Plan out how the timing of activities will fit with your class schedule. Yeast grow most robustly at 30°C. They can be grown at room temperature as well but will grow more slowly. One competition will take approximately 5-7 days, so can be completed over one week in

class. *We've included estimates for the time it'll take for your students' yeast to grow where applicable in italics*.

2. Make caffeine media. Each competition performed will require 3ml each of a low and a high concentration of caffeine media. In our hands, the following concentrations work well. *If all groups progressed to evolve their yeast in 40mM media, this can be changed to do competitions in 40mM and 20mM caffeine, instead of 20mM and 10mM caffeine.*
    - Low dose: 20mM caffeine (3.88g/L). Moderately inhibits growth of ancestral strain. For 4 classrooms (1 competition each), mix 10mL of YPD+G418 containing 0mM caffeine with 10mL YPD+G418 containing 40mM caffeine.
    - High dose: 40mM caffeine (7.77g/L). Moderately inhibits growth of ancestral strain. Mix 10mL of YPD+G418 containing 0mM caffeine with 10mL YPD+G418 containing 40mM caffeine.
3. Prepare tubes containing caffeine media. Each class (6 teams each with a different color) needs one tube each of no caffeine, 20mM, and 40mM media. So for 4 classes, prepare 4 tubes with 2uL YPD+G418, 4 tubes with the low dose (20mM) media made in step 2, and 4 tubes with the high dose (40mM) media made in step 2.

PROTOCOL

Day 1: Mix strains in media with different concentrations of caffeine. Each class needs to do this only once, as a group!

1. Examine the cultures you saved from the end of your +caffeine (not control!) evolution. If yeast have settled at the bottom of the tube (pelleted), gently shake the tube until they are completely resuspended. The liquid sample of yeast (culture) should be dense enough that you cannot see through it, and the two cultures should be comparable in density.
2. Each group will remove 20ul of their evolved culture and mix them together in a single microcentrifuge tube. Make sure to mix well by vortexing or pipetting up and down several times. For each class, there will be six strains of different colors mixed together, for a total of 120uL.
3. Add 5ul of mixed culture to each of the three test tubes prepared with 0mM, 20mM, and 40mM caffeine. Dispose of all pipette tips as biohazardous waste. Put the tubes in the incubator to grow.

    These cultures should be allowed to grow for at least 2 days in the incubator, but leaving them for 3-5 days is also fine, and may lead to more colonies.
4. Prepare a 1:10,000 dilution of the remaining (105uL) mixed yeast culture from the microcentrifuge via serial dilution. Fill two microcentrifuge tubes with 990ul water and label them "dilution 1" and "dilution 2". In the tube labeled "dilution 1", add 10ul of mixed culture and vortex for 5 seconds or invert 10 times to mix. Transfer 10ul from "dilution 1" to "dilution 2" and vortex for 5 seconds to mix.
5. Prepare a 1:50,000 dilution. Label another 1.5mL tube "dilution 3" and fill with 400uL water. Transfer 100ul from "dilution 2" to "dilution 3" and vortex for 5 seconds to mix.

6. Obtain two YPD + G418 agar plates. Label one "Before competition, 1:10,000", and the other "Before competition, 1:50,000." Label with your classroom number to tell your competitions apart from other classes!
7. Use a sterile plate spreader to spread 150ul from "dilution 2" onto the 1:10,000 dilution plate, and 150uL "dilution 3" onto the 1:50,000 plate. *Allow these plates to grow until colonies have formed and their colors can be clearly distinguished (2-3 days at 30°C; 3-4 days at room temperature).*

   Plates can be left at 30°C for 5 days or room temperature for a week without worry. Placing plates in the fridge for a few days can make colors more vibrant and easier to distinguish, as pigments continue to be formed but the yeast colonies do not grow larger. You can store these in the fridge until after the competitions are complete, and count all of the plates at the same time.

Day 4: Plate mixed cultures onto YPD + G418 agar media.

1. Examine the cultures you inoculated on Day 1. If yeast have settled at the bottom of the tube, gently shake the tube until they are completely resuspended. The liquid sample of yeast (culture) should be dense enough that you cannot see through it, though the culture grown in the highest concentration of caffeine may appear less dense than the others.
2. Prepare a 1:10,000 dilution of each mixed yeast culture (0mM, 20mM, 40mM caffeine) via serial dilution. For instance, for the YPD + G418 media condition, fill two microcentrifuge tubes with 990ul water and label them "YPD + G418 dilution 1" and "YPD + G418 dilution 2". In the tube labeled "YPD + G418 dilution 1", add 10ul of mixed culture, and vortex for 5 seconds or invert 10 times to mix. Transfer 10ul from "YPD + G418 dilution 1" to "YPD + G418 dilution 2" and vortex for 5 seconds to mix. You should have 3 tubes of "dilution 2", one for each concentration of caffeine (0mM, 10mM, 20mM)
3. Prepare a 1:50,000 dilution of each mixed yeast culture (0mM, 10mM, 20mM caffeine) via serial dilution. Label a tube "dilution 3" and the concentration of caffeine, fill it with 400uL water, add 100uL of "dilution 2", and vortex for 5 seconds or invert 10 times to mix. You should have 3 tubes of "dilution 3", one for each concentration of caffeine (0mM, 10mM, 20mM).
4. Obtain six YPD + G418 agar plates. Label one "Competition in 0mM caffeine, 1:10,000", and another "Competition in 0mM caffeine, 1:50,000." Repeat this for 20mM and 40mM caffeine for a total of 6 plates, 2 per concentration. Label with your classroom number to tell your competitions apart from other classes!
5. Use a sterile plate spreader or glass beads to spread 150ul from "dilution 2" onto the 1:10,000 dilution plate for the corresponding concentration of caffeine, and 150uL "dilution 3" onto the 1:50,000 plate. Repeat for each concentration. *Allow these plates to grow until colonies have formed and their colors can be clearly distinguished (2-3 days at 30°C; 3-4 days at room temperature). Plates can be left at 30°C for 5 days or room temperature for a week without worry. Placing plates in the fridge for a few days can make colors more vibrant and easier to distinguish, as pigments continue to be formed but the yeast colonies do not grow larger.*

Day 7: Calculate ratio of colors among CFUs as a proxy for relative fitness.

1. For each condition (before competition, competition in 0mM, competition in 10mM, competition in 20mM), determine which of the two plated dilutions (1:10,000 or 1:50,000) is easier to count. A plate with ~100 colonies is reasonable: if the colonies are too close together, it is very challenging to count, but if there are only a few colonies, it is not as informative. You do not have to use the same dilution for each condition, since we are interested in the *relative* number of colonies of each color, not the absolute number.
2. Count the number of colonies on each selected plate. Remember to count from the initial mixture that you plated on Day 2, as well as the competitions you plated on Day 3. Use Table 1 to record your colony counts, per directions in Question 1.
3. Follow directions in Questions 1 and 2 to complete the tables.
4. Answer the questions and extension questions.

TABLE 1: Number of colonies

Color						
Name of strain						
Initial mixture						
0mM caffeine competition						
20mM caffeine competition						
40mM caffeine competition						

TABLE 2: Relative abundance of colors

Color						
Name of strain						
Initial mixture						
0mM caffeine competition						
20mM caffeine competition						
40mM caffeine competition						

QUESTIONS

1. Complete Table 1:
    a. Write the color and strain info (Your group or another group's?) for each strain that you mixed for your competition.
    b. In rows 3-6, record the number of colonies you counted for each strain/color on each plate: the initial mixture you plated on Day 2, and the three competitions you plated on Day 4.
2. Complete Table 2:
    a. Write the color and strain info as in Table 1
    b. In rows 3-6, calculate the relative abundance of each strain for each plate. This is calculated as:

        number of colonies of color A / total colonies

        So if on your initial plate you counted 57 blue (color A) colonies, and 109 total colonies (including blue), the relative abundance of blue colonies is:

        57 blue / 109 = 0.52

        The relative abundances of each color should add to approximately 1 for each plate.
3. Do you see an equivalent number of colonies of each color on the plate you made on Day 2? What does this tell you about your initial mixture of the yeast strains?
4. Does the relative abundance of each color strain differ across the three media conditions you plated from on Day 3?
5. Which strain had the greatest increase in relative frequency from the before competition plate (Table 2 row 3) to the after competition in 0mM caffeine plate (Table 2 row 4)? What does this tell you about the fitness in the absence of caffeine, when the selection pressure has been removed?
6. Which strain had the greatest increase in relative frequency from the before competition plate (Table 2 row 3) to the after competition in 40mM caffeine plate (Table 2 row 6)? What does this tell you about the fitness in the presence of caffeine, when the selection pressure is maintained? (If no strains grew in the 40mM competition, answer this for the 20mM competition, Table 2 row 5!)

EXTENSION QUESTIONS

1. How does your evolved yeast strain compare to those of other groups? Did it do better or worse than other groups'? How do you know?
2. Do yeast with a high fitness in one environment (presence of caffeine) always have a high fitness in other environments (absence of caffeine)? Use data from the competitions to support your answer.
3. Do all yeast adapted to the same environment (evolved in the same dose of caffeine) have the same fitness in that environment? Use data from the competitions to support your answer.

CONTINUED INSTRUCTIONS FOR MULTI-CLASS COMPETITION

SELECT CLASSROOM WINNERS

1. Select the strain that won each classroom's competition. If they are all different colors, continue with the next section! If not, follow the remaining instructions in this section.
2. First choose the strain that won by the largest margin (largest increase in frequency from pre-competition mixture to post-competition mixture).
3. Move on to the next classroom and pick the strain of a different color that won by the largest margin. Continue for the other two classes to get four strains of different colors.

COMPETE CLASSROOM WINNERS

This could be done by one classroom, or by a few student volunteers

1. Before the lab: prepare 3 tubes - 1 each containing 2mL of YPD+G418, 2mL 20mM caffeine (1mL YPD+G418 mixed with 1mL YPD+G418+40mM caffeine), and 2mL YPD+G418+40mM caffeine.
2. Day 1: Follow the instructions for Day 1 of the main protocol, using the four classroom winner strains. You will mix together 20uL of each for a total of 80uL mixture. You will have three tubes for the competition (0, 10, 20mM caffeine) and two plates of the initial mixture (1:10,000 and 1:50,000 dilutions).
3. Day 4: Follow the instructions for Day 4 of the main protocol to plate each competition. You will have six plates - two dilutions (1;10,000 and 1:50,000) for each of the three competitions (0mM, 10mM, 20mM caffeine).
4. Day 7: Count the colonies of each color, and record in the table below. Calculate relative frequencies as in Question 2 instructions.
5. Determine which strain is the overall winner, with the largest increase in relative frequency from before competition to after competition in 20mM caffeine!

TABLE 3: Multi-class competition results

	Number of colonies				Relative frequency			
Color								
Classroom								
Initial mixture								
0mM caffeine competition								
10mM caffeine competition								
20mM caffeine competition								