UNIVERSITY OF CALIFORNIA SAN DIEGO

Towards Quantifying Eco-evolutionary Dynamics in a Two-Species Microbial Community

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by

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The Thesis of Elissa Heredia is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Towards Quantifying Eco-evolutionary Dynamics in a Two-Species Microbial Community

by

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Master of Science in Biology

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One important problem in ecology and evolution is how species interactions affect the evolution of community members, and vice versa, how evolution affects species interactions. To address this problem, researchers observe species interactions and their evolution in simplified model communities in the lab. A previous study carried out in a model two-species microbial mutualism between yeast *Saccharomyces cerevisiae* and alga *Chlamydomonas reinhardtii* found that a single adaptive mutation in yeast can affect yields of both species. My thesis builds on this result in two directions. First, to better understand these effects, I developed an experimental approach for estimating birth and death rates of both species in monoculture. Once extended to

co-cultures, this approach will allow us to study how individual adaptive mutations affect basic life-history traits. Second, I helped carry out a long-term evolution experiment in the yeast-alga community in two conditions, one where yeast is obligately dependent on the alga and another where the dependence is facultative. I found that during the course of evolution, yields of both species repeatably increased in the facultative condition but the changes were more variable in the obligate condition. This work, together with the ongoing sequencing efforts, will help us understand the conditions in which co-evolution is more or less repeatable at different levels of biological organization.

Introduction

Evolution is driven by a mixture of deterministic and stochastic processes or "forces". While natural selection is a deterministic force, evolutionary outcomes are also historically contingent, i.e., they depend on stochastic past events, such as environmental changes or mutations [*Blount et al, 2018*]. Thus, a major question in evolutionary biology is how repeatable evolution is. As Stephen J. Gould of formulated it, if we could "replay life's tape" on Earth, would we see the same life forms evolve the way we know life has evolved [*Gould, 1991*]? Gould's question spurred many investigations in natural and experimental systems (reviewed in [*Venkataram & Kryazhimskiy, 2023; Lässig et al, 2017; Stern & Orgogozo, 2009; de Visser, 2014; Nosil et al, 2020*]).

Studies of individual species in their natural environment give us insights into how the species had evolved in the past [*Venkataram and Kryazhimskiy, 2023; Blount et al, 2018; Lenski, 2017*]. Many such study systems are based on vertebrate species where evolutionary responses rely on existing genetic variation [*Blount et al, 2018*]. For example, researchers tested how differences in predation effects evolution of guppies and found that while some details vary, similar life-history traits evolve in different populations [*Reznick & Bryga, 1987*]. Another experiment included how 3 populations of the invasive lizard, the brown anole (*Anolis sagrei*), adapted to their invasion in the Southeastern United States finding that each population developed similar adaptation patterns to better acclimate to their new environment [*Kolbe et al, 2014*]. However, studying evolutionary repeatability in natural systems is difficult, primarily because no two populations in nature live in strictly identical conditions. This problem can be overcome by studying evolution in a laboratory systems [*Venkataram & Kryazhimskiy, 2023*].

In the lab, researchers typically observe evolution in short-lived, microbial model organisms whose overall population sizes can reach billions of individuals [*Friedman & Gore, 2017; Jessup et al, 2003; Altermatt et al, 2014*]. These microbial model organisms provide a simplified system that allows for the development of a quantitative understanding of how they function and evolve [*Friedman & Gore, 2017; Fredrickson, 2015*]. Commonly used model organisms are the bacteria *Escherichia coli* and yeast *Saccharomyces cerevisiae* [*Venkataram & Kryazhimskiy, 2023; Lenski et al, 1998; Lenski 2017*], among others. These studies have allowed researchers to conduct many evolution experiments, such as Richard Lenski's long term evolution experiment in *E. coli* growing over several decades. This experiment began in 1988 with 12 populations of *E. coli* starting from the same ancestral strain and allowed to propagate to this day. Lenski and his team have found that some traits evolve convergently while other diverge. For example, all populations evolved larger cells, but the exact volume increase and cell shape varied among populations [*Lenski, 2017*].

Most studies of the repeatability of evolution focus on individual species [*Lenski, 2017; Reznick & Bryga, 1987; Kolbe et al, 2014*]. However, in nature, most species belong to ecological communities, where they interact, evolve and adapt not only to the abiotic environment but also to one another. Thus, more recently, researchers began asking how repeatable evolution is at community level properties, such as diversity, composition, etc. [*Venkataram & Kryazhimskiy, 2023; Friedman & Gore, 2017*]. Since addressing this problem in natural system is challenging, some researchers choose to study the repeatability of species coevolution in the lab [*Venkataram & Kryazhimskiy, 2023; Friedman & Gore, 2017; Hom &Murry, 2014; Venkataram et al, 2023; Lawrence et al, 2012; Hu et al, 2022*]. The systems chosen in the lab often are simplified to two or a handful of species to allow researchers to study

specific species interactions in controlled environments instead of attempting to observe complex species interactions in the natural environment, providing expectations of behaviors when these complex systems are eventually studied [*Venkataram et al, 2023; Hom & Murry, 2014*]. Simplified species interaction models in the laboratory also provide insight into how species evolution affects interactions within the community [*Lawrence et al, 2012*]. For example, if one species adapts to environmental changes, they may use another species' by-product, leading to a more efficient, stable community [*Friedman & Gore, 2017*].

There are several different types of species interactions [*Friedman & Gore, 2017*]. Some interactions are antagonistic, such as predatory and parasitic, where one species grows at the expense of another [*Friedman & Gore, 2017; Holland and DeAngelis*]. Other interactions are more beneficial where both or one species benefit [*Holland & DeAngelis, 2010*]. One interesting species interaction is a mutualism where both species benefit due to proximity to one another and both species overall benefiting from their interaction [*Hom & Murry, 2014*]. Mutualisms are often found in stable environments, as this relationship can be fragile and can be affected but environmental change or a change between the interacting species [*Rodrigues et al, 2020*]. Furthermore, species relationships can be density-dependent. For example, as a population's density changes, the relationship between species may change from a mutualistic to competitive or vice versa [*Holland & DeAngelis, 2010*].

While antagonistic interactions have been extensively studied, mutualisms have been somewhat overlooked. Yet, mutualisms are very common [*Bronstein, 2015; Kehe et al, 2021*] Strong mutualisms are thought to stabilize communities by preventing extinctions and promoting diversity at the community level [*Friedman & Gore, 2017; Korolev & Nelson, 2011*]. Mutualisms found in nature such as fig trees and wasps and plants and mycorrhizal fungi are

studied and have been established for a long time [*Venkataram & Kryazhimskiy, 2023; Herre et al, 1996; Jousselin et al, 2003; Rønsted et al, 2005; Cruaud et al, 2012; Segar et al, 2013; Maherali et al, 2016; Frederickson, 2017*]. These well-established mutualisms provide avenues for making general predictions about mutualisms [*Hale & Valdovinos, 2021*], however it is not known exactly how mutualisms originate. It is very difficult to study the beginning this relationship type in nature because it may unfold over thousands of generations [*Hom & Murry, 2014*]. To understand how mutualisms form and whether this process is highly repeatable, researchers recently developed various model systems where microbial species known to produce certain by-products (nutrients) are observed in the laboratory [*Hom & Murry, 2014; Friedman & Gore, 2017; Gore & van Oudenaarden, 2009*]. Under certain conditions, these species can establish cooperation, where they promote the other's growth and eventually utilize each other's by-products [*Hom & Murry, 2014; Friedman & Gore, 2017; Lawrence et al, 2012*].

Our lab uses the model community formed by yeast *Saccharomyces cerevisiae* and alga *Chlamydomonas reinhardtii* [*Hom & Murry, 2014*]. They form a mutualistic relationship when there is limited gas exchange between the surrounding air and culture, and limited supplemental nutrients – nitrite and glucose – in the media. Under these conditions, the alga produces ammonia which yeast utilizes as a nitrogen source while yeast produces carbon dioxide alga utilizes as a carbon source [*Hom & Murry, 2014*]. In this limited resources system, the budding yeast and alga first compete for ammonium, but as it is depleted, the species rely on each other's by-products to survive, characterized as competitive mutualism [*Venkataram et al, 2023*]. Using this model system, we are interested in addressing the question of the repeatability of community evolution.

What mutations within one species lead to the changes seen at the community level? Are these mutations repeatable among similar species communities? Will communities constantly evolve to have the same mutations arise within individual species? All of these questions led to the development of both projects within my research.

The first chapter of my thesis is motivated by the question of *How do adaptive mutations in one species affect the ecological interactions in the community?* To address this question, we need to be able to quantify how species' vital rates (births and deaths) change during their growth cycle. While the ultimate goal is to estimate these rates for both species simultaneously when they grow as a community, here I make the first step towards this goal by developing a method for estimating birth and death rates of yeast and alga in monocultures throughout the 5-day growth. In order to achieve this goal, I carry out time-resolved measurements of the density of live cells and the density of cell-like particles which includes both live and dead cells.

In the second chapter of my thesis, I will address the question: what is the repeatability of evolution at different levels of biological organization? To address this question, former postdoc in the lab Sandeep Venkataram and I set up a long-term yeast-alga co-evolution (YAC) experiment. We set up 20 co-cultures in each of two treatments (40 co-cultures total) over the course of about 1,000 generations. One treatment referred to as "Obligate" lacks supplemental ammonium in the media, fostering an environment where the yeast cannot grow on its own and depends on the alga for ammonia production. The second treatment referred to as "Facultative" has a small amount of supplemental ammonium in the media, fostering an environment where yeast can grow on its own and does not depend on the alga for ammonia production, at least in the initial phase of the growth cycle. We estimated how the yields of both species (i.e., final cell densities at the end of 3-day growth cycle) changed throughout the evolution and observed how

these yield trajectories differed between replicates and between the two treatments. To study evolutionary repeatability at the genome level, I have extracted genomic DNA from 12 time points from 20 evolving communities (10 from the Obligate treatment and 10 from the Facultative treatment) and prepared Illumina sequencing libraries. Sequencing and analysis of these data are still ongoing.

Chapter 1. Estimating birth and death rates in microbial monocultures

Introduction

Birth and death are fundamental processes that govern population dynamics. Thus, they ultimately determine whether a population survives or goes extinct. As such, they are subject to natural selection. Previous work has shown that even a single mutation in one organism can have a dramatic effect on the population dynamics in an ecological community [*Korolev & Nelson, 2011*]. Specifically, Venkataram et al have shown that mutations in yeast can affect how competitive or cooperative the yeast is and thereby influence the yields of both species [*Venkataram et al, 2023*]. However, the same total yield can be a result of very different life-history strategies. For example, a type that grows fast initially when nutrients are abundant but also dies rapidly during starvation could have the same yield as a type that grows continuously and slowly throughout the entire growth-dilution cycle. It is unclear which vital rates were affected by adaptive mutations observed by Venkataram et al to achieve the observed changes in yield. To determine that, we need to be able to measure birth and death rates in our study organisms.

Measuring birth and death rates in microbes is difficult. There have been several attempts to quantify these rates throughout a growth in a single species system. In one such study, Hart et al quantified birth and death rates in fluorescent *Saccharomyces cerevisiae*, distinguishing live and dead cells using a fluorescent reporter [*Hart et al, 2019*]. We address this problem by developing a different approach for obtaining time-resolved estimates of birth and death rates in microbial monocultures that does not require fluorescence. The basic idea of the method is that dead cells do not disintegrate immediately and therefore can be registered in a

particle counter alongside live cells, whereas only live cells can form colonies. Thus, by comparing the counts of total and live cells, we can estimate the number of dead cells at any point in time. Then, using such temporally resolved live and dead cell counts and a simple model of the birth-death process, we infer the underlying birth and death rates. We applied these methods to both yeast and alga monocultures growing in the minimal medium CYM. To estimate birth and death rates in a microbial monoculture, we grew a microbial population (yeast or alga) over a 5-day period and measured the total number of cell (using a particle counter) and the number of live cells (using plating on solid agar; see Materials and Methods). We then applied equations (3)–(5) in Materials and Methods to estimate how the birth and death rates changed over the growth cycle.

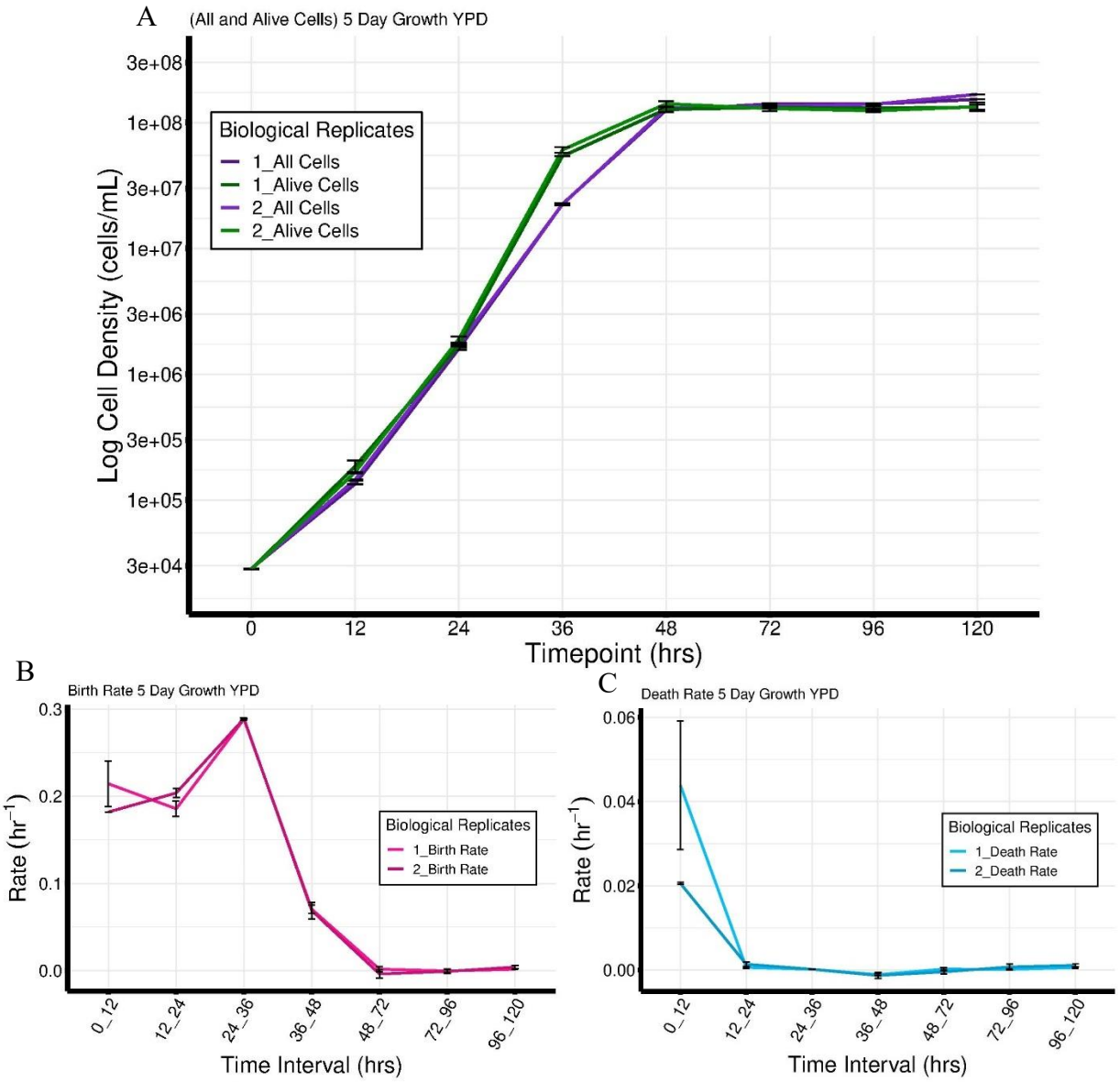

Figure 1: Total and alive cell densities with birth and death rates of yeast in the rich medium YPD. A. Total density of yeast cells (both live and dead together, purple) measured by a Coulter counter and the density of live yeast cells (green) measured via plating and colony counts transformed in log10. **B.** Birth rates estimated via equation (5). **C.** Death rates estimated via equation (4). In all panels, lines of the same color represent two biological replicates. Two technical replicates per biological replicate are shown as error bars.

Results

Yeast growth dynamics in rich medium

It is possible that plating efficiency is less than 100%, i.e., not every live cell forms a visible colony on solid agar. To test for plating efficiency, we grew yeast in the rich YPD medium where cell death should be negligible during exponential growth. Therefore, the total and live cell densities should be very similar to one another in this rich medium.

We found that during the first 24 hours of growth, when the yeast population grows exponentially, cell densities measured by the particle counter and by plating are almost the same, as expected (Figure 1A). Moreover, the two estimates remain essentially indistinguishable from one another for the first 72 hours, well after the point where growth ceases. These observations strongly suggest that our yeast plating efficiency is in fact close to 100%.

Interestingly, we find that after 72 hours, live cell densities begin to decline slightly whereas total cell densities continue to increase suggesting that population turnover continues in these nutrient-replete conditions albeit at a vastly decreased rate (Figure 1B and 1C).

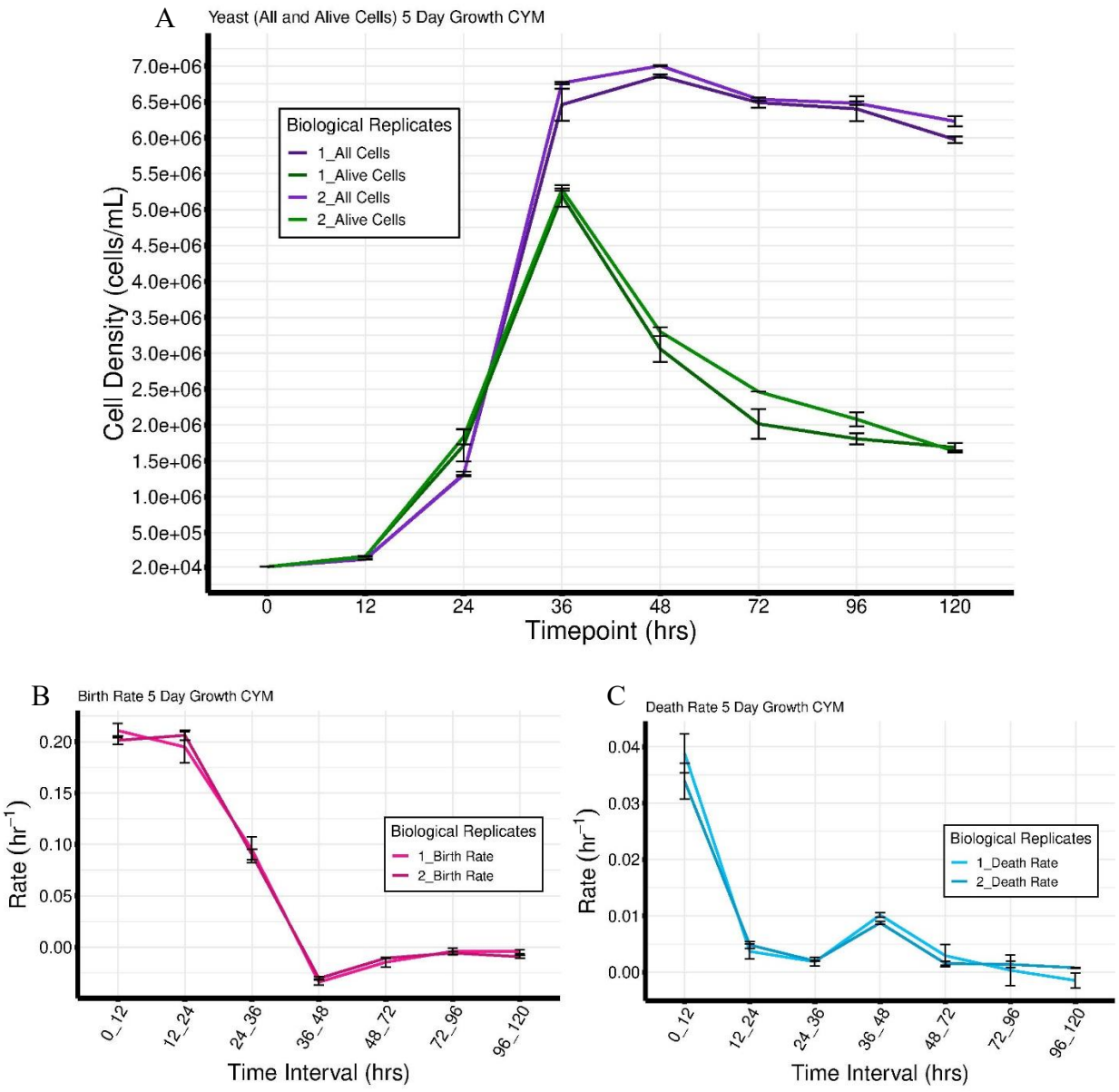

Figure 2: Total and alive cell densities with birth and death rates of yeast – alone in CYM. **A.** Total density of yeast cells (both live and dead together, purple) measured by a Coulter counter and the density of live yeast cells (green) measured via plating and colony counts. **B.** Birth rates estimated via equation (5). **C.** Death rates estimated via equation (4). In all panels, lines of the same color represent two biological replicates. Two technical replicates per biological replicate are shown as error bars.

Yeast growth dynamics in minimal medium

Next, we estimated birth and death rates for yeast in the minimal medium CYM. We find that the total cell count tracks the live cell count for approximately the first 36 hours of growth, but these curves dramatically diverge after that (Figure 2A). Live cell count peaks at 36 hours and plumets afterwards whereas the total cell count continues to increase and peaks at 48 hours. Between 48 and 120 hours, total cell count declines by about 13%. The latter observation suggests that dead cells (slowly) disintegrate and are no longer registered by our particle counter.

Next, we used these data to estimate the birth and death rates of the yeast in CYM. The birth rate stays approximately constant during the first 24 hours of the growth cycle and ceases by 36 hours (Figure 2B). In contrast, the death rate spikes during the 36–48-hour interval and declines after that (Figure 2C), suggesting that bulk of cell deaths occur during the transition from rapid exponential growth to stationary phase.

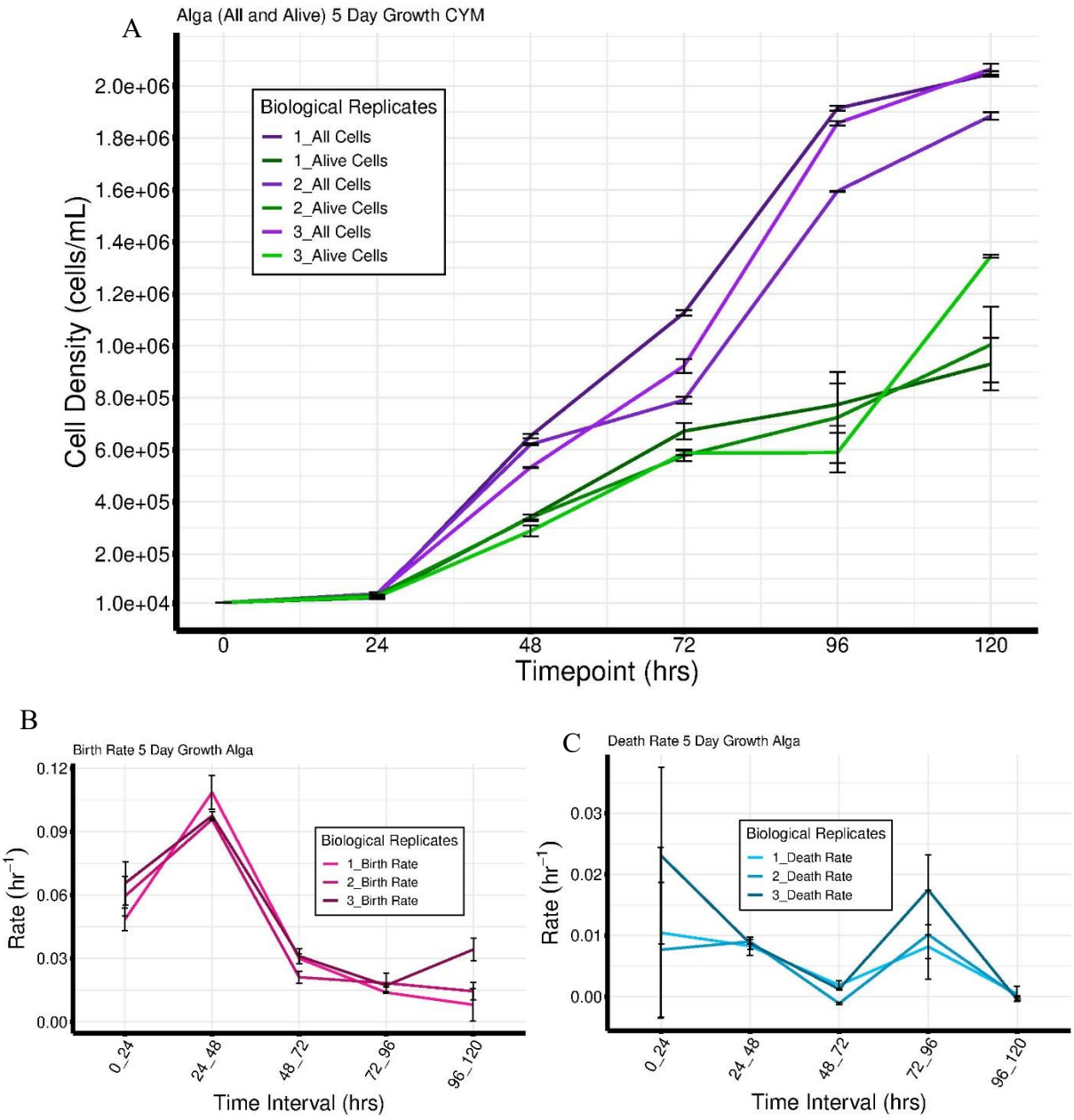

Figure 3: Total and Alive cell densities with birth and death rates of alga – alone in CYM. **A.** Total density of yeast cells (both live and dead together, purple) measured by a Coulter counter and the density of live yeast cells (green) measured via plating and colony counts. **B.** Birth rates estimated via equation (5). **C.** Death rates estimated via equation (4). In all panels, lines of the same color represent three biological replicates. Two technical replicates per biological replicate are shown as error bars.

Alga growth dynamics in minimal medium

Next, we estimated birth and death rates for alga in the minimal medium CYM. We find that the total cell count is steady with the live cell count for about the first 48 hours of growth, but the curves diverge afterwards (Figure 3A). Live cell count continues to increase after 48 hours to the end of the cycle but at a slower rate than the initial 48 hours. Between 24 and 96 hours, the total cell density increases faster than the live cell count, but slows down during the last 24 hours. These observations suggests the dead cell count is also increasing.

We used this data to estimate the birth and death rates of the alga in CYM. The birth rate increases during the first 48 hours of growth, but drastically decreases by 72 hours, remaining low until spiking at the very end (Figure 3B). On the other hand, the death rate decreases, remaining below the birth rate until 72 hours, the peaks at 96 hours and decreases again at the end of the growth (Figure 3C). The observations between 72 and 120 hours of growth demonstrates that as nutrients deplete, there are more deaths occurring than births.

Discussion

We developed a simple method for estimating birth and death rates in microbial monocultures. Using growth in rich medium YPD, we found that plating efficiency is close to 100%. However, we also observed small turn-over in rich medium once the limiting nutrient (carbon) is exhausted. Throughout all experiments, the cell density estimates are reproducible across biological replicates for both species. Interestingly, there is a difference between yeast and alga life history strategies in CYM. Yeast grows quickly and experiences peak death when nutrients are exhausted. Alga grows slower but dies more or less at a constant rate throughout the growth.

As expected in yeast, once the population reached the stationary phase, the overall growth rate declined rapidly, likely due to the media nutrients depleting as the cell density increases. The decline in growth based on nutrient availability was also seen in the Hom and Murry paper in multiple co-cultures of yeast and alga in media with varying levels of key nutrients – glucose for yeast and nitrite for alga. In cultures with less glucose, the yeast did not grow very well whereas in cultures with less nitrite available, the alga grew poorly [*Hom & Murry, 2014*]. The growth rate dependability upon nutrient availability in limiting media was also seen in another study with several bacteria species grown together finding that when the bacteria were in a limiting environment, they would work together, utilizing different nutrients and metabolizing waste products [*Lawrence et al, 2012*]. Nutrient availability affects species whether they are grown in isolation from other species or grown in communities.

In calculating the birth and death rates, our estimators work reasonably well both during exponential and stationary phases. However, several birth rate estimates are negative in the stationary phase, which is not biologically possible. This indicates that our estimator can be

improved by more accurately incorporating measurement errors. A similar problem was noted by Hart et al who's involved utilizing nutrient availability and fluorescent measurements to determine the death and birth rates, however, they found that depending upon their counting method, the rates would change from one another [*Hart et al, 2019*]. These methods overall need further development to estimate birth and death rates more accurately.

In the future, the next step would be to grow the yeast and alga in the CYM media together using the same methods to measure all and alive cell densities of both species. There would most likely be a need to test other methods that would help organize the measured cells by species and cell category. Such methods include sequencing, fluorescence for the alga as alga has chlorophyll that can be measured and cell counting by the hemocytometer. The yeast and alga cells are physically different from one another with yeast being smaller than alga, alga being green and being able to swim throughout the media. Introducing these methods to untangle the co-cultures would provide their own challenges but may provide valuable information for either or both species.

Materials and Methods

Media

YPD (Yeast extract, Peptone, Dextrose)

Prepare one bottle of YPD (Yeast extract, Peptone, Dextrose) with 10g yeast extract and 20g peptone in 900mL of nanopure (Milli-Q) water. Prepare a second bottle with 20g Dextrose in 100mL of nanopure water. Autoclave both bottles, mix them together and store at room temperature.

YPD-Agar Plates (Yeast extract, Peptone, Dextrose-Agar Plates)

Prepare one bottle of YPD-Agar Plates (Yeast extract, Peptone, Dextrose-Agar Plates) with 10g yeast extract, 20g peptone, and 20g agar in 900mL of nanopure water. Prepare a second bottle with 20g Dextrose in 100mL of nanopure water. Autoclave both bottles, mix them together and pour ~20mL of volume per plate. Allow plates to solidify before storing upside-down at room temperature.

0.5mM NH4+ CYM (Chlamydomonas-Yeast Medium)

Prepare one bottle of 0.5nM NH4+ CYM (Chlamydomonas-Yeast Medium) with 40mL 25X CYB Salts (or 1mL Calcium, 1mL magnesium, and 1mL Trace Elements (A red liquid stored at 4°C)), 10mL 1M KNO2 (potassium nitrite), 133uL 3.74M NH4+, and 900mL of nanopure water. Autoclave, allow to cool to 60°C, then add 50mL 40% Dextrose, 1mL 1000X Phosphate Stock (P Stock stored at room temperature), and 1mL 1000X Vitamin Stock (stored at -80°C). Store at room temperature.

TAP (Tris-Acetate-Phosphate)

Prepare one bottle of TAP (Tris-Acetate-Phosphate) with 40mL 25X CYB Salts (or 1mL calcium, 1mL magnesium, and 1mL trace elements (A red liquid stored at 4°C)), 5mL 200x tris-

acetate, 4mL 250x nitrogen stock, and 950mL of nanopure water. Autoclave, allow to cool to 60°C, then add 1mL 1000X Phosphate Stock (P Stock stored at room temperature).

TAP-Agarose (Tris-Acetate-Phosphate-Agarose)

Prepare one bottle of TAP (Tris-Acetate-Phosphate) with 40mL 25X CYB Salts (or 1mL calcium, 1mL magnesium, and 1mL trace elements (A red liquid stored at 4°C)), 5mL 200x tris-acetate, 4mL 250x nitrogen stock, 10g agarose, and 950mL of nanopure water. Autoclave, allow to cool to 60°C, then add 1mL 1000X Phosphate Stock (P Stock stored at room temperature). Pour 15-20mL of volume per plate, allow to solidify, and store upside-down at room temperature.

Species and Strains

CC1690 Alga (Chlamydomonas reinhardtii) Revival from LN2 Stocks

Day 1 Using freezer gloves, remove 2mL cryotube stock of Algae from LN2 tank and thaw in cold water. Centrifuge tube at 750xg for 3 minutes, remove supernatant to remove methanol and resuspend pelleted cells in 1mL PBS. Inoculate algae into 50mL of TAP media in 250mL non-baffled flask with a cap. Cover entire flask in foil and place on shaker at room temperature at 125rmp.

Day 2 Remove flask from shaker and dilute culture 1:100 into growth media – typically 50mL 0.5mM NH4+ CYM in 250mL non-baffled flask with a cap. Can also grow in 10mL 0.5mM NH4+ CYM in 50mL non-baffled flask with cap. – Place back on shaker at 125rmp in light with no foil covering the new flask.

Day 7 If adding other strains or yeast, thaw from freezer and inoculate into a separate flask.

Day 12 Culture should be saturated or close to saturation. Inoculate algae at 10^4 cells/mL into growth media under experimental conditions. Yeast/other strains diluted 1:100 into growth media under experimental conditions.

Day 17 Initial precultures are complete. Dilute yeast and algae into second precultures both alone and in community as appropriate for the experiment. Inoculate algae at 10^4 cells/mL and dilute yeast 1:100.

Day 22 Dilute precultures 1:100 to begin measurements from this day and onwards. Can keep Day 2 flasks for 2 weeks then dilute 1:100 and keep freshly diluted flasks for 2 more weeks. After this, bleach all algae stocks and start new revival if needed.

Yeast (Saccharomyces cerevisiae) Revival from -80 Freezer

Using freezer gloves, remove 1mL cryotube from -80°C freezer. Under a flame to prevent contamination, scrape top of frozen cells with 100uL pipette tip (if cells have melted, take a small volume from the tube). Place cells into 20uL PBS and place 1mL cryotube into freezer. Inoculate 20uL PBS plus yeast cells into growth medium of choice (50mL 0.5mM NH4+ CYM in 250mL non-baffled flask with cap or 50mL YPD in 250 non-baffled flask with cap). Allow to grow until preculture has reached or is near saturation. Dilute 1:100 or to 10^4 cells/mL can begin experiment.

Experimental Designs

5-Day Yeast-Alone in YPD

Before measurements Make preculture of 50mL YPD media in a 250mL flask (with a cap) with yeast. Allow to grow for ~5 days/ until fully saturated. Place preculture flask on shaker under lights at 125rpm to mimic Yeast-Alga growth together.

Day 0 Morning Take a new, clean, autoclaved 250mL flask (with a cap) and place 50mL of YPD into flask with volume of preculture diluted to ~10^4 cells/mL. (Used particle counter on the yeast preculture to determine how much volume of preculture to add to the fresh YPD media.) Place flask on shaker under lights to mimic Yeast-Alga growth together.

Day 0 Night After ~12 hours of growth, take 1mL sample from growth flask for particle counter measurements and plate 100uL at 1:80 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 1 Morning After ~24 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:700 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 1 Night After ~36 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:11,200 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 2 Morning After ~48 hours of growth, take 10uL sample for particle counter and plate 100uL at 1:60,000 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Image Day 0, 12 hour plates and count colonies grown.

Day 3 Morning After ~72 hours of growth, take 10uL sample for particle counter and plate 100uL at 1:70,000 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Image Day 1, 24 hour plates and count colonies grown.

Day 4 Morning After ~96 hours of growth, take 10uL sample for particle counter and plate 100uL at 1:70,000 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Image Day 1, 36 hour plates and count colonies grown.

Day 5 Morning After ~120 hours of growth, take 10uL sample for particle counter and plate 100uL at 1:50,000 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Image Day 2, 48 hour plates and count colonies grown. In the following days, image Day 3, 72 hour plates image Day 4, 96 hour plates and image Day 5, 120 hour plates. Count colonies grown for each plate. Bleach cultures when experiment is complete.

5-Day Yeast-Alone in CYM

Before Measurements Make preculture of 50mL CYM media in a 250mL flask with yeast. Allow to grow for ~5 days/ until fully saturated. Place preculture flask on shaker under lights at 125rpm to mimic Yeast-Alga growth together.

Day 0 Morning Place 50mL of CYM into new, cleaned, autoclaved 250mL flask and add volume of preculture diluted to ~10^4 cells/mL. (Used particle counter on the yeast preculture to determine how much volume of preculture to add to the fresh CYM media). Place flask on shaker at 125rmp under lights to mimic Yeast-Alga growth together.

Day 0 Night After ~12 hours of growth, take 1mL sample for particle counter and plate 100uL at 1:70 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 1 Morning After ~24 hours of growth, take 1mL sample for particle counter and plate 100uL at 1:600 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 1 Night After ~36 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:1,600 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 2 Morning After ~48 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:3,000 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Image Day 0, 12 hour plates and count colonies grown.

Day 3 Morning After ~72 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:1,600 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Image Day 1, 24 hour plates and count colonies grown.

Day 4 Morning After ~96 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:1,600 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to not disturb culture growth. Image Day 1, 36 hour plates and count colonies grown.

Day 5 Morning After ~120 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:800 dilution on a YPD plate. Place plates in 30C incubator. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Image Day 2, 48 hour plates and count colonies grown. In the following days, image Day 3, 72

hour plates, Day 4, 96 hour plates and Day 5, 120 hour plates. Count colonies grown for each plate. Bleach all cultures when experiment is complete.

5-Day Alga-Alone in CYM

Before Measurements Make preculture of 50mL CYM media in a 250mL flask with 1mL of live alga stock. Allow to grow for ~5 days/ until fully saturated. Place preculture flask on shaker under lights at 125rpm to mimic Yeast-Alga growth together.

Day 0 Morning Place 50mL of CYM into new, clean, and autoclaved 250mL flask with volume of preculture diluted to ~10^4 cells/mL (~500uL). (Used particle counter on the alga preculture to determine how much volume of preculture to add to the fresh CYM media). Place flask on shaker at 125rpm under lights to mimic Yeast-Alga growth together.

Day 1 After ~24 hours of growth, take 1mL sample for particle counter and plate 100uL at 1:15 dilution on a TAP plate. Place plates on the bench under lights. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 2 After ~48 hours of growth, take 1mL sample for particle counter and plate 100uL at 1:300 dilution on a TAP plate. Place plates on the bench under lights. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 3 After ~72 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:400 dilution on a TAP plate. Place plates on the bench under lights. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 4 After ~96 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:900 dilution on a TAP plate. Place plates on the bench under lights. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed.

Day 5 After ~120 hours of growth, take 100uL sample for particle counter and plate 100uL at 1:100 dilution on a TAP plate. Place plates on the bench under lights. Place the flask back onto the shaker immediately after removing samples to limit time cultures are disturbed. Two weeks after starting Day 1 Plates, image Day 1, 24 hour plates and count colonies grown. In the following days, image Day 2, 48 hour plates, image Day 3, 72 hour plates, image Day 4, 96 hour plates and image Day 5, 120 hour plates. Count colonies grown for all plates. Bleach all cultures when experiment is complete.

Model of growth for inferring birth and death rates

Equations for inferring birth and death rates

We model the growth process deterministically as follows. If n_t is the number of live cells at time t, we assume that at any point in time, the population grows or shrinks exponentially with rate $r = b - d$, where b and d are the per capita birth and death rate, respectively. Mathematically,

$$n_t = n_0 e^{rt}, \tag{1}$$

where n_0 is the initial number of live cells. Assuming that dead cells do not disappear, the number of dead cells k_t changes with time according to the differential equation

$$\dot{k}_t = d n_t,$$

whose solution is

$$k_t = k_0 + \frac{d n_0}{r}(e^{rt} - 1), \tag{2}$$

where k_0 is the initial number of dead cells. From equation (1), we obtain the estimate of the growth rate as

$$\hat{r} = \frac{1}{t} \ln \frac{n_t}{n_0}. \tag{3}$$

To estimate death rate, we can first estimate the current number of dead cells as $k_t = m_t - n_t$, where m_t is the combined total number of live and dead cells at time t. Then, using equation (2), we obtain the estimate of the death rate as

$$\hat{d} = \hat{r}\frac{k_t - k_0}{n_0(e^{\hat{r}t} - 1)}, \tag{4}$$

and we estimate the birth rate as

$$\hat{b} = \hat{r} - \hat{d}. \tag{5}$$

Measurements for Estimating Birth and Death Rates

Particle (Coulter Counter) – Total Cell Density

Total cell density was measured using the particle (coulter) counter and multiplying the measurement by the dilution factor. Under the flame, take a separate cuvette and fill with 10mL *buffer* to serve as the blank. Take a sample of the liquid culture (typically 10uL for high density cultures but adjust for expected lower density cultures) and place in a cuvette filled with 10mL of *the buffer*. Invert the blank cuvette, evenly dispersing particles, place in the machine and have probe in the cuvette. Press start, allowing machine to count particles. The coulter counter counts particles – assumed to be cells – by taking up a sample of the buffer through the aperture and counting the disruption the cell causes in the current. The blank should be below 50 particles. However, if it is above this threshold, clean the probe and run the blank again or run a new blank. After the blank is read, invert the culture sample cuvette several times and place in the machine with the probe in the cuvette. Press start and record the number of particles counted. Multiply this number by the dilution factor (typically 1000 for samples with 10uL sample volume but adjust if using a different sampled culture volume) to get the total cell density. Discard cuvette samples and clean cuvettes with DI water before placing them upside down to

dry over night. The particle counter is assumed to estimate the total cell density since it does not distinguish between alive and dead cells, counting all particles that pass through the aperture.

Live Cell Plating – Alive Cell Density

Alive cell density was measured by counting the number of colonies that grew on a plate with growth media and multiplying by the appropriate dilution factor. Under the flame, take a sample of the liquid culture and dilute the sample into PBS in appropriate dilution to get approximately 200 cell colonies to grow on the place. (This dilution depends on when in the growth cycle the sample is taken as there are assumed to be more alive cells during the beginning and middle of the growth than there is towards the end.) Under the flame, take a plate with solid media in it and gently spread ~10-20 glass beads on the surface of the media. Place 100uL of diluted culture sample on the surface of the plate, close the lid, and gently shake the plate on the bench to disturb glass beads to spread the cells. After fully spreading cells, allow plate to absorb moisture and shake glass beads into 70% ethanol. Cover plates with lid and place into 30°C incubator for three days to allow for growth. After three days, image plates, count colonies, and multiply by dilution factor to get estimated alive cell density. Live cell plating estimates alive cells based on the assumption that each colony grown on the plate grew from a single alive cell as these cells are assumed to be the only cells that can reproduce.

Inferring Dead Cell Density

Dead cell density cannot be directly measured using the instruments in the lab. Instead it was inferred by subtracting the estimated alive cell density (plating) from the total cell density (coulter counter) at each sampling timepoint. The dead cells are assumed to no longer be able to reproduce and can only increase as the alive cells die, becoming dead cells.

Chapter 2. Long Term Co-Evolution

Introduction

An important question in evolutionary biology is how repeatable evolution is. Many researchers have studied this question in both natural and laboratory systems [*Hom & Murry, 2014; Venkataram et al, 2023; Lawrence et al, 2012; Hu et al, 2022; Herre et al, 1996; Jousselin et al, 2003; Rønsted et al, 2005; Cruaud et al, 2012; Segar et al, 2013; Maherali et al, 2016 Frederickson, 2015*]. However, it remains unclear how species interactions influence the repeatability of evolution at various levels of biological organization, from the genome to community level properties [*Fisher et al, 2019*]. Previous work done by Venkataram et al in the yeast alga system found that adaptive mutations within the yeast had diverse genetic and ecological effects. They also found that in the presence of alga, these ecological effects differed than without alga and altered the mutations fitness benefits. Mutations in one species can affect the overall community while enhancing the relationship between species and altering both species yields [*Venkataram et al, 2023*].

These results suggest that community-level properties, such as species yields, can change repeatably during adaptive evolution. To test this hypothesis, we set up a long-term co-evolution experiment in two conditions, which we refer to as Obligate and Facultative. In the Obligate condition, yeast must rely on the alga for growth and survival, since it is unable to metabolize the only nitrogen source (nitrite) available in the medium [*Hom & Murry, 2014*]. Furthermore, alga also highly benefit from the presence of yeast in this condition, since by metabolizing glucose yeast supplies additional dissolved carbon dioxide, which the alga uses as carbon source. In the Facultative treatment, the yeast and alga still highly benefit from each other [*Venkataram et al,*

2023] but both can survive in each other's absence, due to the addition of ammonium in the media, which is an additional nitrogen source that yeast can metabolize.

To understand how the interaction between the species affects the repeatability of evolution at the community level, we maintained 20 replicate co-cultures in each treatment for about 1,000 generations and measured how the yields of both species change over time.

Results and Discussion

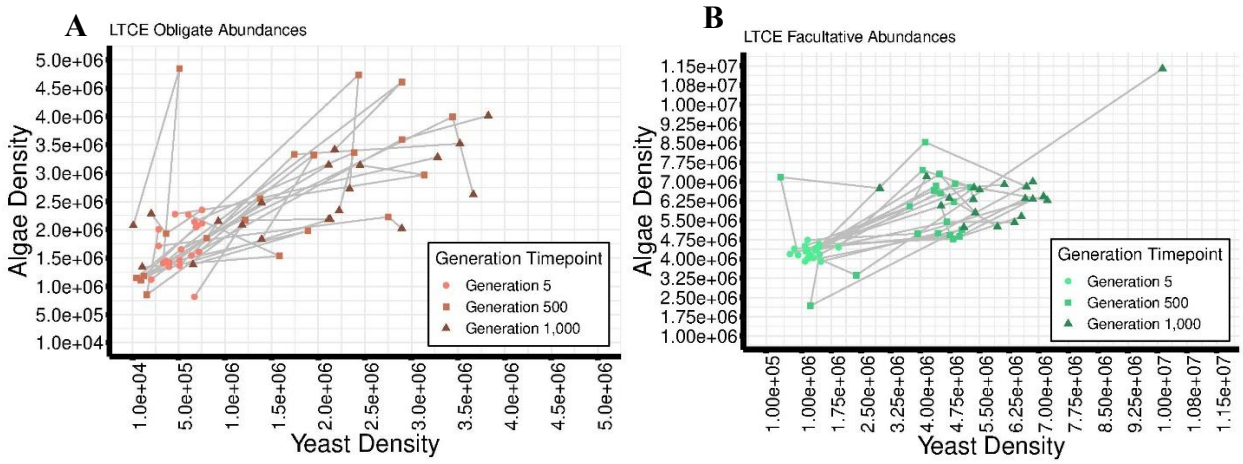

Figure 4: Abundances of yeast and alga during yearlong growth. A. The abundances of yeast and alga in Obligate treatment approximately 5 generations, 500 generations, and 1,000 generations after initial growth by subculturing to propagate each culture. **B.** The abundances of yeast and alga in Facultative treatment approximately 5 generations, 500 generations, and 1,000 generations) after initial growth (Figure 4B). There were 20 biological replicates for each treatment.

Results

To study the repeatability of evolution at the community level, we set up a long-term yeast-alga co-evolution experiment in two treatments. In the obligate treatment, no ammonia is added to the CYM medium, which makes yeast fully dependent on the ammonium provided by the alga [*Hom & Murry, 2014*]. In the facultative treatment, we added 0.5 mM of ammonia to the CYM medium, which allows yeast to grow independently of the alga [*Hom & Murry, 2014*]. In both treatments, alga strongly benefits from the presence of yeast [*Hom & Murry, 2014*]. We propagated 20 replicates of each type of co-cultures (40 co-cultures total) for about 1,000 generations and measured yields of both species, defined as cell densities at the of the growth cycle, approximately every 39-40 generations (see Methods for details).

In the obligate treatment, the initial yeast and alga yields were 5.3×10^5 cells/mL and 1.7×10^6 cells/mL on average, with the measurement noise measured as standard deviation around these means being 1.7×10^5 cells/mL and 4.3×10^5 cells/mL, respectively (Figure 4A). At the end of the co-evolution experiment, the yields of both species have on average increased to 1.9×10^6 cells/mL and 2.5×10^6 cells/mL for and the standard deviation increased to 1.2×10^6 cells/mL and 7.1×10^5 cells/mL yeast and alga, respectively (Figure 4A). In the facultative treatment, the initial yeast and alga yields were1.2×10^6 cells/mL and 4.3×10^6 cells/mL with the measurement noise of 2.7×10^5 cells/mL and 2.2×10^5 cells/mL, respectively (Figure 4B). At the end of the co-evolution experiment, the yields of both species on average increased to 6.0×10^6 cells/mL and 6.5×10^6 cells/mL for yeast and alga respectively and the standard deviation increased to 1.5×10^6 cells/mL and 1.3×10^6 cells/mL (Figure 4B). Thus, evolution led to fairly repeatable yield increases in Facultative co-cultures, consistent with the results of Venkataram et al obtained in a short-term

experiment [*Venkataram et al, 2023*], whereas the yield dynamics in the Obligate co-cultures were more chaotic.

Discussion

These observations suggest that the type of ecological interaction can influence the repeatability of evolution at the ecological level. The mechanistic reasons for why the eco-evolutionary dynamics are less repeatable in the obligate regime are not yet clear. One hypothesis is that yeast and alga growth dynamics are more tightly coupled in the obligate regime and thus their yields are more sensitive to small phenotypic changes that may be induced by mutations that spread and fix in one or both species. Another possibility is that adaptation is faster in the obligate regime, and such that these co-cultures were able to explore a large space of genetic and phenotypic variation than facultative co-cultures. To probe these and other potential explanations for the observed differences in evolutionary repeatability, we are in the process of sequencing these co-cultures.

Overall, this work suggests that the external environment can modulate not only species interactions but, through these interactions, it can also influence the evolutionary dynamics and their repeatability.

Materials and Methods

Media

0mM NH4+ CYM (Chlamydomonas-Yeast Medium)

Prepare one bottle of 0nM NH4+ CYM (Chlamydomonas-Yeast Medium) with 40mL 25X CYB Salts (or 1mL Calcium, 1mL magnesium, and 1mL Trace Elements (A red liquid stored at 4°C)), 10mL 1M KNO2 (potassium nitrite), and 900mL of nanopure water. Autoclave, allow to cool to 60°C, then add 50mL 40% Dextrose, 1mL 1000X Phosphate Stock (P Stock stored at room temperature), and 1mL 1000X Vitamin Stock (stored at -80°C). Store at room temperature.

0.5mM NH4+ CYM (Chlamydomonas-Yeast Medium)

Prepare one bottle of 0.5nM NH4+ CYM (Chlamydomonas-Yeast Medium) with 40mL 25X CYB Salts (or 1mL Calcium, 1mL magnesium, and 1mL Trace Elements (A red liquid stored at 4°C)), 10mL 1M KNO2 (potassium nitrite), 133uL 3.74M NH4+, and 900mL of nanopure water. Autoclave, allow to cool to 60°C, then add 50mL 40% Dextrose, 1mL 1000X Phosphate Stock (P Stock stored at room temperature), and 1mL 1000X Vitamin Stock (stored at -80°C). Store at room temperature.

0.5mM NH4+ CYM-Agarose Plates (Chlamydomonas-Yeast Medium – Agarose Plates)

Prepare one bottle of 0.5nM NH4+ CYM (Chlamydomonas-Yeast Medium) with 40mL 25X CYB Salts (or 1mL calcium, 1mL magnesium, and 1mL trace elements (A red liquid stored at 4°C)), 10mL 1M KNO2 (potassium nitrite), 133uL 3.74M NH4+, 10g agarose and 900mL of nanopure water. Autoclave, allow to cool to 60°C, then add 50mL 40% Dextrose, 1mL 1000X Phosphate Stock (P Stock stored at room temperature), and 1mL 1000X Vitamin Stock (stored at -80°C). Pour 15-20mL of volume per plate, allow to solidify and store upside-down at room temperature.

TAP (Tris-Acetate-Phosphate)

Prepare one bottle of TAP (Tris-Acetate-Phosphate) with 40mL 25X CYB Salts (or 1mL calcium, 1mL magnesium, and 1mL trace elements (A red liquid stored at 4°C)), 5mL 200x tris-acetate, 4mL 250x nitrogen stock, and 950mL of nanopure water. Autoclave, allow to cool to 60°C, then add 1mL 1000X Phosphate Stock (P Stock stored at room temperature).

Strains

CC1690 Alga (Chlamydomonas reinhardtii) Revival from LN2 Stocks

Day 1 Using freezer gloves, remove 2mL cryotube stock of Algae from LN2 tank and thaw in cold water. Centrifuge tube at 750xg for 3 minutes, remove supernatant to remove methanol and resuspend pelleted cells in 1mL PBS. Inoculate algae into 50mL of TAP media in 250mL non-baffled flask with a cap. Cover entire flask in foil and place on shaker at room temperature at 125rmp.

Day 2 Remove flask from shaker and dilute culture 1:100 into growth media – typically 50mL 0.5mM NH4+ CYM in 250mL non-baffled flask with a cap. Can also grow in 10mL 0.5mM NH4+ CYM in 50mL non-baffled flask with cap. – Place back on shaker at 125rmp in light with no foil covering the new flask.

Day 7 If adding other strains or yeast, thaw from freezer and inoculate into a separate flask.

Day 12 Culture should be saturated or close to saturation. Inoculate algae at 10^4 cells/mL into growth media under experimental conditions. Yeast/other strains diluted 1:100 into growth media under experimental conditions.

Day 17 Initial precultures are complete. Dilute yeast and algae into second precultures both alone and in community as appropriate for the experiment. Inoculate algae at 10^4 cells/mL and dilute yeast 1:100.

Day 22 Dilute precultures 1:100 to begin measurements from this day and onwards. Can keep Day 2 flasks for 2 weeks then dilute 1:100 and keep freshly diluted flasks for 2 more weeks. After this, bleach all algae stocks and start new revival if needed.

Yeast (Saccharomyces cerevisiae) Revival from -80 Freezer

Using freezer gloves, remove 1mL cryotube from -80°C freezer. Under a flame to prevent contamination, scrape top of frozen cells with 100uL pipette tip (if cells have melted, take a small volume from the tube). Place cells into 20uL PBS and place 1mL cryotube into freezer. Inoculate 20uL PBS plus yeast cells into growth medium of choice (50mL 0.5mM NH4+ CYM in 250mL non-baffled flask with cap or 50mL YPD in 250 non-baffled flask with cap). Allow to grow until preculture has reached or is near saturation. Dilute 1:100 or to 10^4 cells/mL can begin experiment.

Experimental Design

LTCE Facultative and Obligate in CYM

Transfer Prep – Every 3 Days Label sterilized room-temp 250ml Erlenmeyer flasks with beaker cap with washable marker. Under the flame, loosen caps to easily remove with one hand. Fill each flask with 50mL of fresh media (0mM NH4+ or 0.5mM NH4+).

Transfer - Every 3 Days Remove saturated cultures from the shaker. Vortex each culture briefly (3 seconds) to resuspend the cells. Under a flame, transfer 1ml of culture to fresh media in flask corresponding to culture number. (Transfer immediately after vortex, do not let cultures sit after vortexing). Bring transferred cultures back to the shaker and start the shaker. Ensure the lights are on, shaker is at 125 RPM and no flask will tip or fall over.

New Timepoint Prep - Every 3 Weeks Label 2ml screw cap cryotubes as “Culture ID – Transfer ID” on the side and caps with “Culture ID” for four times as many cultures. Label

1.5ml nonsterile Eppendorf tubes with “Culture ID – Transfer ID” on the caps for two times as many cultures. Label sterile 50ml conical with “Culture ID” for each culture. Prepare 20% glycerol, nonsterile, in a conical tube needing 2ml per culture. Aliquot sterile 40% glycerol into conical tubes needing 2ml per culture. Prepare 6% sterile methanol media in conical tubes needing 2mL per culture. (Use PPE with handling methanol and keep away from flame as it is flammable). Aliquot sterile PBS into conical tube needing 1ml per culture. Under the flame, slightly loosen the caps of all 2ml cryotubes and arrange 0.65ml tubes into tube rack for two times as many cultures. Label 0.65ml tubes “culture ID”. Dispense 300ul PBS to each 0.65ml tube assuming 1:900 final dilution for plating, **or** 400ul PBS if 1:1600 final dilution for plating. Dispense 900ul of 6% methanol media into half of the screw cap cryotubes. (Ensure that 0mM NH4+ media is dispensed before dispensing 0.5mM NH4+ methanol media). Dispense 900ul of 40% glycerol into other half of screw cap cryotubes.

New Timepoint – Every 3 Weeks (~Every 39-40 Generations) Remove flasks from shaker and vortex each culture for 3 seconds to resuspend cells. Under the flame, transfer 900ul of culture to corresponding 2ml screw cap cryotubes. Store 6% methanol cryotubes in the dark for 4 hours. Store 40% glycerol cryotubes in -80°C as glycerol stocks. Prepare CYM-agarose plates with labels for each culture and 5-10 sterile glass beads per plate. Vortex each culture for 3 seconds to resuspend cells. Under the flame, dilute 10ul of culture into first dilution PBS 0.65mL tube and vortex tube for 3 seconds. Under the flame, serial dilute first PBS tube (10ul) second PBS tube. Under the flame, plate 100ul of 2nd PBS tube onto CYM-agarose plate and shake plates to spread cells. Wait 2 minutes to let plates absorb moisture. Under the flame, dump out beads from plate into 70% ethanol in a beaker. Store plates upside down in 30C incubator. Vortex each culture for 3 seconds to resuspend cells. Under the flame, aliquot 200ul

of each culture into a well of 96-well black side clear flat bottom plate. Measure fluorescence of each well at 535nm excitation, 640nm emission.

Vortex each culture for 3 seconds to resuspend cells. Aliquot 20ml of each culture into corresponding 50ml conical tubes. Centrifuge all conical tubes at max speed for five minutes. Remove supernatant into a reservoir with bleach. Aliquot 2ml of 20% glycerol into each conical tube. Pipette up and down ten times to resuspend cell pellet. Aliquot 1ml of resuspended, concentrated cells into corresponding 1.5ml centrifuge tubes. Store 1.5mL centrifuge tubes in -20°C as DNA stocks. Prepare Mr. Frosty containers by ensuring isopropanol is up to the line marking. After 4 hour incubation is complete put all 2ml methanol cryotubes into Mr. Frosty containers. Incubate Mr. Frosty containers at -80°C for 1 hour to slowly reduce the temperature of the tubes. After 1hr incubation, transfer tubes from Mr. Frosty containers directly into LN2 freezer.

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