Experimental Evolution of Increased Size and Complexity in *Anabaena variabilis*

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Dedication

This thesis is dedicated to my family. I would not be who I am or where I am without their endless love and support.

Abstract

The evolution of multicellularity has occurred more than 25 times in the history of life, facilitating transformative changes in biological diversity. Previously, we have shown that the evolution of multicellular traits can readily be observed in laboratory populations across a broad diversity of model unicellular organisms such as yeast, chlamydomonas, and *Escherichi coli*. Cyanobacteria are the oldest multicellular organisms, dating back 3.5 billion years, and many species appear morphologically unchanged, suggesting they have remained primitively multicellular. Is this because they are incapable of evolving increased complexity? The model prokaryote, *Anabaena variabilis*, is a filamentous cyanobacteria, predating fossil records. It is of order nostacales, existing as single strands or loosely associated mats, and has three distinct cells types. A propagation regime for rapid settling was used to select for increased size advantage. The initial response to selection resulted in a dramatic size increase, from microscopic strands to inseparable macroscopic aggregates. With settling selection, *A. variabilis* also became more complex; growth rate increased, two distinguishable and heritable morphologies developed, and growth and reproduction patterns changed. This shows that *Anabaena*, although remaining primitively multicellular for billions of years, rapidly responds to selection, evolving increased size and complexity.

Table of Contents

List of Tables

List of Figures

Introduction

Organisms constantly evolve and adapt (Van Valen 2009). So why have some remained phenotypically unchanged for billions of years? Anabaena is a model organism in biotechnology with implications for synthetic biology and nitrogen fixation, as well as use in bioproducts and biofuels (Heidorn et al. 2011). I am looking to answer if *Anabaena* can rapidly respond to directed evolution, as an alternative to genetic engineering and some of the issues involved, such as instability of vectors, and the redundancy of genes in the genome.

Multicellularity is important; it is the basis of complex life seen today. The evolution of multicellularity cannot be isolated to a single occurrence. It has persisted at least 16 independent times throughout eukaryotic lineages (King 2004; Schlichting 2003; Simpson 2011), and has occurred at least 25 times (with some loss) throughout the history of life; once for metazoan, and multiple times in plants, fungi and Eubacteria. The transition has facilitated crucial transformative changes in biological diversity (Grosberg and Strathmann 2007).

Surveying the diversity of life, multicellular organisms are ubiquitous. Many of the common organisms seen today are extraordinarily intricate, containing differentiation of tissues and organs (Schlichting 2003). However, multicellular organisms are clones of cells that typically express different phenotypes, despite having the same genotype (Bell and Mooers 1997). What truly defines a multicellular organism is when selection transitions from a single cell to a group of cells, defining an individual (Michod 2007).

Bonner (1998) argues that size comes first and then changes that continue to provide fitness advantages follow. Increased size provides many evolutionary advantages and can be accompanied by complexity. Size can be easily defined as space occupied, but the definition for complexity varies. Complexity can be defined by cell differentiation, division of labor, spatial patterning or morphology, changes in genome, and changes in life history such as growth and reproductive patterns (Bonner 1998; Bell and Mooers 1997; Grosberg and Strathmann 2007; Libby and Rainey 2013). In this study, I focus on changes in morphology and life history in experimentally evolved *Anabaena*.

Multicellularity is interesting because it is one of the major transitions in the course of evolution (Bonner 1998). Previously, we have shown that (given strong selection) the evolution of simple multicellular traits can readily be observed in laboratory populations across a broad diversity of model unicellular organisms such as yeast, chlamydomonas, and *Escherichi coli* (Ratcliff et al. 2012; Ratcliff et al. 2013; Personal Communication with Xiao Yi). *Sacchromyces cerevisiae*, also known as baker's yeast, was selected for increased size, and responded with primitive multicellularity. Clusters rapidly evolved and displaced their unicellular ancestor in ten replicate populations within 21 to 28 transfers, with one cycle of selection for rapid settling per transfer. Some yeast genotypes come together to form aggregates (Tarnita et al. 2013), but that was not the evolutionary observed response. The mechanism for cluster formation was clonal, an adhesion of the daughter cell to the parent (Ratcliff et al. 2012).

With continued selection, the multicellular yeast evolved complex adaptations such as a change in reproduction patterns to form daughter clusters which resemble the parent cluster. They did this by evolving higher rates of programmed cell death, so that sub-clusters can break off, opposed to single cell reproduction, which was how the ancestor reproduced (Ratcliff et al. 2012).

A similar evolutionary outcome was observed with the unicellular algae, and model organism, *Chlamydomonas reinhardtii*, which, unlike unicellular yeast, has no prior multicellular ancestor. When put under selection for larger size, one population evolved a multicellular stage as early as 46 transfers. They also formed multicellular clusters clonally. However, unlike the yeast, they developed a novel multicellular life cycle of distinct alternating phases; dispersal and growth (Ratcliff et al. 2013). Similar ongoing research with the model prokaryote, *E. coli*, suggests that the simple prokaryote can also rapidly evolve a multicellular form under the appropriate selective conditions (Personal communication with Xiao Yi). In all of these experiments, there is little potential for intercellular competition within the multicellular individual, due to kin relationships and cooperation among cells composing the multicellular organism.

The first evidence of the transition to simple multicellularity by clonal aggregation is cyanobacterial fossils (Libby and Rainey 2013). Cyanobacteria are the

oldest multicellular organisms, dating back 3.5 billion years, and many species appear morphologically unchanged since that time. The genus *Anabaena* is a filamentous planktonic cyanobacteria of the order Nostacales (Hoek 1995). It is the genus found preserved in pre-fossil stramatolites, and it predates fossil records (Astafieva 2013). *Anabaena* species are photoautotrophs and true heterotrophs, able to capture light energy and fix carbon, and use fructose as a carbon and energy source in the dark. They exhibit cell differentiation, having three distinct cell types: photosynthetic, heterocysts for nitrogen fixation, and akinetes for storage of starch during environmental stress and spores for possible reproduction in extremely environmentally stressed conditions. In the wild, *Anabaena* species exist as single strands or loosely associated mats, which are easily dispersed into single strands with dilution or aggravation, during cyanobacteria blooms (Thiel et al. 2014). *Anabaena* is considered simple prokaryotes that exhibit primitive multicellularity, and fossil records suggest it has remained primitively multicellular for billions of years (Bonner 1998).

Anabaena variabilis was chosen for this experiment because it has a fifty year history as a model prokaryotic organism, frequently used to study photosynthesis, nitrogen fixation, and cell differentiation. *A. variabilis'* 6.4 Mbp genome has been sequenced and consists of a large circular chromosome and a linear genetic sequence that is not found in other species. It has a typical doubling time of 16 hours in ideal heterotrophic laboratory conditions, it can be found in highly diverse and extreme environments, and has many applications to current biotechnology (Heidorn et al. 2011; Thiel et al. 2014).

Anabaena species, along with other cyanobacteria, are attractive for various bioproducts, including renewable biofuel (Wang et al. 2012; Heidorn et al. 2011). Biofuels of interest include ethanol, butanol, biodiesel, and hydrogen. It is also of interest for bioproducts such as bioplastics, and biomass for vitamins and food sources. *Anabaena* also plays critical roles in large scale bioprocesses, such as bioremediation, biofertilization, aquaculture, and production of engineered high value products (Heidorn et al. 2011).

It is commonly used in biotechnology due to its manipulatable genome and fast growth rate, in comparison to other plants and algae with doubling times ranging from days to weeks (Wang et al. 2012; Heidorn et al. 2011). There is also a wide range of genetic tools and biobricks available, with over 1 billion dollars invested, in the United States alone, since 2002 (Wang et al. 2012). Current applications in biotechnology involve synthetic ecology approaches and the production of diverse bioproducts.

Anabaena has great potential for synthetic ecology, because of its multifunctional abilities to fix nitrogen and carbon and by acting as a platform for subsequent advances. Synthetic biology approaches use standardized, well characterized, biological parts and modeling of genetic and metabolic networks within cells, based on hierarchical network approaches (Heidorn et al. 2011). For applications using *Anabaena*, attention is directed to engineering for chemical capture, solar energy, and carbon dioxide capture (Wang et al. 2012).

Anabaena, like several species of cyanobacteria, is known for the ability to form mutualistic relationships with other organisms, or a symbiosis. This requires a host loss of function and symbiont contribution to compensate for the loss. The best characterized of Anabaena symbiotic relationships is that of *Anabaena azollae* and the plant Azolla, where the cyanobacteria gains carbon and atmospheric nitrogen sources from the plant in exchange for fixed nitrogen. The cooperation and ability to form these symbiotic relationships provides much interest for synthetic ecology (Ray et al. 1978).

Using experimental evolution of *Anabeana variabilis*, I am looking to answer several questions in this thesis; 1. Why have *Anabaena* have remained primitively multicellular for billions of years? 2. Is *Anabaene* capable of responding to selection for increased size? If so, how do they respond to this selection? 3. Is *Anabaena* capable of evolving increased complexity?

Chapter One

Experimental Evolution in *Anabaena variabilis*: Increased size in response to settling selection

Introduction

Larger size has many advantages. It can provide protection from predators, due to gape or other prey handling limitations (Stephens and Krebs 1986). It can also provide protection from environmental perturbations, by the generation of an internal environment that is isolated from the external conditions, which can be particularly harmful and potentially mutagenic (such as UV irradiation). Larger size also can increase storage reserves for circumstances when nutrients are limited. Another benefit is increased potential for finer scale adaptation, due to differentiation and division of labor among component parts (Grosberg and Strathmann 2007; King 2004; Bonner 1998).

However, larger size is not always beneficial. Some disadvantages to larger size include increased costs of reproduction, generation time, and the occurrence of mutations that can accumulate as individuals grow. The ecological circumstances are critical in determining the evolutionary benefits of size, as well as for other traits (Libby and Rainey 2013). In particular, access to and the availability of resources, are frequently identified as factors limiting individual size. Given the diversity of individual organismal size, and the frequency of changes in size over evolutionary time for many species, the almost unchanging constancy of size in some species is surprising.

Cyanobacteria of the genus *Anabaena* appear morphologically unchanged from their fossil ancestors of 3.5 billion years ago, suggesting they have stayed primitively multicellular. *Anabaena* is a simple, filamentous, prokaryote with some cellular differentiation. They have three distinct cell types: photosynthetic cells, heterocysts for nitrogen fixation, and akinetes for stress storage and reproduction (Hoek 1995; Thiel et al. 2014; Bonner 1998). Under stress situations, *Anabaena* can exhibit false branching, where two filaments emerge from the same sheath. This suggests that, *Anabaena* may have a genetic predisposition for increased size (Hoek 1995; Michod 2007), and such a

genetic predisposition can aid in adaptation (Michod 2007). However, this does not limit responses to selection to only cyanobacteria that exhibit false or true branching, a new mutation is likely to arise that has an advantage for increased size and requires less energy output by the *Anabaena* strand.

In this chapter, I investigate the evolutionary potential for increased size in *Anabaena varabilis*. I investigate this using experimental evolution, where previous experiments with *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii* have shown dramatic increases in size following settling selection. By imposing settling selection, an increase in size becomes favored (Ratcliff et al. 2012; Ratcliff et al. 2013). Although selection for rapid settling is not widespread in nature, it is experimentally controllable for laboratory selection (Ratcliff et al. 2012). Benefits of using experimental evolution include a controllable and stable environment, the elimination of the influx of genotypes into the population by starting from an isogenic strain, evaluating the repeatability by running experiments in parallel, and removing organism interactions such as competition (Lenski and Travisano 1994).

Methods

***Anabaena variabilis*.** The initial culture of *Anabaena variabilis* was obtained from Brett Barney PhD of Bioproducts and Biosystems Engineering at the University of Minnesota. The culture was streaked for purity with three rounds of single colony selection on BG-11 plates. The final isogenic culture was grown in nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light, as adapted from Yoon et al. (2008). Aliquots were preserved with a final solution of 8% DMSO in BG-11 and labeled as the ancestor, or transfer 0.

Settling Experiment. Settling selection was imposed to create an advantage for increased size. Ten replicate populations, inoculated with the initial isogenic culture of *Anabaena variabilis*, were grown in 10-ml aliquots of nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light (Yoon et al. 2008). Every 72 hours 1.5 ml of sample was removed and centrifuged at 100*g* for 30 seconds. The bottom 100 µL was transferred to a new 25- x 150-mm culture tube containing fresh media, as performed by Ratcliff et al. (2012). Archives for -80^{o}C storage were taken every 7 transfers (21 days), for 96 transfers (about 10 months), and preserved with a final solution of 8% DMSO in BG-11 Media.

Non-Settling Experiment. To control for any possible lab adaptations, ten replicate populations, inoculated with the initial isogenic culture of *Anabaena variabilis*, were grown in 10-ml aliquots of nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light **(**Yoon et al. 2008). Every 72 hours, 100 µL was transferred without centrifugation. Archives for -80^{o}C storage were taken every 7 transfers (21 days), for 96 transfers (about 10 months), and preserved with a final solution of 8% DMSO in BG-11 Media.

Size Distributions. To determine area, images of each replicate were taken on an Olympus IX70 inverted microscope, using the 10x objective, and phase contrast illumination after 72 hours of growth. Cultures were votexed to break up any loosely associated strands. ImageJ was used to measure area and data were analyzed in JMP.

Bradford Assay Growth Assessment. To determine growth rate by measuring protein content, freezer samples were thawed and 100 µl were inoculated into a 25- x 150-mm glass culture tube. Immediately after inoculation a 100 µl aliquot, time 0, was taken from each sample and frozen at -30^{o}C for analysis once sample collection is complete. Aliquots were taken of each tube every 24 hours for 72 hours. To measure protein concentration the Bradford Protein Assay procedure was adapted from Zor and Selinger (1996). After collection was complete, aliquots were thawed, vortexed, 5 µl of each were placed in replicates of two into a 96 well plate, including two 5 µl replicates of BG-11 media as a blank. 145 µl of water and 150 µl of Coomassie Blue Reagent was added to each well. The 96 well plate was inserted into a Molecular Devices Emax Precision Microplate Reader and read at 405nm and 600nm, simultaneously. Samples reading above 0.5 were diluted and reread; samples reading below 0.008 were run with more sample volume. The final protein concentration was calculated against a standard calculated curve accurate for 0.008 to 0.5 mg/ml and converted to µg/ml. Growth rate was calculated as an average µg/ml increase over 72 hours. Protein concentration at 72 hours, independent of growth rate, was normalized by subtracting time 0 readings.

Settling Rate. To measure the increase in settling rate, or speed of which *Anabaena* falls out of solution, cultures were grown from freezer stocks in 25- x 150-mm glass culture tubes for 72 hours. Samples were added into cuvettes, agitated by lightly shaking, and placed into a Cary 300 bio spectrophotometer to measure absorbance. BG-11 Media was used as a blank. Each sample was run for 3.5 minutes under the kinetics function to record absorbance decrease over time as cultures settled. The lamp was turned on a minimum of 15 minutes before data was collected. Settings were set as wavelength 600nm, bandwith 2, average time 0.5, Y-min at 0.1, Y-max at 1.5. The absorbance data was analyzed in JMP and fit to a linear curve, using the negative slope as the value for settling rate.

Dry Weight. To assess biomass by weight, cultures were grown in replicates of three in pre-weighed 25- x 150-mm glass culture tubes for 72 hours. They were then placed in a 40^{o}C incubator for three days to ensure complete evaporation of media. The tubes were again weighed and biomass calculated by subtracting the initial weight of the tube.

Growth Rate by Area. To determine growth rate as an increase in area of *Anabaena*, per sample, images were taken on an Olympus IX70 inverted microscope, using the 10x objective, and phase contrast illumination at inoculation, 24, 48, and 72 hours of growth. A total of 30 photos, per time point, per tube, were taken. Nine photos per slide were taken at predetermined positions. The images were analyzed for area in ImageJ and Microsoft Excel was used to fit a line to the area versus time data, the resulting slope was used as growth rate.

Results

Anabaena variabilis increases in size within the first 45 transfers under settling selection (Figure 1.1). *A. variabilis* cells and small multicell filaments are microscopic and their presence is evident by a green pigmentation in the water or media, as seen in the ancestor and non-settling replicates. In the replicates under settling selection, the *A. variabilis* became macroscopic. Visible conglomerations were seen as early as 10 transfers (10 rounds of settling selection) in three of the settling replicates. At transfer 14, they were visible in five of the replicates, and by transfer 21, all ten settling replicates exhibited macroscopic clumps of *Anabaena*, even after vortexing.

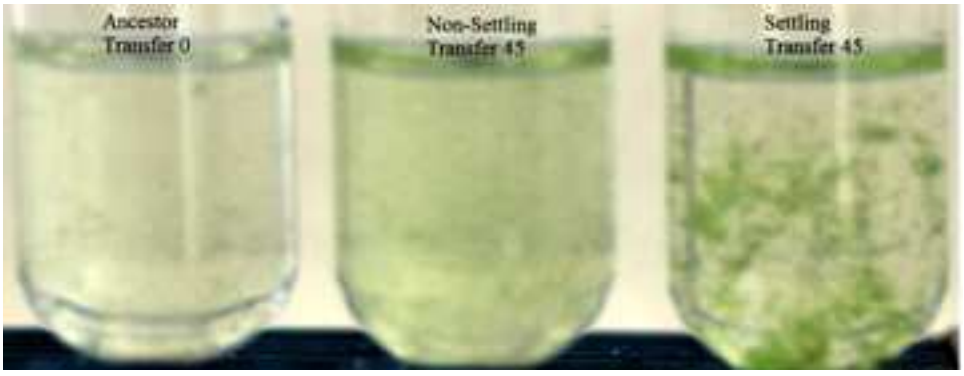

Figure 1.1. Macroscopic images of transfer 45. Macroscopic images of the ancestor (transfer 0) (left), a replicate of the non-settling selection population, transfer 45 (middle) and a replicate of the settling selection, transfer 45 (right). Images were taken after

vortexing to break up any loosely associated strands. Increases in density can also be macroscopically observed by pigmentation.

As suggested in Figure 1.1, density increased over the course of the selection experiment. Slight increases were also seen in the non-settling replicates. Figure 1.2 shows transfer 0 through 45, following heavy votexing to break up large clumps and create a homogenous solution in which density can easily be seen.

Figure 1.2. Macroscopic image of density across transfers. Density can be seen through pigmentation intensity as shown across settling transfers.

Image analysis of microscopic images confirmed these apparent clump-size increases. Transfer 0, 21, and 45 of the settling replicates were assessed for size, as shown in Figure 1.3. With each transfer, size and organism count increases, which can be seen by the frequency count on the y-axis. The larger clump fraction of the population that starts to emerge at transfer 21 becomes dominant by transfer 45. There is a huge size shift between transfer 0 (the ancestor), transfer 21, which was when all ten replicates of the settling selection contained visible conglomerations, and by transfer 45 there are no longer small strands in the populations.

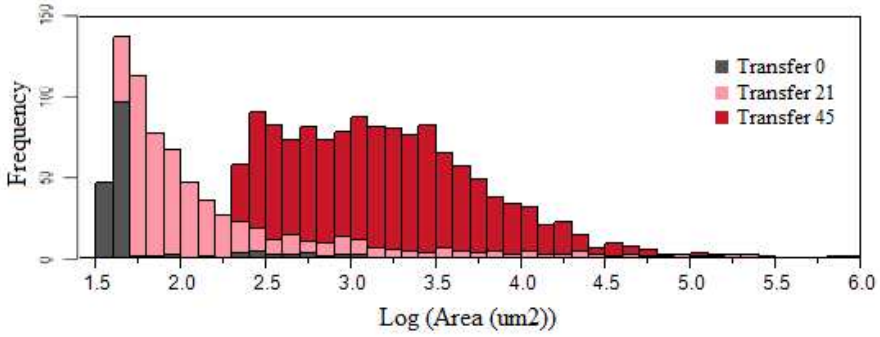

Figure 1.3. Size distribution. Size distribution at transfer 0 (ancestor), transfer 21 and transfer 45 of settling selection. Area was measured on whole *Anabaena* pieces in the microscope field of view.

To visualize the dramatic increase in size over the first 45 transfers, microscope images of transfer 0, 21, and 45 were compared. Figure 1.4 illustrates the average size of *Anabaena* in the settling replicates across transfers. The ancestor contains a few short strands, while transfer 21 contains a clump that easily fits within the field, and transfer 45 contains a clump that barely fits within the field of view.

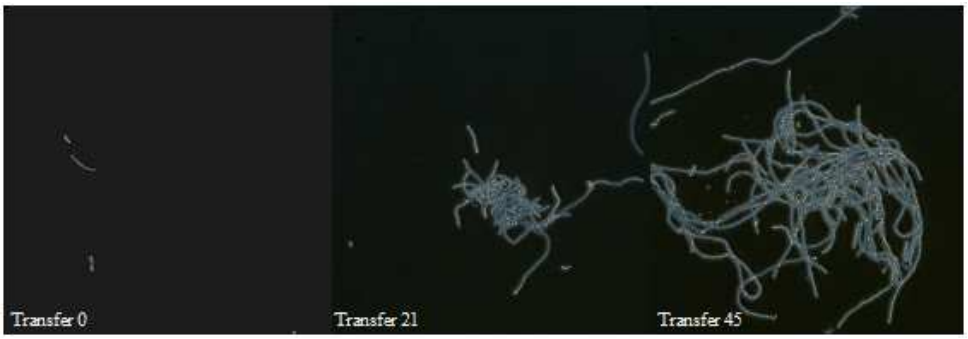

Figure 1.4. Microscopic images of settling transfers. Images, taken at 10x, depicting average size of transfer 0, transfer 21, and transfer 45 of settling selection.

Did larger clusters represent an increase in total biomass, or simply increased aggregation? Another method to measure growth in dense cultures is to use protein concentration by Bradford's Assay as a proxy for biomass. Figure 1.5 illustrates the average protein concentration across the ten replicate settling populations over transfers. Transfer 0 = 47.1 µg/ml, transfer 7 = 47.7 µg/ml, transfer 14 = 49.1 µg/ml, transfer 21 = 51.0 µg/ml, transfer 28 = 55.8 µg/ml, transfer 35 = 59.9 µg/ml, transfer 42 = 69.8 µg/ml, and transfer 45 = 75.1 µg/ml.

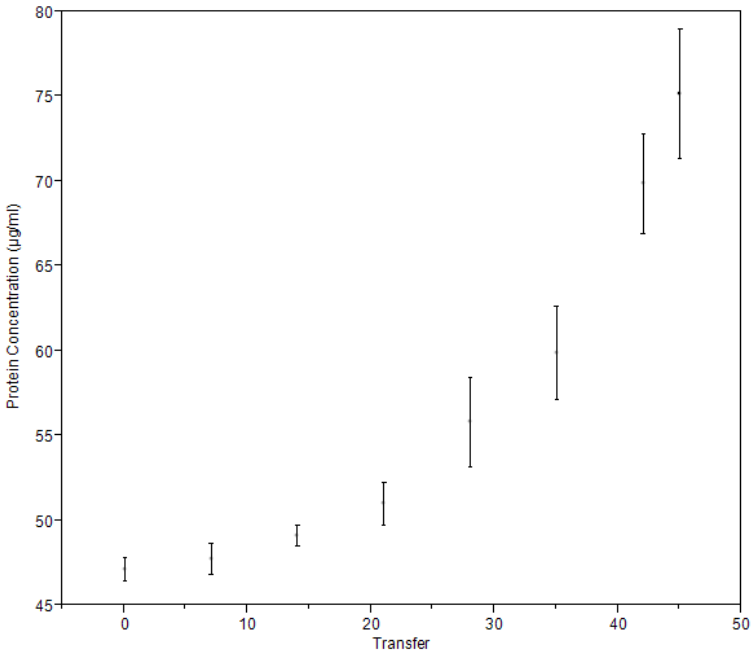

Figure 1.5 Biomass (protein concentration) by transfer number. Measurement of growth and density through mean protein concentration, in µg/ml, after 72 hours, as measured by Bradford's Assay, across transfers. The error bars depict standard error.

Populations selected for rapid settling did indeed increase the rate at which they fall out of solution over time. This can also be seen by Figure 1.1, where the visible clusters in the settling transfer 45 replicate are already starting to fall out of solution before the image was able to be acquired. Settling rates were measured using a spectrophotometer across transfers from the settling selection. Figure 1.6 shows the increased density and increased rates from the ancestor to transfer 45.

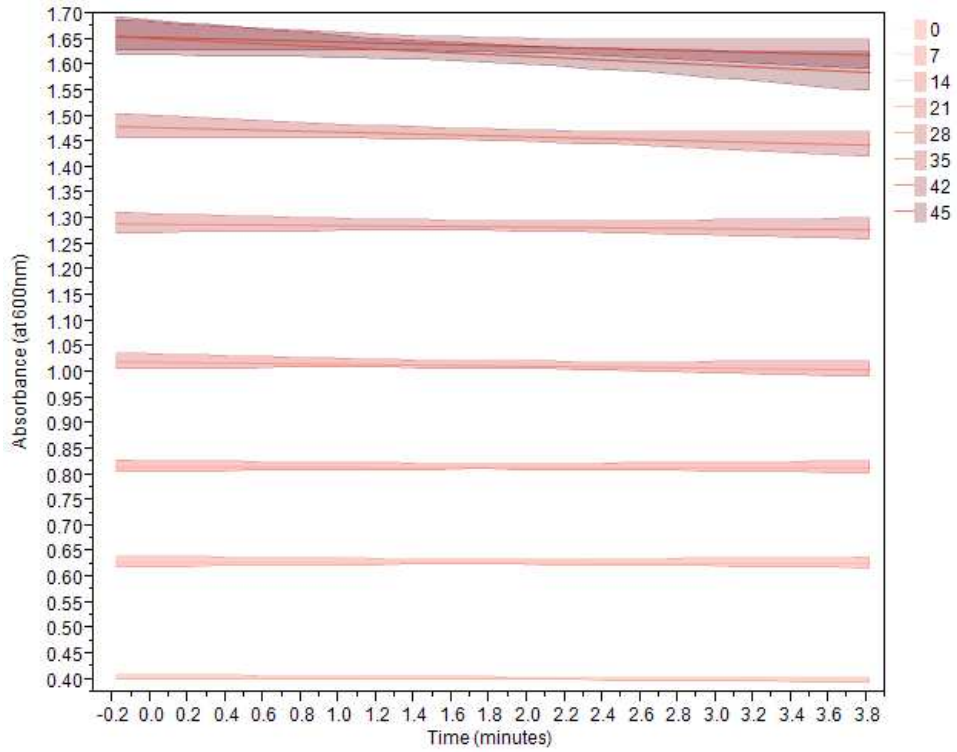

Figure 1.6 Settling rates across transfers. Settling rates were recorded on a spectrophotometer for 3.6 minutes across settling populations from transfers 0, 7, 14, 21, 28, 35, 42, and 45, indicated by gradation of red. The shaded region is the 95% confidence of fit to a straight line.

The rate of settling was estimated as the slope from a best fit least squares regression analysis of the spectrophotometric data. With continued selection, settling rates increase. Different y-intercepts are reflective of absolute starting absorbance and overall saturation of culture after 72 hours. The equations and settling rates that were fit for each transfer can be found in Table 1.1.

Transfer	Equation	Settling Rate
0	y= -0.00015x+0.4038	0.00015
7	y= -0.0006x+0.6295	0.0006
14	y= -0.0004x+0.8157	0.0004
21	y= -0.0039x+1.0206	0.0039
28	y= -0.0039x+1.2903	0.0039
35	y= -0.0091x+1.4791	0.0091
42	y= -0.0091x+1.6552	0.0091
45	y= -0.0174x+1.6529	0.0174

Table 1.1. Settling equations and rate. Line equations fit from Figure 1.6. The negative slope was used as settling rate.

The expected response to settling selection is increased size, which I expected would tradeoff with growth rate within the population. The relationship is shown in Figure 1.7. The x-axis shows the log mean area in square microns of the *Anabaena* strands or clumps and the y-axis shows the log mean dry weight of a 10ml sample in mg. The non-settling population does see an increased growth rate due to lab adaptations, but the settling selection does see an increase in growth rate that is greater than that of the non-settling.

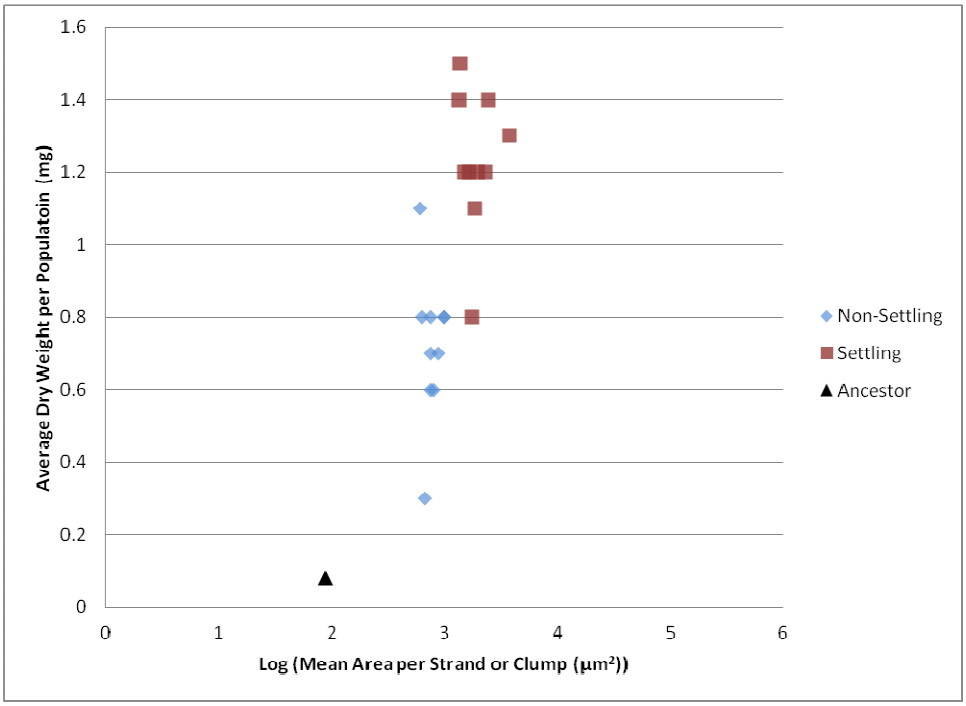

Figure 1.7 Dry weight versus size. Transfer 0, the ancestor (black triangle), transfer 45 settling (red square), and transfer 45 non-settling (blue diamond). Average log mean area per clump, based off of 30 images, averaging 100 measurements, in comparison to the average dry weight of a 10ml sample, replicated three times. Each mark represents one population.

As the average size of *Anabaena* increases so does biomass. The ancestor has both low biomass and small size. The non-settling replicates have increased biomass per population and area per strand in comparison to the ancestor, but all non-settling replicates are smaller than the settling replicates. Dry weight is lower overall in the non-settling replicates, as compared to the settling replicates, but there are some populations that are comparable to one another as far as biomass per population and density.

Discussion

With settling selection, increased size evolved rapidly in *Anabaena variabilis*. In developmental terms, the initial transition to multicellularity is easy (Grosberg and Strathmann 2007). In principle, there are two ways that they could be acquiring this large size; adhesion of cells post replication, or by strand aggregation (Tarnita et al. 2013). In

either case, there is strong selection on an emergent property, the size of the individual. It allows for precise identification of a new level, the clump. It is general enough to incorporate both ecological and genealogical levels as aggregates form a number of new species (Simpson 2011). It is suggestive by the images in Figure 1.3 that selection has moved from strands, to clumps, creating a new level of selection, and redefining the individual. Although there was a genetic predisposition for the possibility of false branching, only one possible occurrence was observed, indicating this is not how increased size was acquired.

Settling replicates increased in density, biomass per ml, which could be seen macroscopically by media pigmentation, as well as through protein concentration and biomass assay. The non-settling replicates also increased in density and biomass. This is presumably due to adaptation to lab environments as typically seen (Lenski and Travisano 1994; Yoon et al. 2008). Prior to settling selection, this strain was grown in continuous culture at full saturation in a bubble column reactor, a different selective environment.

The direct observation of increased settling rate by large clumps, as well as the simultaneous increase in size and settling rate, indicates that the increased size is an adaptation to settling selection. Adaptation is a feature that enhances fitness, and all natural selection, such as a change in environment, increases adaptation (Van Valen 2009).

For filamentous bacteria, spectrophotometers are not recommended for density measurements due to non-homogenous growth mode of cells (Heidorn et al. 2011). This was especially true as the *A. variabilis* became macroscopic over the course of selection. Originally, attempts for estimated growth rates were performed by spectrophotometer, but it was hard to obtain stable and accurate readings, so results were replicated with protein concentration. The spectrophotometer was then used to measure settling rate. Settling rate and absolute absorbance increased with each transfer.

The evolution of increased size allows for acquisition of new specialized functions (Michod 2007). This increase in settling rate was seen across the settling replicates. When selecting for rapid settling for increase in size, there was increase in

settling rate. Although this was expected due to the selection regime, it is contrary to natural conditions, because cyanobacteria have the ability to control their buoyancy for access to light (Hoek 1995). Their light source was not limited in growth conditions. Shaking cultures kept the *Anabaena* suspended and mixing, and therefore settling, or sinking, during growth between settling selections, was not costly to their fitness.

The increased sizes were clearly adaptive for the settling replicates, without an apparent fitness cost, which was surprising. Costs create tradeoffs, which drive transitions, such as the initial advantage of group formation and the need of cell specializations for nutrient distribution or reproduction (Michod 2007). It was expected that the settling populations would be larger per strand or clump, but possibly have lower biomass per population than the non-settling transfers. Large size can be costly in terms of increased generation time, or slower growth rate, increased resource need, and resource distribution. It takes longer for clumps to reach ideal size for reproduction; this can be due to limitations of resource distribution (Michod 2003). However, Figure 1.7 indicates that the growth rate in the settling replicates is the same and faster. This could be because the size reached at this point does not limit the previously mentioned factors enough to have a costly effect, or because the adaptation is more advantageous to fitness. The newly formed clumps could also be growing from several points, therefore not limiting growth to two points per individual as in the strands of the non-settling treatment and ancestor.

Increases in final density measurements, such as increased absorbance and dry weight, could be partially attributed to lab adaptation and amount of material transferred each time. As the density increases, and more clumps are able to settle to the bottom of the tube and survive the transfer, more initial biomass is transferred to inoculate the fresh media. This effect is minimal and normalized by running both settling and non-settling treatments. Protein concentration was normalized to control for this. Growth rate, normalized by time zero measurements, taken immediately after inoculation, will give more accurate assessment in future experiments. This would then allow measurements of growth rate with regard to how much biomass was transferred into the fresh media.

It can be hypothesized by the increased optical density, protein concentration, and biomass, seen in the settling replicates that the growth rate has increased enough that their increased size is due to aggregation by adhesion of offspring. Clonal development has less intracellular competition, and cyanobacteria were the first evidence of this (Grosberg and Strathmann 2007). Individual strands are the result of the linear growth of cells, and the importance of clonal development in the clumps allows for increased cooperation and limits competition for resources, such as light, allowing and sharing access. Organisms incorporate both ecological and genealogical levels to form new species (Simpson 2011). How a species is defined is continually argued. At what point does evolution result in a new species? Could the settling replicates of *A. variabilis* be evolving into a new species? Further experiments are needed to test this hypothesis and the aggregation of clonal strands in clumps.

Conclusion

Although primitively multicellular and morphologically constant for billions of years, *A. variabilis* is capable of rapidly evolving with selection. When settling selection for increased size is applied, they rapidly evolve a dramatic increase in size, accompanied by an increase in settling rate. Increase in growth rate is seen across the measurements of density, protein concentration, and biomass.

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Chapter Two

Distinct Morphologies for Increased Size and Complexity in *Anabaena variabilis*

Introduction

Most extant multicellular organisms develop by failed separation of the products of cell division. Aggregative development of unrelated cells only occurs in a few groups of terrestrial or semi-terrestrial microorganisms (Bonner 1998). *Anabaena variabilis* is aquatic, and following this observation, the evolution of increased multicellular complexity might occur by the accumulated products of cell division. Nevertheless, fitness tradeoffs shape the evolution of increased multicellular complexity, and although increased size can be beneficial, it typically has costs in terms of reduced reproduction. These costs involve increased generation time as it takes more time to reach maturity, complexity in the mechanisms associated with reproduction, and increased occurrence of deleterious mutations (Michod 2003). In experimental evolution of multicellularity with yeast, observed fitness tradeoffs were associated with growth rate and complex reproductive mechanisms (Ratcliff et al. 2012; Grosberg and Strathmann 2007; Michod 2007). Interestingly, the complexity cost involved a mode of cellular differentiation by replicative altruism, as increased rates of programmed cell death evolved that improved growth rates (Ratcliff et al. 2012). Given the long evolutionary history of multicellularity in *Anabaena*, it was unclear how increased size was achieved, and if it involved new modes of differentiation besides those already present.

Complexity increases with size, independent of phylogeny (Bell and Mooers 1997). Complexity can be defined as the diversity of specialized cell types, arrangement of cells, and genetic makeup (Bell and Mooers1997; Grosberg and Strathmann 2007). Even the simplest of multicellular organisms exhibit division of labor and spatial pattering (Libby and Rainey 2013). This can be seen in *Anabaena* through the three different cell types; photosynthetic cells, heterocysts for nitrogen fixation, which are

spaced every 8-10 cells for efficient fixed nitrogen distribution, and akinetes for nutrient storage and stress reproduction (Libby and Rainey 2013; Thiel et al. 2014).

Increases in complexity can also be seen by the passing of fitness components from one level of organization to a higher, more inclusive level that represents the new emerging organism as a whole (Simpson 2011). The modes by which this occur remain unclear, but necessarily involve transitions in heritability from individual cells to inclusive wholes. Heritability, the resemblance between parents and offspring, is crucial for the evolution of complexity. Low heritability limits the potential for selection to maintain the trait, even if it is strongly beneficial. How does heritability itself evolve, when the traits under selection involve reorganization of fitness components for survival and reproduction (Michod 2003)?

I showed in, Chapter 1, that *Anabaena* rapidly responds to selection by increasing in size. How do they achieve dramatic size increases, and does increased complexity evolve as a result?

Methods

***Anabaena variabilis*.** The initial culture of *Anabaena variabilis* was obtained from Brett Barney PhD of Bioproducts and Biosystems Engineering at the University of Minnesota. The culture was streaked for purity with three rounds of single colony selection on BG-11 plates. The final isogenic culture was grown in nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light, as adapted from Yoon et al. (2008). Aliquots were preserved with a final solution of 8% DMSO in BG-11 and labeled as the ancestor, or transfer 0.

Settling Experiment. Settling selection was imposed to create an advantage for increased size. Ten replicate populations, inoculated with the initial isogenic culture of *Anabaena variabilis*, were grown in 10-ml aliquots of nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light (Yoon et al. 2008). Every 72 hours 1.5 ml of sample was removed and centrifuged at 100*g* for 30 seconds. The bottom 100 µL was transferred to a new 25- x 150-mm culture tube containing fresh media, as performed by Ratcliff et al. (2012). Archives for -80^{o}C storage were taken every 7 transfers (21 days), for 96 transfers (about 10 months), and preserved with a final solution of 8% DMSO in BG-11 Media.

Non-Settling Experiment. To control for any possible lab adaptations, ten replicate populations, inoculated with the initial isogenic culture of *Anabaena variabilis*, were grown in 10-ml aliquots of nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light **(**Yoon et al. 2008). Every 72 hours, 100 µL was transferred without centrifugation. Archives for -80^{o}C storage were taken every 7 transfers (21 days), for 96 transfers (about 10 months), and preserved with a final solution of 8% DMSO in BG-11 Media.

Bradford Assay Growth Assessment. To determine growth rate by measure of protein content, freezer samples were thawed and 100 µl were inoculated into a 25- x 150-mm glass culture tube. Immediately after inoculation a 100 µl aliquot, time 0, was taken from each sample and frozen at -30^{o}C for analysis once sample collection is complete. Aliquots were taken of each tube every 24 hours for 72 hours. To measure protein concentration the Bradford Protein Assay procedure was adapted from Zor and Selinger (1996). After collection was complete, aliquots were thawed, vortexed, 5 µl of each were placed in replicates of two into a 96 well plate, including two 5 µl replicates of BG-11 media as a blank. 145 µl of water and 150 µl of Coomassie Blue Reagent was added to each well. Plate was inserted into a Molecular Devices Emax Precision Microplate Reader and read at 405nm and 600nm, simultaneously. Samples reading above 0.5 were diluted and reread; samples reading below 0.008 were run with more sample volume. The final protein concentration was calculated against a standard calculated curve accurate for 0.008 to 0.5 mg/ml and converted to µg/ml. Growth rate was calculated as an average µg/ml increase over 72 hours. Protein concentration at 72 hours, independent of growth rate, was normalized by subtracting time 0 readings.

Settling Rate. To measure the increase in settling rate, or speed of which *Anabaena* falls out of solution, cultures were grown from freezer stocks in 25- x 150-mm glass culture tubes for 72 hours. Samples were added into cuvettes, agitated by lightly shaking, and placed into a Cary 300 bio spectrophotometer to measure absorbance. BG-11 Media was used as a blank. Each sample was run for 3.5 minutes under the kinetics function to record absorbance decrease over time as cultures settled. The lamp was turned on a minimum of 15 minutes before data was collected. Settings were set as wavelength 600nm, bandwith 2, average time 0.5, Y-min at 0.1, Y-max at 1.5. The absorbance data was analyzed in JMP and fit to a linear curve, using the negative slope as the value for settling rate.

Analysis of Morphologies. I used two approaches to assess the evolution of morphology in the selected populations; objective assessment through measurements and double blind evaluation by laboratory volunteers. To start, frozen transfer 45 archives were grown in 25- x 150-mm glass culture tubes for 72 hours. 10 µl were placed on a hemocytometer and imaged on an Olympus IX70 inverted microscope, using the 4x objective, phase contrast illumination. Nine photos per slide were taken at predetermined positions, centering the main strand or conglomeration. A total of 30 photos per tube were taken. To control for any

possible bias in data collection and analysis, images were renamed with a randomly generated 6-digit number.

Objective Assessment. The *Anabaena* in each image was measured with the ImageJ and analyzed in JMP for an ANOVA test using the parameters of integrated density, circularity, and hole size. In ImageJ integrated density is defined as sum of the values of the pixels in the image or selection, circularity is a shape descriptor in respect to a perfect circle. Hole size was achieved by measuring each image twice, once with the function include holes, once without, the two values were then subtracted to determine the size of the holes.

Double blind evaluation. A standardized rubric for categorization was made and taught to seven participants who were then shown each image and asked to place each under the category of tangle, cluster, extended chain length, or no visible change. (Figure 2.2 shows an example of these, and the rubric is included in the appendix.) Overall agreement was measured in JMP and plotted in graphs for representative data.

Heritability of Morphologies. To determine if assigned mechanisms were genetically based, and therefore heritable, individual clumps were isolated: cultures were grown for 72 hours, then a 10 µl sample was placed in a well of a 96 well plate. 200 µl of BG-11 media was used to dilute the well, and the well was then mixed with a pipette tip. 10 µl from that well was moved into the next, and diluted. This was done under a microscope at high magnification until an individual, typical of either a cluster or tangle mechanism, was isolated. The resulting cluster or tangle was then imaged on an Olympus IX70 inverted microscope, using the 10x objective, and phase contrast illumination, and carefully pipetted into a 25- x 150-mm glass culture tube with 10 ml BG-11 Media. They were grown for 72 hours and 10 photos per replicate were taken on a hemocytometer at predetermined positions, centering the *Anabaena*. Each image was assigned a random 6-digit number and then assessed for morphology using the parameters of integrated density, circularity, and hole size.

Results

I hypothesized in Chapter 1 that growth rate increases with transfer number in the replicates maintained with settling selection, in which increases in settling rate were also observed. This is counter to the expectation that growth rate trades-off with size since more resources are invested to increase settling rates. To assess if growth rate is actually increasing, growth rate over 72 hours (as protein concentration per ml) was measured for each replicate at eight points over the course of selection. Simultaneously, settling rate was also measured for the settling replicates on a spectrophotometer as the loss of

absorbance over 3.5 minutes. I observed that the longer the populations underwent selection, the higher the initial biomass for settling, and the higher the absolute growth rate of the population. Results are shown in Figure 2.1.

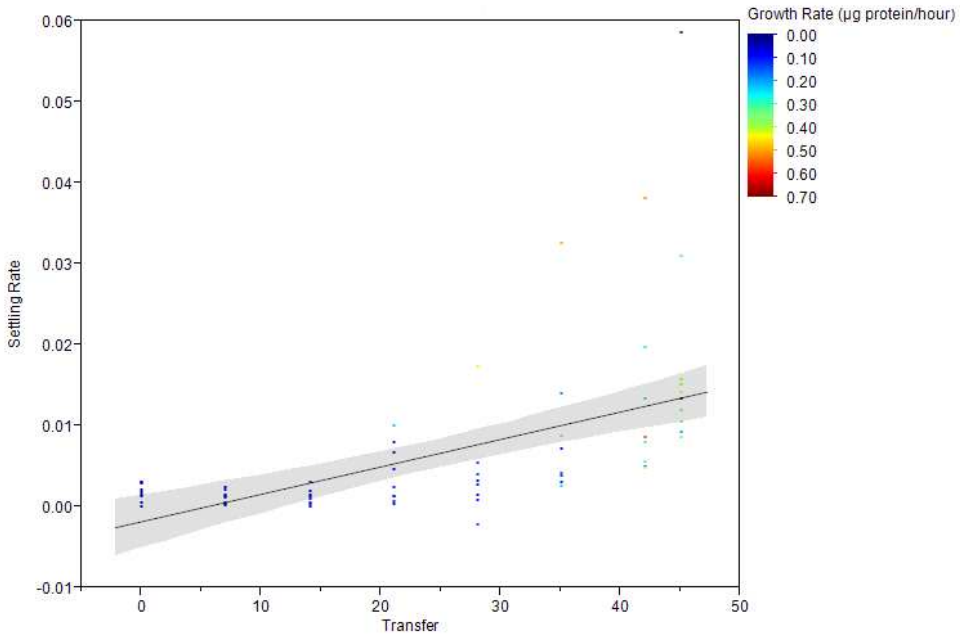


Figure 2.1. Settling rate over transfers. Settling rate, measured by absorbance lost over time, by transfer number, shown in comparison to absolute growth rates, measured by protein concentration, and across ten populations. The shaded region shows 95% confidence interval.

An analysis of variance of the data indicates several statistically significant effects. Unsurprisingly, growth rate and settling rate differ statistically significant from the ancestor, and growth rate is positively associated with settling rate ($p < 0.001$).

The settling replicates of *Anabaena* are able to increase both growth and settling rate by developing morphologies, depicted in Figure 2.2. Transfer 0, the ancestor, shows no change in morphology, for it exists as single filamentous strands in shaking liquid culture. Longer strands were seen as early as 7 transfers in, this morphology was labeled length. Increased length was seen across all ten settling replicates.

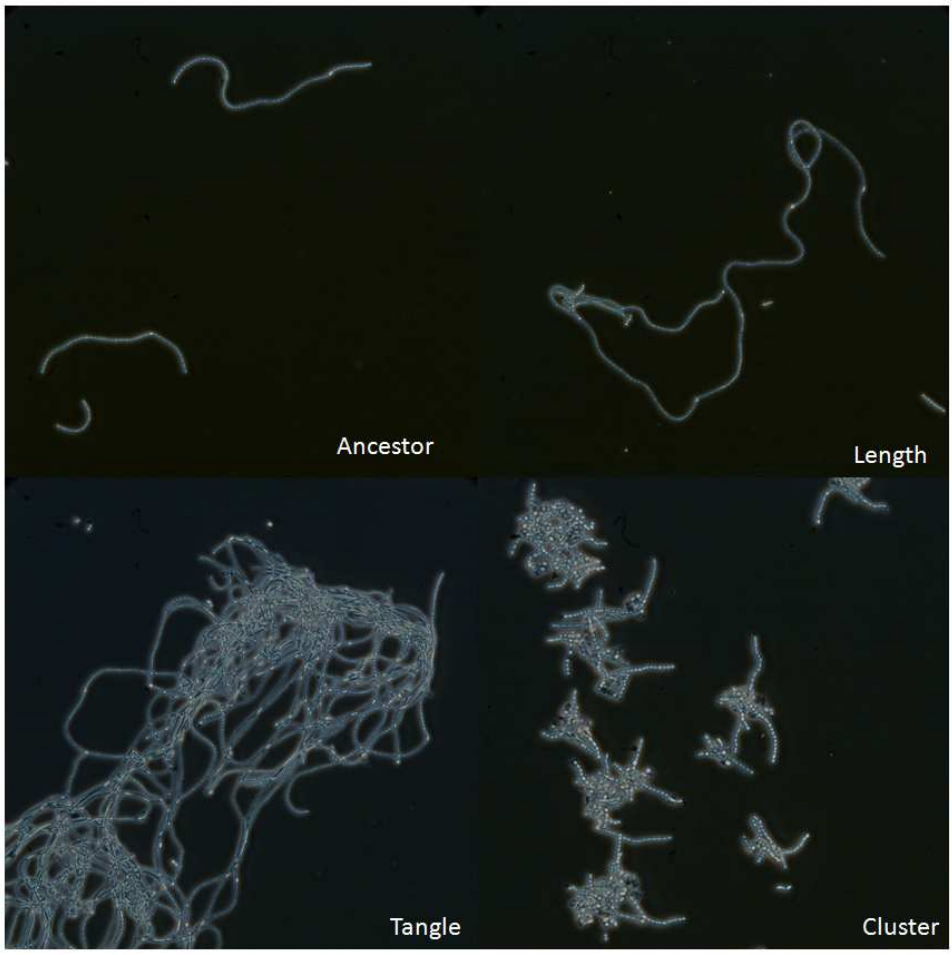


Figure 2.2. Types of Morphologies. Example of mechanisms types given to assessors. Images shown were taken in phase contrast at 10x after vortexing. Upper left is the ancestor, and an example of no change, upper right is length, lower left is a tangle, and lower right shows clusters.

After 14 transfers, large groups became visible in culture tubes, even after vortexing. At 21 transfers, each settling replicate appeared to have one of two morphologies for larger size, as exemplified in Figure 2.2, and single strands of greater length were no longer seen. One morphology, termed tangle, is where strands get long, and become entangled with one another. The other morphology, termed cluster, is where strands bend and break to associate with one another, for a size benefit.

To assess if these two apparently different morphologies were measurably distinct, microscope images were measured in ImageJ and analyzed in JMP by an

ANOVA. Results are in Table 2.1. Parameters used to distinguish between the two mechanisms were integrated density, circularity, and hole size. The hypothesis that they are not distinguishable was rejected with a p-value <0.001.

Analysis of Variance			
Souce DF	**Sum of Squares**	**Mean Square**	**F Ratio**
Model 19	1.7485E+12	9.20E+10	3.661
Error 312	7.8424E+12	2.51E+10	**Prob > F**
C. Total 331	9.5909E+12		<.0001

Table 2.1. ANOVA of measured morphologies. Analysis of variance test of measured values for tangles and clusters in ImageJ. The degrees of freedom represented in the model take into account how the experiment was setup, non-settling controls were excluded.

In ImageJ integrated density is defined as sum of the values of the pixels in the image or selection, and circularity is a shape descriptor in respect to a perfect circle. Hole size was achieved by measuring each image twice, once with the function include holes, once without, the two values were then subtracted to determine the size of the holes.

A principal component chart (Figure 2.3) was created to illustrate the parameters used and the differences between tangles and clusters. Parameters entered were integrated density, hole area, and circularity.

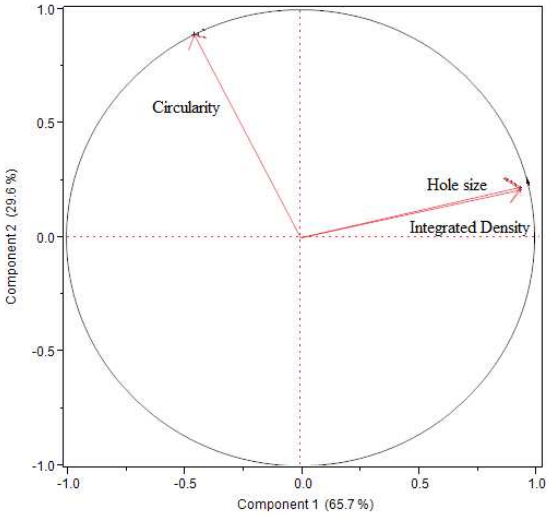


Figure 2.3. Principal components. Chart shows factors used to determine between clusters and tangles. Measurements of circularity, integrated density, and hole size were taken in ImageJ.

The principal components generated and used are shown in Table 2.2. The first two principals generated were significant.

	Principal 1	Principal 2	Principal 3
Circularity	-0.45439	0.8908	0.00327
Integrated Density	0.93771	0.22135	-0.26777
Hole Area (um^2)	0.94012	0.20977	0.26867

Table 2.2. Principal component matrix. Shown above are the values for the principal component matrix. Principal one and two are significant to distinguish between tangles and clusters.

To visualize these principal components, Figure 2.4 was generated. It shows the two significant principal components on the axes, and the populations are represented in color. Populations are grouped together.

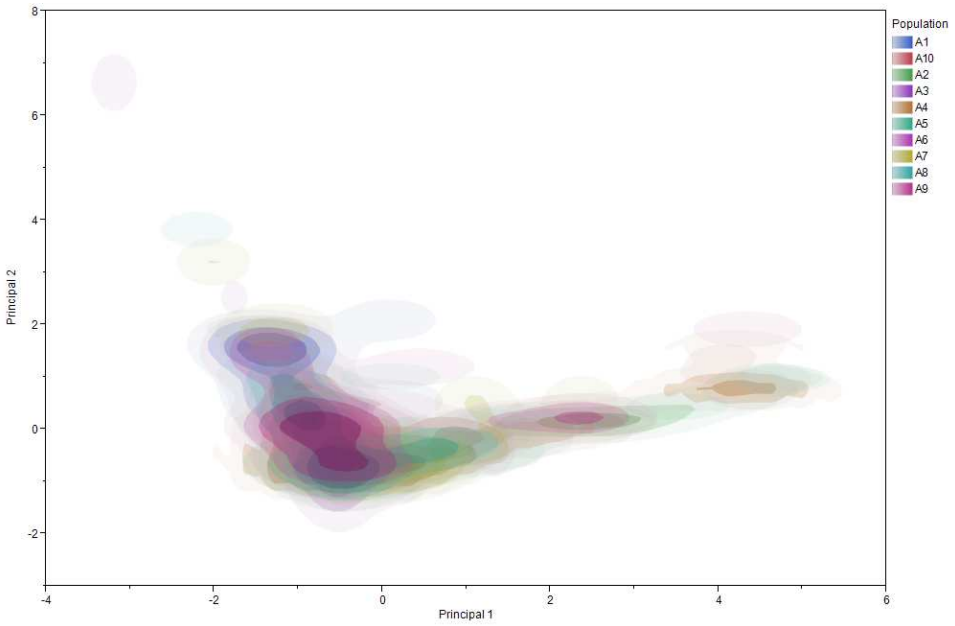


Figure 2.4. Principal component graph. Populations typically include tangles or clusters of certain specification. Shading indicates density.

To assess if these morphologies are recognizable by others, seven volunteers were given a rubric with basic instructions and the images in Figure 2.2. They were then shown the images of both settling and non-settling treatments, in a random, unbiased manner, and asked to categorize each image as either no morphology seen, length, tangle, or cluster. An analysis of variance test was run between the non-settling and settling selection replicates, and the p-value was less than 0.001. However, an analysis of variance test with random effects was run to determine the assessors' overall ability to correctly identify between tangles and clusters in comparison to ImageJ, the p-value was 0.0406 indicating that there is a recognizable difference. Figures 2.5 and 2.6 illustrate the agreement across assessors for tangle and cluster identification, respectively. Some populations have more agreement than others. For example, assessors agreed that population 4 was mostly tangles, but disagreed more on some other populations.

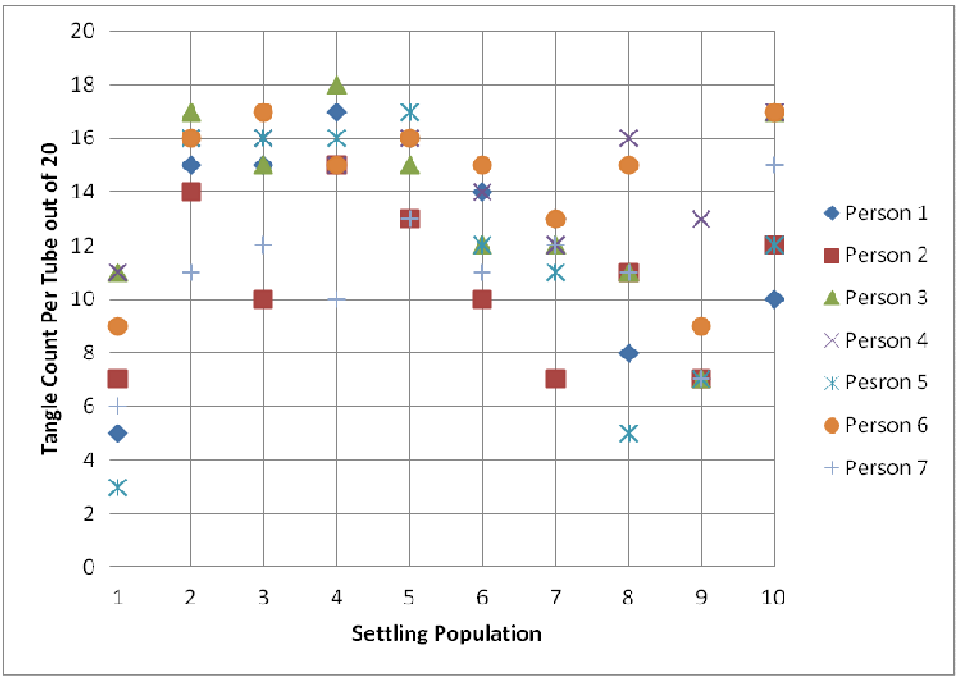


Figure 2.5. Subjective assessment of Tangles. Overall Agreement of Tangles in each population. Assed by seven lab members using a rubric for determination between cluster or tangle. Each population consisted of 30 images.

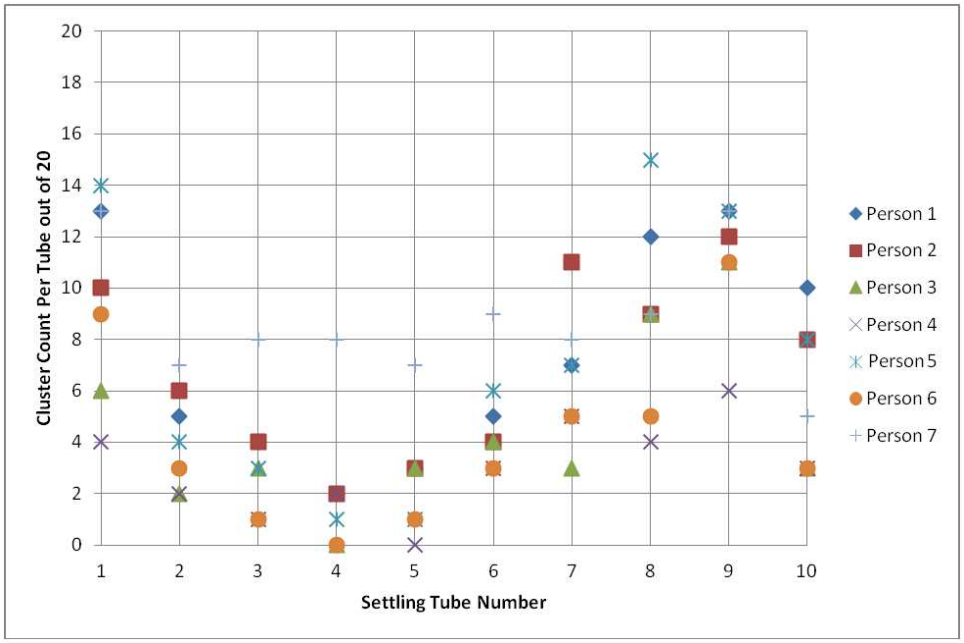


Figure 2.6. Subjective assessment of Clusters. Overall Agreement of Clusters in each population. Assed by seven lab members using a rubric for determination between cluster or tangle. Each population consisted of 30 images.

To test if morphological complexity is heritable, a single clump, of distinguishable morphological category was isolated from seven of the populations that contained easily identifiable tangles or clusters. Three populations (1, 7, and 8) were not easily distinguishable by objective or subjective measures. Images were taken and classified as either a tangle or cluster by ImageJ parameters of integrated density, circularity, and hole size. After 72 hours, images of the offspring were taken and classified as a cluster or tangle by ImageJ parameters. Resulting offspring counts are displayed in Table 2.2.

Starting Phenotype	Tangle	Cluster
Tangle Offspring	49	3
Cluster Offspring	1	47

Table 2.3. Morphology heritability. A single clump of determined phenotype was isolated and grown for 72 hours. 5 of each, tangle and cluster, were assessed. 10 images of each were taken at predetermined positions and classified as either a tangle or cluster.

Of the isolate tangle phenotypes, 98% of the offspring were classified as tangles, and of the cluster phenotype, 94% of the offspring were classified as clusters.

Discussion

Settling selection was performed on *Anabaena variabilis* with the expectation of the evolution of increased size. Observed was the evolution of two morphologies, which suggest that there are at least two mechanisms by which increased size could have evolved.

The development of morphologies was not an effect of an over saturated culture, since when grown to saturation in the ancestor and non-settling replicates and vortexed, this is not seen. This is not the phenomena known as “Nostoc balls.” Classifications for

"Nostoc Balls" include an extracellular investment of a thick gelatinous sheath, which surrounds aggregates of helical filaments in a ball formation. They are found on terrestrial environments and correspond to microcolonies aggregating in the presence of fluid for moisture (Bazzichelli et al. 1986).

The growth rate of *Anabaena variabilis,* selected for setting selection, has the greatest effect on settling rate, with a p-value of 0.001. There is an expected tradeoff between growth rate and settling rate, as discussed in Chapter 1. Most features have costs (Van Valen 2009). So clumps that invested more energy into forming morphologies for settling, and less into rapid relative growth, had the fitness advantage. Ecological circumstances are critical in evolution (Libby and Rainey 2013). However, it appears that either growth rate provided a greater fitness advantage, or individuals who had evolved and survived rapid settling, came to evolve increased growth rates.

Absolute growth rate has a significant effect on settling rate, with a p-value of less than 0.001, further proving that adaptation to settle under imposed conditions is more advantageous to fitness than possible costs. Features are maintained despite costs because features are adaptive (Van Valen 2009). The combination of transfer and growth rate also has a significant effect on settling selection, with a p-value of less than 0.001, which confirms that growth rate increases with transfer, which aids in the increase of settling rates.

One possible down side to increased growth in *Anabaena*, for use in biofuels, is that it may oppose cell maintenance of lipid storage, which is important for biofuel production (Bull and Collins 2012). This is plausible, but would be expected to be seen with the change in protein concentration.

A filamentous strain of cyanobacteria can only grow from two points, as seen in the ancestor and increased length morphologies. This could explain the increase in growth rates in terms of protein concentration, but not the rapid acquisition of size (Hoek 1995). By transfer 7, increased growth rate was already measureable, and when examined under the microscope, longer strands had developed across the ten settling replicates. Within a shaking culture, strands can only get so long before breaking, and there proves to be two distinct ways around this. By transfer 21, each of the ten settling replicates

formed either tangles or clusters. Increased size comes with increased mutation rates (Michod 2003). Evolution is based off advantageous mutations, so in a highly selective environment, this may be a benefit, aiding in the development of morphologies.

The Darwinian individual is now the tangle or cluster. The unit of selection changed from a strand to a clump (Libby and Rainey 2013). This was achieved by the passing of dominate fitness components from one level of organization to a higher, more inclusive level (Simpson 2011). The traits passed from single strands to clumps include increased growth rate and adhesion.

Parameters used by ImageJ to categorize tangles and clusters were integrated density, circularity, and hole size. The analysis of variance test had a p-value of less than 0.001, meaning that measureable differences are present. This was further exemplified by Figure 2.4, where two regions are formed. Despite overlaps, populations are found in distinct regions of the classifications.

When shown to assessors, there were no discrepancies of which images had higher level associations such as tangles or clusters. Zero photos from non-settling selection were categorized as a tangle or cluster. However, telling the difference between tangles and clusters was not quite as easy, but still had great results. The p-value was 0.0406, which is significant, demonstrating that human-recognizable differences between the two morphologies exist. Patterns of assessors' markers in Figures 2.5 and 2.6, illustrate individuals' tendencies to favor one morphology over the other when the image could be considered ambiguous. They also suggest that tangles are more common.

An essential trait for evolution is heritability between adults and offspring (Libby and Rainey 2013). It also includes acquisition of heritable variation in fitness at a new level (Michod 2003). Both of these can be demonstrated by the variation of two distinct morphologies and their heritability. Table 2.3 is highly suggestive that the fate of becoming a tangle or cluster is genetic. The exact mechanism is not yet known, but it could be predicted to be group level production (Libby and Rainey 2013). One possible mechanism is altruistic reproduction through programmed cell death (Grosberg and Strathmann 2014). This was seen in the yeast experiments, aiding the development of a juvenile stage (Ratcliff et al. 2012).

Conclusion

There is no apparent persistent tradeoff between relative growth rate and settling rate and growth rate can be an indicator of settling rate. Increased growth rate and size are acquired through the development of two distinct morphologies, increasing complexity and becoming the Darwinian individual. The morphologies of tangles and clusters are measurably distinct and recognizable across seven different assessors. The mechanistic morphologies formed are heritable, further increasing complexity.

Chapter Three

Increased Complexity of *Anabeana variabilis* Through Experimental Evolution

Introduction

Natural selection for one feature often brings changes in others (Van Valen 2009). In selection for rapid settling for increased size in *Anabaena variabilis*, size increased, accompanied by changes in growth rate and settling rate, which lead to an increase in complexity through changes in morphology. These changes were likely facilitated by changes in their pre-existing life history. Increased complexity includes changes in reproductive patterns; variation promotes an origin of altruistic interaction with the group (Michod 2007).

Ancestral *A. variabilis* reproduces by fragmentation, in which a section of the chain will break off and float away, or separate from a dead cell (Yoon et al. 2008). Do settling selected clumps reproduce? If so, is this random fragmentation or something more organized? Changes in settling selected *Anabaena* have already resulted in increases in complexity through morphology, suggesting a fitness reorganization involving changes in reproduction strategies and changes in life history. Evolution of larger size can increase the costs of reproduction, and the responses to selection reflect the balance between survival and reproduction. The larger an individual needs to be for survival, the more costly it is to reproduce a large daughter cell, as it creates a decrease in parental size, but if the daughter is not large enough, survival of both clumps is at risk (Michod 2007).

Organized reproduction can be triggered, or repressed, by a cue or signal for division of cells or clumps. Common triggers for cell division in lab cultures include time, media change which affects the availability of nutrients or dilutes chemical cues or density, or attaining an optimal or terminal size (Hoek 1995). All three are possible in the settling selected *Anabaena*.

Large size can be costly for fitness in terms of reproduction and increased generation time, and increased resource need (Michod 2003). The settling selected *Anabaena* experience increased absolute growth rates and higher density, suggesting possible nutrient shortages or chemical cues for the repression of division in the media. *Anabaena* are photosynthetic, and large clumps may exceed a size where there is adequate light exposure for nitrogen fixation, causing them to reach a terminal size and trigger reproduction. Cyanobacteria, including *Anabaena*, can develop circadian rhythms. It is a way of cell wide regulation for energy conservation and enhances fitness of cells in periodically variable environments (Heidorn et al. 2011). *Anabaena* is both a photoautotroph and true heterotroph, able to capture energy from light, and in the dark, using fructose as a carbon and energy source (Thiel et al. 2014). Although the *Anabaena* for this experiment is grown under constant light, is may be able to respond to the stimulus of the time of transfers. Under our selection environment, with transfers every 72 hours, I hypothesized that the optimal time to divide would be at 72 hours, as inoculation into fresh media occurs. Parent clumps would still be large enough to survive transferring and could break off multiple daughter clumps that would have 72 hours to grow in maximum resources.

Methods

***Anabaena variabilis*.** The initial culture of *Anabaena variabilis* was obtained from Brett Barney PhD of Bioproducts and Biosystems Engineering at the University of Minnesota. The culture was streaked for purity with three rounds of single colony selection on BG-11 plates. The final isogenic culture was grown in nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light, as adapted from Yoon et al. (2008). Aliquots were preserved with a final solution of 8% DMSO in BG-11 and labeled as the ancestor, or transfer 0.

Settling Experiment. Settling selection was imposed to create an advantage for increased size. Ten replicate populations, inoculated with the initial isogenic culture of *Anabaena variabilis*, were grown in 10-ml aliquots of nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light (Yoon et al. 2008). Every 72 hours 1.5 ml of sample was removed and centrifuged at 100*g* for 30 seconds. The bottom 100 µL was transferred to a new 25- x 150-mm culture tube containing fresh media, as performed by Ratcliff et al. (2012). Archives for -80^{o}C storage were taken

every 7 transfers (21 days), for 96 transfers (about 10 months), and preserved with a final solution of 8% DMSO in BG-11 Media.

Non-Settling Experiment. To control for any possible lab adaptations, ten replicate populations, inoculated with the initial isogenic culture of *Anabaena variabilis*, were grown in 10-ml aliquots of nitrogen free liquid BG-11 Media (pH 7.4) in 25- x 150-mm glass culture tubes at 22.5^{o}C, shaking at 250 RPM under constant light **(**Yoon et al. 2008). Every 72 hours, 100 µL was transferred without centrifugation. Archives for -80^{o}C storage were taken every 7 transfers (21 days), for 96 transfers (about 10 months), and preserved with a final solution of 8% DMSO in BG-11 Media.

Size Change During 72 Hour Growth Cycle. To visualize how *Anabaena* is growing and dividing throughout the normal 72 hour cycle, ancestor and transfer 45 freezer stocks were inoculated into 10ml of BG-11 Media in 25- x 150-mm glass culture tubes and grown under constant light. Images were taken on an Olympus IX70 inverted microscope, using the 10x objective, and phase contrast illumination after inoculation, and 6, 24, 48, 72, 78, 96 and 120 hours of growth. The cultures were subjected to their normal transfer regime at 72 hours, immediately after the aliquot was taken. The images were analyzed for area in ImageJ and analyzed in JMP.

Growth from Suspension of Single Cells. In order to fully break up larger clusters and visualize the overall growth process, without the disruption or cue of transferring, the settling and non-settling replicates were digested down to single cells following methods developed by Ratcliff et al. (2012). Cultures were grown from freezer stock for 72 hours. A 1.5 ml aliquot was placed into a micro-centrifuge tube. Large aggregated clumps were broken down by vortexing on high for 5 minutes, 3 times, until the solution appeared homogenous. Individual cells were obtained by enzymatically digesting *Anabaena* samples in 1,000 units of lyticase and 2% β-glucuronidase/arylsulfatase (Roche) for 2 hours at room temperature, then vortexing on high for 5 minutes. The presence of single cells was confirmed with microscopy, and more time or vortexing was performed if needed. Cells were centrifuged and washed with BG-11 Media. The sample was subjected to 1 min of centrifugation at 100g to separate cells from debris, and the bottom 100 µl was transferred to 10 ml of fresh media in a 25- x 150-mm glass culture tube. 100 µl aliquots were taken immediately, then at 6, 24, 48, 72, 78, 96, 120, and 144 hours. Aliquots were immediately imaged on an Olympus IX70 inverted microscope, using the 10x objective, and phase contrast illumination. Nine photos per slide were taken at predetermined positions. A total of 30 photos, per time point, per tube, were taken. Area was analyzed in ImageJ. This experiment was repeated 3 times, independently.

Effects of Transfer Time. To see if cell reproduction is triggered by transferring, transfers were conducted early, on time, or late. Replicates of settling population 1 at transfer 45 were grown from freezer stock and

transferred after either 66, 72, or 78 hours of growth. 100 µl aliquots were taken and immediately imaged on an Olympus IX70 inverted microscope, using the 10x objective, and phase contrast illumination at 65, 67, 69, 71, 73, 75, 77, and 79 hours of growth. A total of 30 photos, per time point, per tube, were taken. Nine photos per slide were taken at predetermined positions. Area was measure in ImageJ and analyzed in JMP.

Results

All results in this chapter were obtained using populations after 45 transfers.

When we follow *Anabaena variabilis* through two rounds of their regular 72 hour transfer regime, imaged in Figure 3.1, it is apparent that the non-settling replicates do not experience much change in size. Alternatively, the settling selected populations are the largest at the time of transfer (0 hours) then appear to divide, based on the decreases in overall clump size, and grow until transferring again. Images were taken after vortexing to break up loosely associated strands.

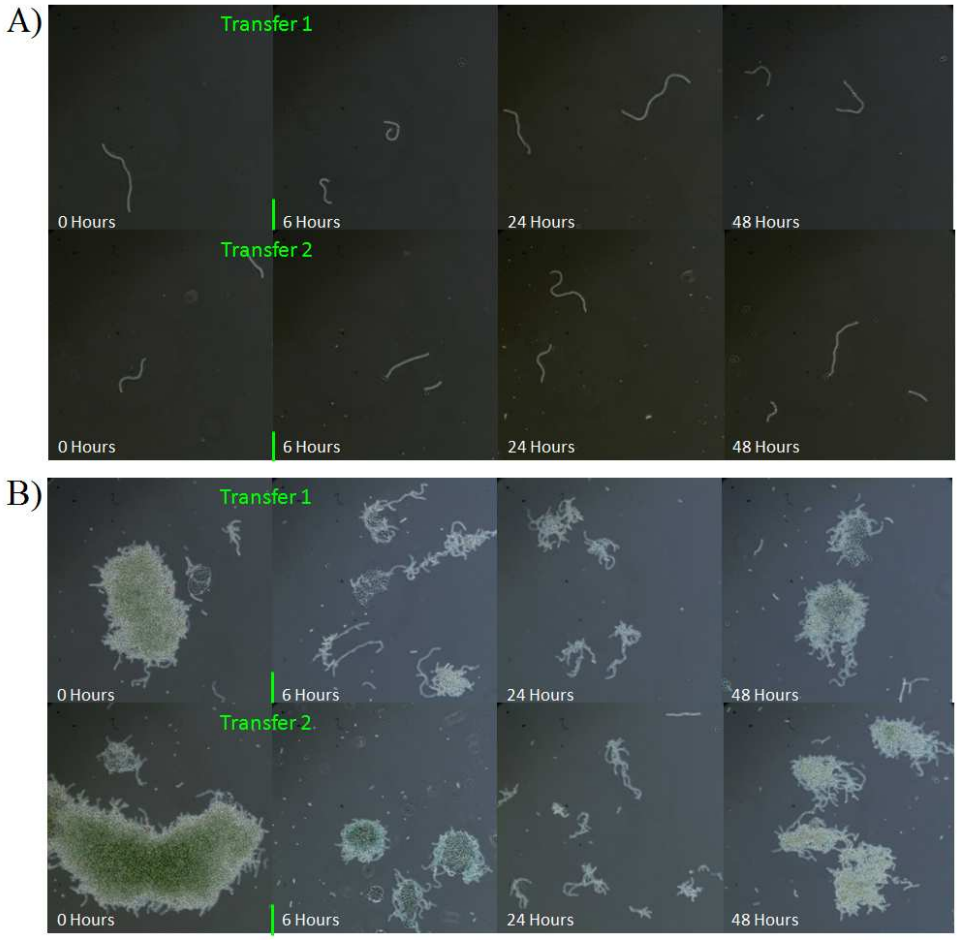


Figure 3.1. Images of treatments over transfers. A) Non-settling replicates typical size and formation through two regular transfers, with 0 hours being immediately before transferring. B) Settling replicates average size and formation through two regular transfers, with 0 hours being immediately before transferring.

The images were measured in ImageJ and are graphed in Figure 3.2.

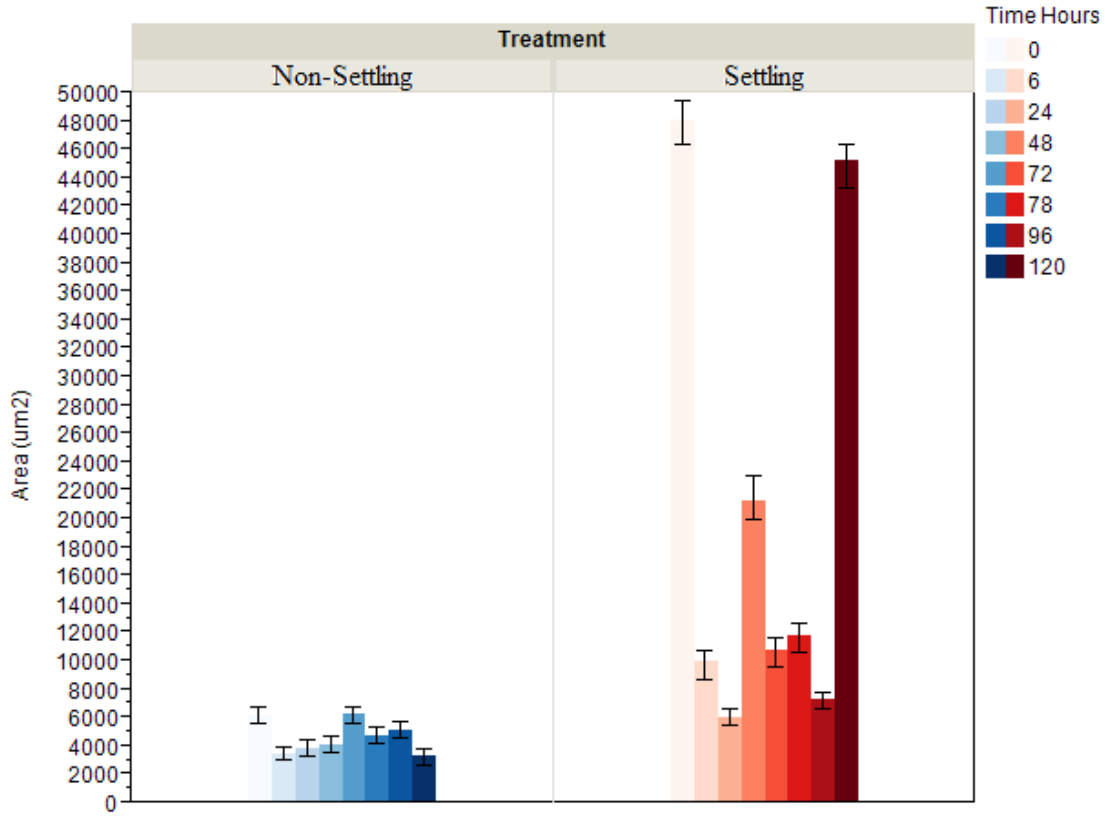


Figure 3.2. Size of treatments over transfers. Average size through two regular transfers. Time 0 and 72 are immediately before transferring. Error reflects the standard error of the variation among the ten populations of each non-settling and settling treatment, where the average total number of measurements per population is approximately 500.

The non-settling replicates have a small size range, in comparison to the settling transfers, and are largest at 0 and 72 hours. Transfer points are at 0 and 72 hours. The settling selected replicates are largest at time 0, 48, and 120.

To test the hypothesis that 1) cluster division was triggered by reaching an optimal size, or 2) that division depended on time since last transfer, *Anabaena* was grown for 144 hours, without transfer and replenishment, from single cells, which were therefore unable to immediately divide at time 0. This was conducted three independent times. The growth is imaged in Figure 3.3.

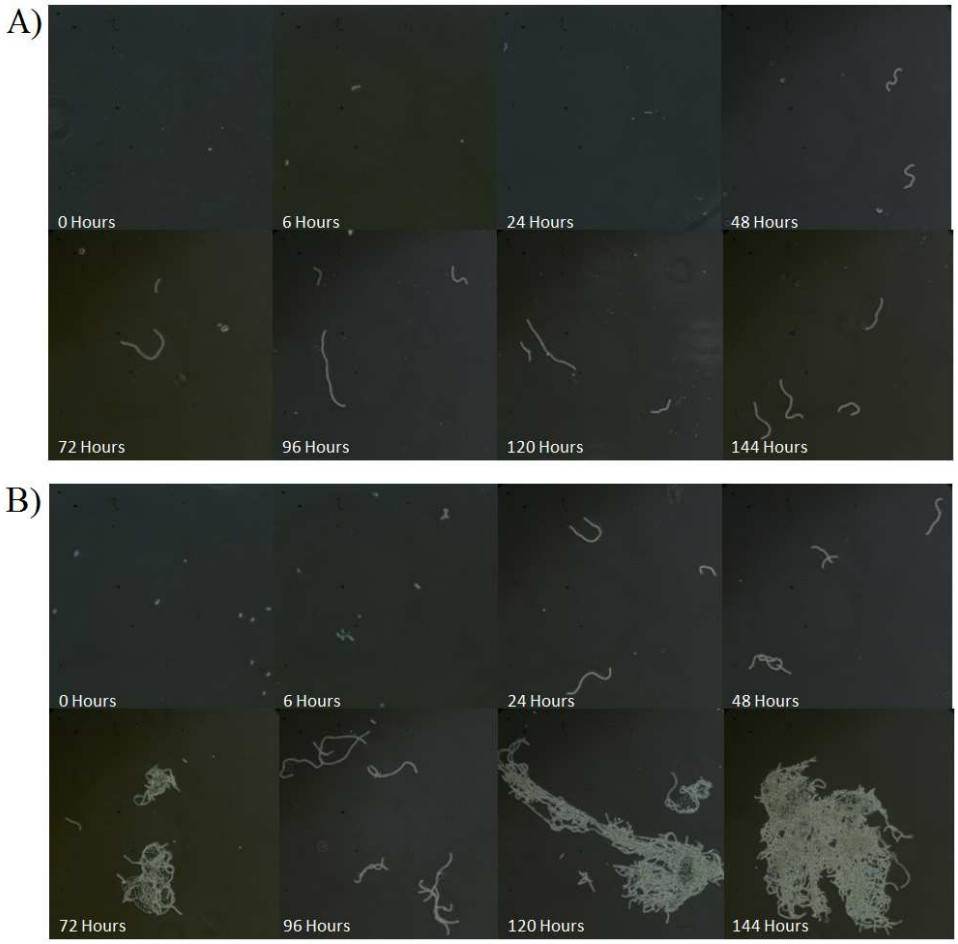


Figure 3.3. Images of growth from single cells. A) Non-settling replicates average size and formation, over 144 hours, without transferring, starting from single cells obtained by digestion in lyticase and 2% β-glucuronidase/arylsulfatase. B) Settling replicates average size and formation, over 144 hours, without transferring, starting from single cells obtained by digestion in lyticase and 2% β-glucuronidase/arylsulfatase.

The non-settling replicates start as single cells, and grow into single strands, of slightly varying lengths. The settling replicates also start as single cells, but soon start taking the shape of larger, more complex, morphologies, as expected. Average size for both treatments is graphed in Figure 3.4.

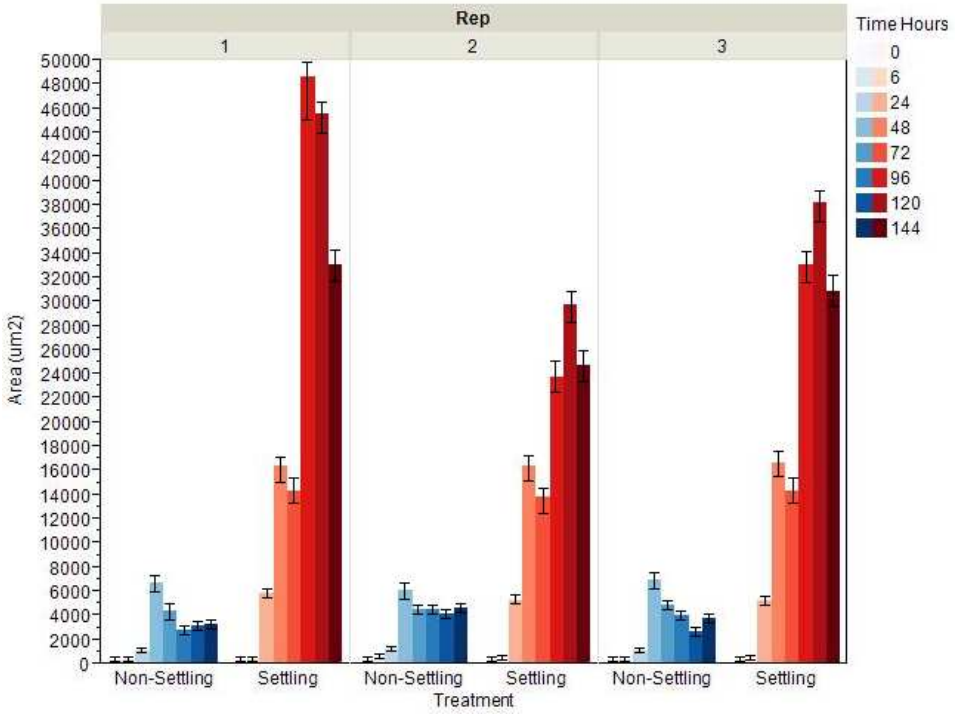


Figure 3.4. Size of single cells over time. Average size over 144 hours (the duration of two regular, 72 hour, transfers, but without transferring). Grown from single cells obtained by digestion in lyticase and 2% β-glucuronidase/arylsulfatase. Error reflects the standard error of the variation among the ten populations of each non-settling and settling treatment, where the average total number of measurements per population is approximately 500.

The non-settling and settling replicates increase in size until 48 hours and then a slight decrease to 72 hours. The non-settling are largest at 48 hours, while the first replicate experiment of the settling selected is largest at 96 hours, with 120 hours almost as large, and in the second two replicate experiments, settling selected is largest at 120 hours. All three replicate experiments share a decrease in size after 120 hours in the settling selected treatment. Size decreases at 72 and 144 hours are interpreted as clump division.

To determine if 1) cluster division was triggered by reaching an optimal size, or 2) that division depended on time since last transfer, the size data for the settling

replicates grown from single cells are compared to those observed through a normal transfer regime in Figure 3.5.

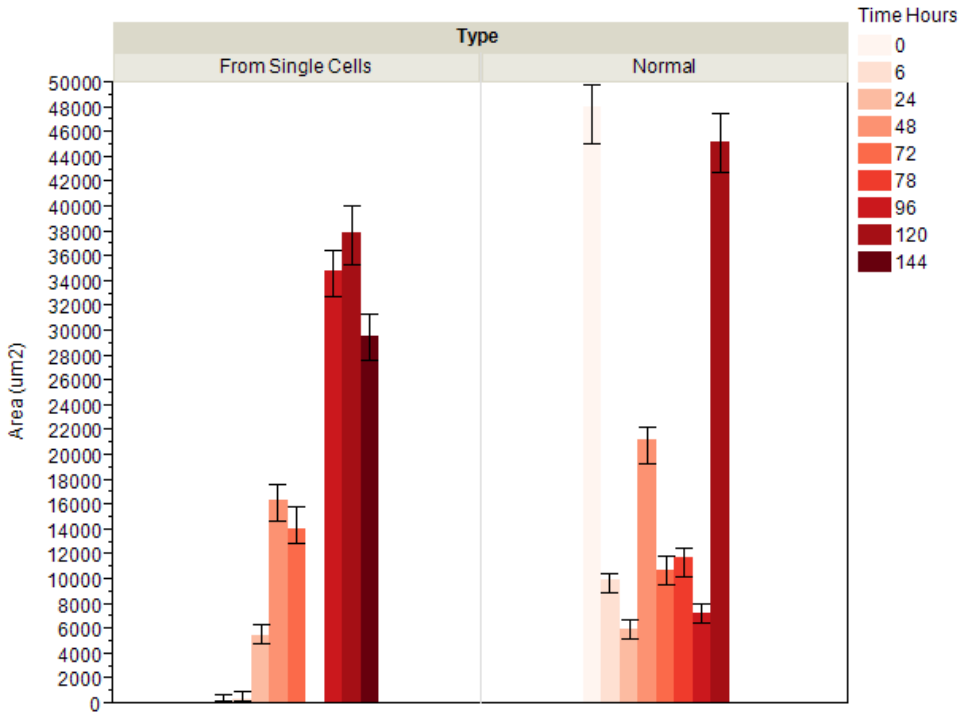


Figure 3.5. Size of single cells and settling treatment. Settling replicates over time from single cells obtained by digestion in lyticase and 2% β-glucuronidase/arylsulfatase without any transfers (left) and a normal sample through two regular transfers (right). Error reflects the standard error of the variation among the ten populations of treatment, where the average total number of measurements per population is approximately 500.

Comparing settling selected *Anabaena* across the two treatments, they share a decrease in average size from 48 to 72 hours, and an increase between 96 and 120 hours. Time 78 was not collected for the growth from single cell analysis. Time 0 and 72 of the normal replicate are immediately before transferring.

To test the hypothesis that cluster division is triggered by reaching some specific size, Figure 3.6 shows the data collected from growth from single cells and normal transfer replicates to compare growth rates over average size (average per clump in μm^2).

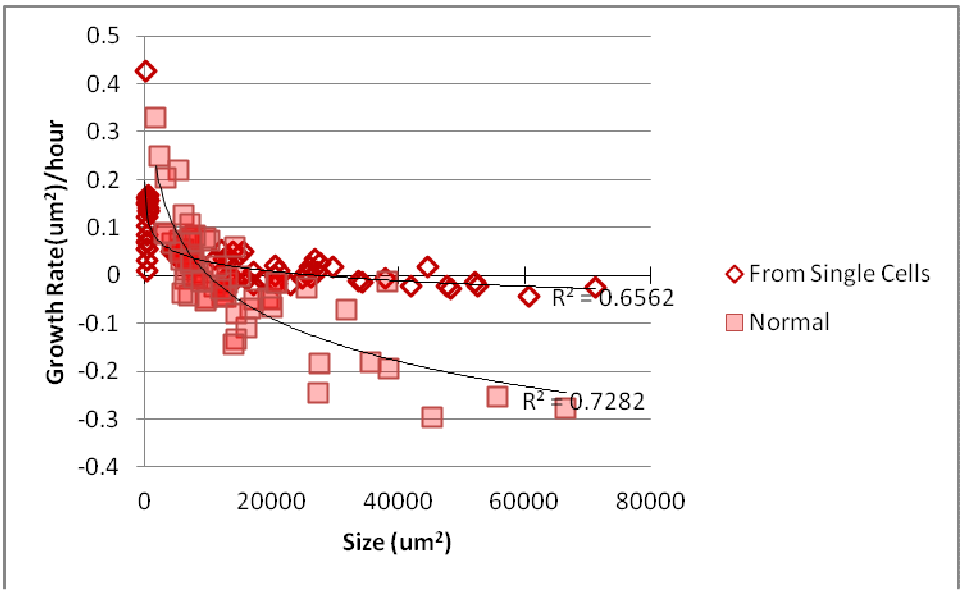


Figure 3.6. Growth rate by size. Growth rate by size across settling populations grown normally and from single cells obtained by digestion in lyticase and 2% β-glucuronidase/arylsulfatase.

Positive values indicate net growth while negative indicates division. A logarithmic line was fitted to the data and the R^2 value is 0.6562 for the single cell population and 0.7282 for the normal populations. Both have rapid growth rates at small size that decrease with large size. However, the replicates grown from single cells do not see the same overall decrease in growth rate that the transfer replicates do. We can define "size at reproduction" as when the curve crosses the zero line, which is greater for replicates grown from single cells. Figure 3.7 shows the growth rate of these treatments over time.

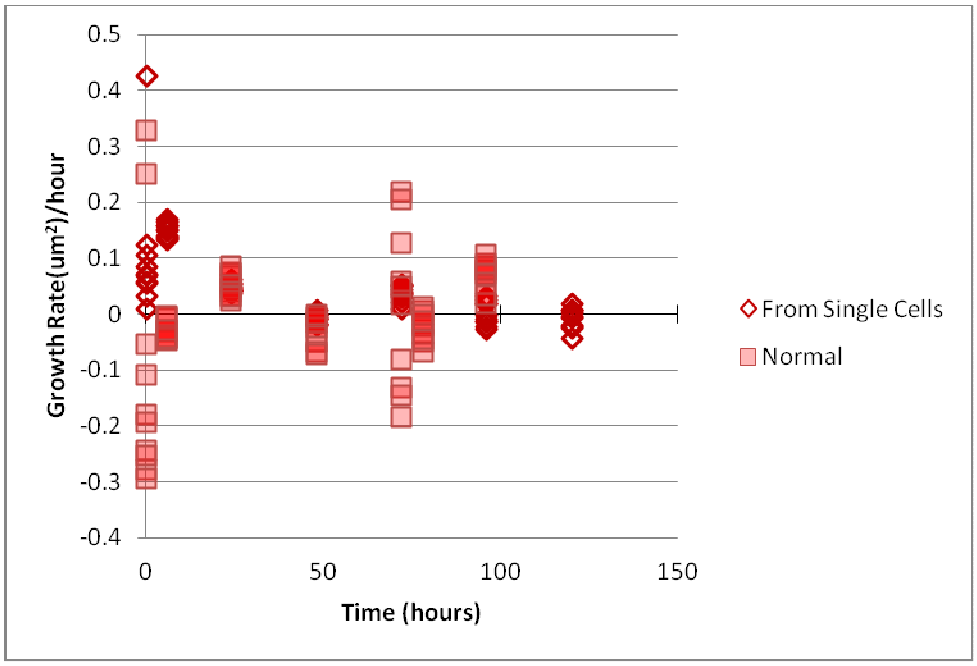


Figure 3.7. Growth rate over time. Growth rate over time across settling populations grown normally and from single cells obtained by digestion in lyticase and 2% β-glucuronidase/arylsulfatase. Positive values indicate growth while negative indicates division.

During selection, transfers occur at 72 hours. At 48 hours there is an overall decrease in growth rates, and at 72 hours there is a wide range of growth rates. Both treatments experience lower growth rates after the 72 hour mark.

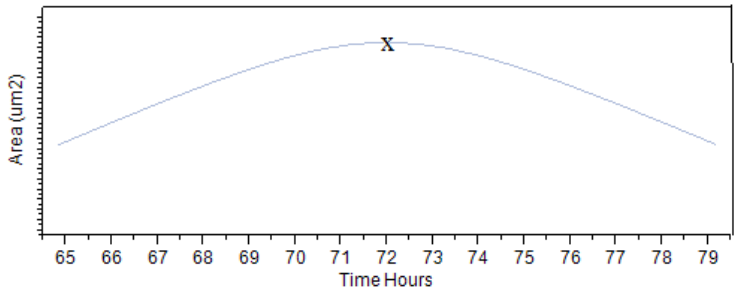
Rather than being triggered by cluster size or time, cluster division could naturally be triggered by media change or could have evolved to be triggered by media change during the fifth month of settling selection. There are three qualitative predictions of the effect of media change in normal settling replicates. These predictions, and results of the experiment, are illustrated in Figure 3.8.

1. When the transfer (and media change) occurs at the normal time of 72 hours, *Anabaena* grows until 72 hours, and then starts to divide (Figure 3.8A).
2. If division is triggered by transfer, with media change, and transfer is imposed earlier than normal, *Anabaena* will grow until the transfer, possibly not reaching

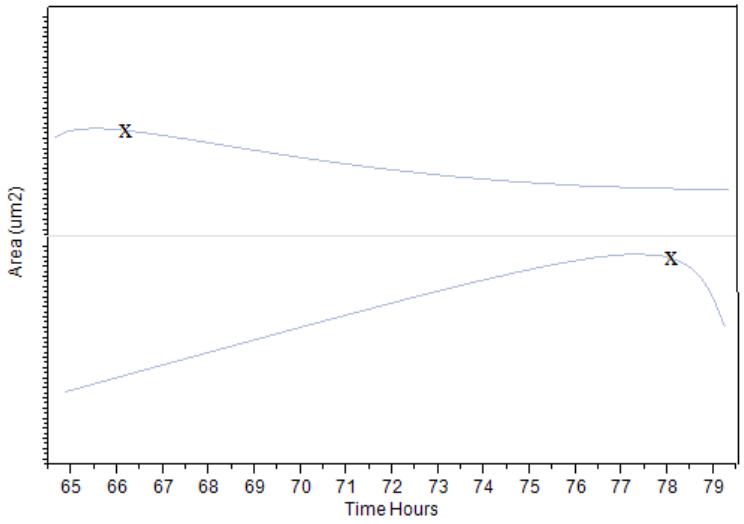
full size, and start to divide immediately after transfer, shifting the curve to the left (Figure 3.8B).

3. If division is triggered by transfer, with media change, and transfer is imposed later than normal, Anabaena will continue to grow until transfer, exceeding normal growth size, and then rapidly divide, shifting the curve to the right (Figure 3.8B).

A)


Area (um2)
65 66 67 68 69 70 71 72 73 74 75 76 77 78 79
Time Hours

B)


Area (um2)
65 66 67 68 69 70 71 72 73 74 75 76 77 78 79
Time Hours

C)

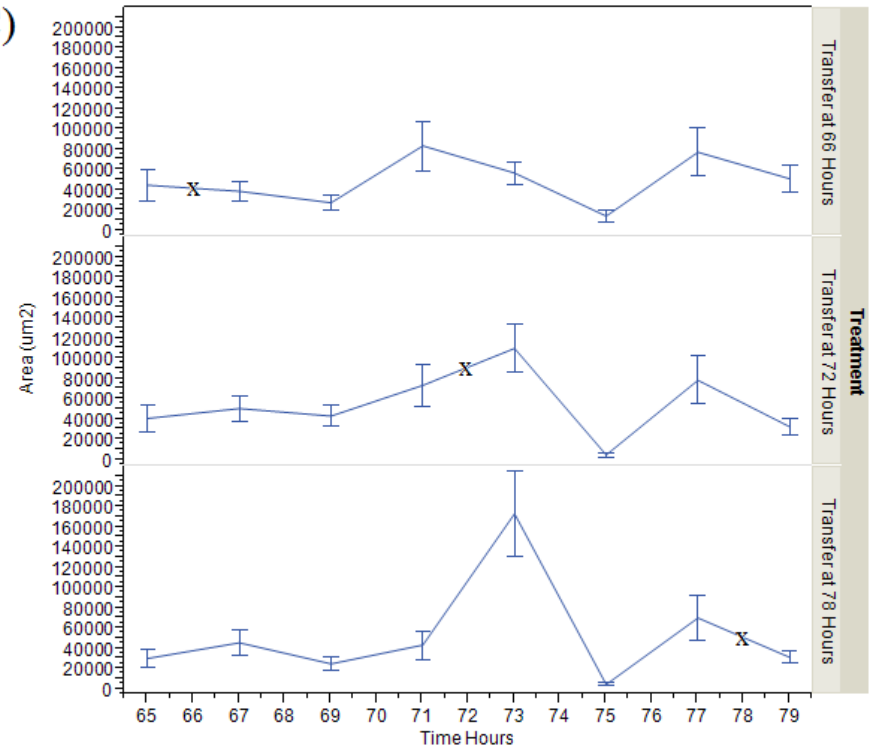

Area (um2)
200000 180000 160000 140000 120000 100000 80000 60000 40000 20000 0
Transfer at 66 Hours
Transfer at 72 Hours
Transfer at 78 Hours
Treatment
65 66 67 68 69 70 71 72 73 74 75 76 77 78 79
Time Hours

Figure 3.8. Effects of transfer time on size. A) Qualitative prediction of average size of *Anabaena* over time during normal proposed transfer regime with transfer occurring at 72 hours and B) Qualitative predictions of average area if i) If division is triggered by transfer and a transfer is imposed earlier than normal, and ii) If division is triggered by transfer and a transfer is imposed later than normal. Black x denotes when the proposed transfer would take place. C) Mean area over time with transfers at 66 hours, six hours before normal transfer regime, 72 hours, normal transfer regime, and at 78 hours, six hours after normal transfer regime. Error bars reflect standard error.

Actual results are shown in Figure 3.8C. When transfer is imposed at 66 hours, division starts one to two hours earlier than when it is imposed at the 72 hour transfer time, which I hypothesized, the population had become adapted to. When transfer is imposed at 78 hours, average size is larger than both 66 and 72 hour transfer, but division starts at the same time as 72 hour transfer.

Discussion

Under natural conditions, *Anabaena* reproduces by fragmentation. This is where single floating strands break off from one another, typically from a dead or stressed cell (Yoon et al 2008). However, with a change in the level of selection to tangles and clusters, there should also be a change in reproduction (Grosberg and Strathmann 2014). There are several proposed hypotheses of how the derived *A. variabilis* are persisting under settling selection conditions. One hypothesis is simply increased survival; where the largest of the clumps is sufficiently large to survive to the next transfer. However this strategy ignores any selective benefits of increased reproduction (Michod 2007), and substantial fitness increases could potentially be obtained by increasing the production of viable offspring (Simpson 2011). Simply splitting in half would avoid reduced settling selection survival, however, this is risky. In a population that is rapidly evolving for larger size, the production of only one offspring does not give good odds for continuation of the specific genome (Michod 2003). Or they could divide asymmetrically into several smaller pieces, of an optimal size, which would probably be able to grow large enough to survive settling transfer. This would increase reproductive output.

The images in Figure 3.1 and 3.3 indicate that there is a change in the mode of reproduction. *A. variabilis* goes from single filament fragmentation, to dividing into several smaller clumps that then grow until the next transfer. The juvenile clumps resemble the adult. The change in reproduction from single strands to small clumps also reflects a change in their life history. Fitness components have clear associations with different life history (Simpson 2011).

Reproduction may not rely on only one trigger. It could be a combination of media change, terminal size, or time (Hoek 1995; Simpson 2011; Michod 2007). Large size can be costly for fitness in terms of reproduction, especially in an organism which relies on availability and access to light (self shading) for nutrients and nutrient (diffusion) limitations, suggesting that reproduction may be triggered by reaching some optimum or terminal size (Michod 2003). However, in Figure 3.5, the replicates started from single cells go through divisions, marked by size decreases, at 48 and 120 hours, without having reached the average size that the replicates from normal transfers divide at. This is further shown in Figure 3.6, which suggests that an optimal or terminal reproduction size has not yet developed. This is seen by the size at reproduction, defined as where the curve crosses the zero line, which is different between the two treatments of growth from single cells and growth through the normal transfer regime.

Since media change and size do not seem to have a direct impact on reproduction, time appears to be the most influential factor. Transfers were conducted every 72 hours (±1 hour). In Figure 3.2, Figure 3.4, and Figure 3.5 the settling selected replicates are largest at time 0, 48, and 120, which is right before the times of transfer, almost as if they are somehow anticipating the transfer and dividing before fresh nutrient availability to give offspring the best opportunity for rapid growth rate. This is further shown in Figure 3.7, where the growth rate for each treatment is concentrated at each time point. Greater variation among replicates is only seen for transfer times 0 and 72, where *Anabaena* in the tube is both growing to the maximum size, but also starting to divide. Although time as a trigger has promising data, Figure 3.8 suggests that media change, or transfer time, has a minor effect on division as well. When the transfer was imposed six hours early, average size decreased 1-2 hours early.

It is highly plausible that time is the trigger for division. *Anabaena* has been shown to develop circadian rhythms (Heidorn et al. 2011). Developing a rhythm to the selection scheme would be a subsequent selection that operates to stabilize evolution (Schlichting 2003). It could also be seen as a strategy that determines ecological specialization (Simpson 2011).

Conclusion

Settling selected *Anabaena* evolve a change in their reproductive pattern and life history as they transition from filament fragmentation of single strands to division into several smaller clumps, resembling the parent. Despite possible costs of larger size, the selected *Anabaena* does not appear to reach an optimal or terminal size for division. There is a possible interaction of media change or transfer on reproduction. However, despite growth in constant light, the evolved *Anabaena* has developed a reproductive cue that directly correlates with time. This could suggest the development of a new mechanism for detection of various rhythms, such as anticipated nutrient availability.

Conclusion

Adaptation only occurs as an outcome of selection. Despite remaining primitively multicellular with constant morphology for billions of years, *Anabaena* is capable of rapid adaptive responses to selection. The initial response included a dramatic increase in size, which fits with the idea that the direction of selection for size change depends solely on ecological opportunity. Results also fit with the idea that size comes first and then changes that continue to provide fitness advantages follow, increasing complexity (Bonner 1998). There was a transition in Darwinian individuality, from a single strand to a clump, creating a higher group level production (Libby and Rainey 2013).

With continued selection, two distinct morphologies for increased size and growth rate were developed: tangle and cluster. The tangle and cluster morphology have proven to be heritable. Life history changed from fragmentation of short strands to reproduction of juvenile clumps, where a large clump breaks into several. The main trigger for reproduction appears to be time. This is despite growth in constant light, and could suggest the development of a new mechanism for detection of various rhythms, such as anticipated nutrient availability.

Future directions include the sequencing of the settling selection replicates genomes. Major transitions often only require a few mutational steps, and changes in a few genes (Grosberg and Strathmann 2007). Genetic barriers may be less restrictive than ecological barriers and rapid transitions do not require extensive expansion of genome complexity (Ratcliff et al. 2012; Ratcliff et al. 2013). This could help explain why *Anabaena* evolves rapidly in selective laboratory conditions, yet has remained primitive and unchanged in the natural environment. The tradeoff among increased size, relative growth rate, density, biomass, and settling rate is still not apparent. Fitness tradeoffs drive transitions (Michod 2007). Although the overall evolved process of reproduction is known, whether the clump dissociates by a single strand fragmentation within the parent, or across several strands, or by an entirely different process is unknown.

Heterocyst spacing and frequency are determined by genetic and environmental factors such as the availability of fixed nitrogen. Heterocysts occupy 5-10% of the mass in *Anabaena variabilis* (Heidorn et al. 2011). It would be interesting to see if changes in

complexity changed the count, spacing, or arranged them more toward the center of mass, where there is not as much light to optimize fixation and photosynthetic levels.

There are implications for biotechnology. Evolutionary biotechnology takes the principles of natural evolution and harnesses them in a goal-directed way to synthesize desired results. This allows for novel organisms and substances to be made that may never survive in the wild, or simply the optimization of existing ones. It allows problems associated with genetics and "traditional" methods to be solved (Schuster 1996). Some issues with *Anabaena variabilis* are that it includes multiple genes that code for same function, so knock outs and targeting by biobricks becomes difficult. It has low transformation efficiency, and all around improvement is needed on current genetic tools (Wang et al. 2012).

Cyanobacteria are hard to use for biomass due to cost of harvest and energy for harvest. To make harvest easier auto-floatation was induced by increasing hydrogen levels (Chen et al. 2014). Another method is to use algae floccation. This is where a chemical to induce floc is added; the algae clump together, sink, and then are removed. Evolutionary changes quickly produce populations that are resistance to flocculation and escape harvest. One way to reduce selection is by rotating harvest schemes (Bull and Collins 2012). The settling selected strains of *Anabaena* have evolved to settle out of solution. If this transition proves to be stable with reversed or relaxed selection, it would have huge implications. Even if the transition is not stable long term, it could be combined with other methods for increased efficiency. The only concern would be that the huge increase in growth rate opposed the maintenance of lipid storage that is desired for fuels (Bull and Collins 2012). This is something that would need to be analyzed in the settling evolved populations.

There are also implications for synthetic ecology, especially in the formation of a symbiosis between organisms. Symbiosis is the co-aggregation of multiple clones or species that create reliability on one another (Grosberg and Strathmann 2007). *Anabaena* exists in symbiosis with plants, such as Azolla. The *Anabaena* provides the Azolla with fixed nitrogen, and the Azolla provides the *Anabaena* with carbon sources. Nitrogen is

essential to life, but the majority of nitrogen needs to be fixed in order to be used, creating an opportunity for beneficial interactions (Rai et al. 2002).

Although most of the analysis was conducted after transfer 45, I did continue to selection experiment through transfer 96. It appeared as though all major qualitative transitions had evolved by transfer 45, but continued increase in size, density, and settling rate were observed. It can be assumed that the continued adaptations provided additional increase in complexity (Van Valen 2009). Transfer 96 populations could be studied to further prove if the lowest level of selection dominates due to higher heritability and shorter generation time (Simpson 2011).

I would like to investigate how stable the transition to tangles and clusters is. What happens when selection is reversed or relaxed? I started evolving replicate populations for reversal of increased size selection, selecting for the smallest, and removal of settling selection, where a set volume is transferred, from transfer 96 isolates. After 21 transfers there were still macroscopic clumps in both treatments. Can evolution be reversed?

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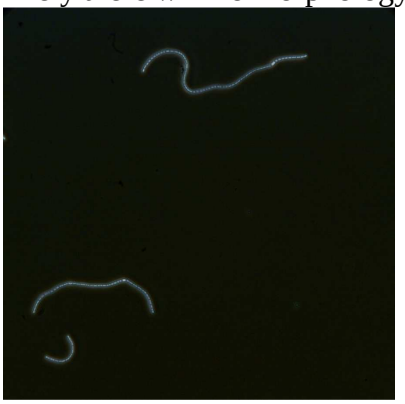
Appendix

Identifying Mechanisms in *Anabaena* at Transfer 45

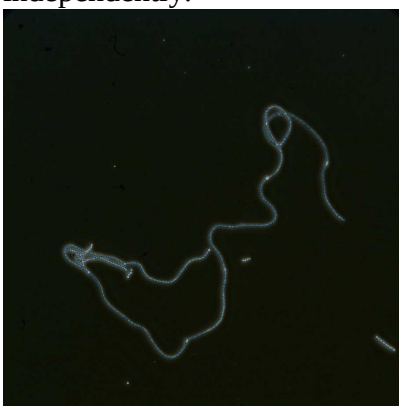
There are 600 images; 300 from non-settling populations and 300 from settling populations. Please look at each image and fill in one selection on the excel sheet for morphology present as either none, length, tangle, or cluster, using the rubric below. Feel free to scroll through the pictures first to familiarize yourself with the data. Use your best judgment to mark the morphology that you feel fits best with the image. Only one selection per image may be made. All images must be categorized.

Morphologies:

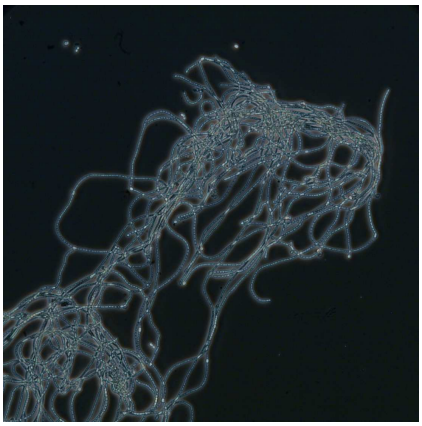
None: Non-settling populations are included in this analysis for a control, so it is very likely there will no morphology observed in an image.



Length: This will be hard to distinguish from none. Single strands are long, but still exist independently.



Tangle: Long strands winding within themselves and others, be careful to distinguish from a cluster.



Cluster: Small strands formed together into a cluster, be careful to distinguish from a tangle.