Investigating the Evolutionary Changes in Crabtree-negative Yeasts During a Long-term Evolution Experiment

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0.1 Abstract

The Crabtree effect is a metabolic strategy that allows yeast to ferment in the presence of oxygen. This is of interest as not all yeasts display this strategy, and nearly 100 years after its discovery it is still unclear what the overall benefit is. Two key theories attempt to explain the emergence of this phenomenon, the make-accumulate-consume theory and the rate/yield trade-off theory. The aim of this thesis was to investigate whether a trade-off between rate and yield develops in Crabtree-negative yeasts over the course of 1500 generations in a high sugar environment. Chapter Two demonstrates that growth rate is more likely to increase than decrease while growth yield is more likely to decrease than increase in the isolate-derived populations of yeast. We find that species that started out relatively fast, changed little while the slower species had more significant gains in growth rate. With growth yield, the species with initially high yield lost more significantly than the already low yield species. This could suggest there is an overall optimum growth rate and growth yield, that the species are evolving towards. In Chapter Three, ethanol production was measured using colorimetric tests and no change was observed to support the development of the Crabtree effect in these populations after 1500 generations. In Chapter Four growth yield was investigated using flow cytometry and it was found that several yeast populations both increased in cell size and decreased in growth yield. This is an interesting observation that has been observed in several previous experimental evolution experiments. In Chapter Five, as cell size is often associated with ploidy changes, DNA content was measured using DAPI and SYTOX DNA stains, detected by flow cytometry. This did not provide any statistically significant conclusions but highlighted the importance of employing further techniques to analyse the DNA content of these populations. This thesis has illustrated the importance of studying the competitive behaviours of microorganisms in isolation, where selfish traits appear to thrive.

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0.4 Abbreviations

ADH	Alcoholic dehydrogenase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CFU	Colony-forming units
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ESS	Evolutionary stable strategy
FACS	Fluorescence activated cell sorter
FSC	Forward-scattered light
GTP	Guanosine-5'-triphosphate
HPLC	High-performance liquid chromatography
HXT	Hexose transporter
MAC	Make-accumulate-consume
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
PBS	Phosphate-buffered saline
RPM	revolutions per minute
RYT	rate/yield trade-off
SM	synthetic minimal

TCA	Tricarboxylic acid
WGD	Whole genome duplication
YPA	Yeast peptone agar
YPD	Yeast extract peptone dextrose

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CHAPTER ONE

Introduction

Rick: ''Are you sure you want to be playing around with that thing?''

Evelyn: ''It's just a book. No harm ever came from reading a book.''

-The Mummy.

1.1 Introduction

To understand evolution, it is necessary to consider the ecological circumstances an organism is evolving in, i.e. its niche in a particular environment. The occupation of a niche appears to be driven by resource supply and competition for such, and is thus an important ecological factor driving the evolution of organisms (Spor et al. 2009b). With the potential of organisms to occupy the same or overlapping niche, different strategies are implemented by organisms to dominate the niche over another competitor (Nowak 2006; Hardin 1960).

Microorganisms are particularly useful in the study of evolution towards a given niche due to their short generation times and well-established methods for studying their genomes (Kussell 2013). This allows for in depth real-time investigation of evolution in action in controlled situations. Yeasts provide an interesting system for studying resource use and competition in particular, due to the various pathways they can utilise to metabolise their preferred carbon sources.

One strategy of interest is that of respiro-fermentative metabolism as exhibited by *Saccharomyces cerevisiae.* As outlined further below, this strategy known as the Crabtree effect, is not as energy efficient as those that are purely respiratory. However, this strategy has developed independently and persisted in two yeast lineages which suggests it has some selective advantage over more efficient strategies (Rozpędowska, Hellborg, et al. 2011). This thesis aims to address whether the selective advantage is due to a trade-off between growth rate and growth yield, resulting in a faster metabolic pathway that can be utilised to outcompete more efficient competitors for the same niche.

1.2 Metabolism

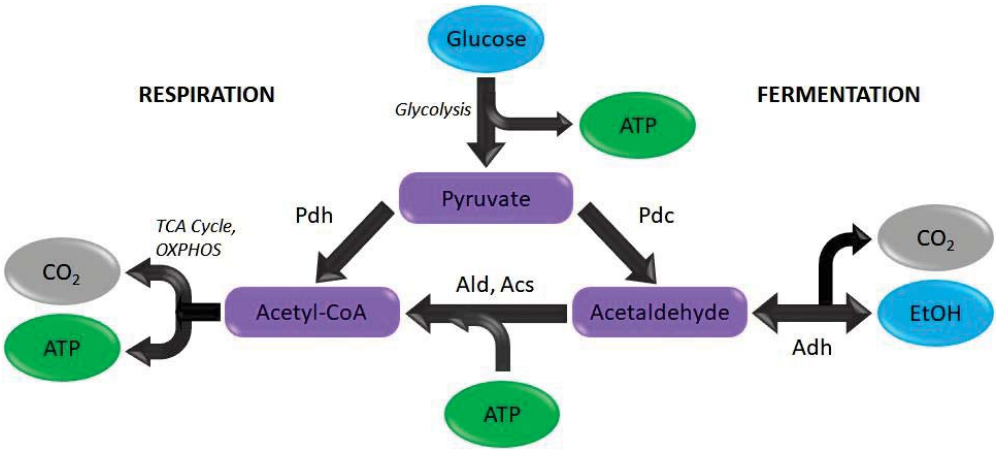


Figure 1.1: Yeast Energy Metabolism. Adapted from Pfeiffer & Morley (2014). Glucose is converted into pyruvate via glycolysis which produces 2 ATP. Pyruvate can then be processed through two pathways, respiration or fermentation. In respiration pyruvate is converted in acetyl-coenzyme A by pyruvate dehydrogenase (Pdh), which is then oxidised to carbon dioxide in the TCA cycle and oxidative phosphorylation (OXPHOS) to produce ATP. In fermentation, pyruvate is converted in to acetaldehyde by pyruvate decarboxylase (Pdc) and then converted into ethanol by alcohol dehydrogenase (Adh) producing carbon dioxide as a waste product. Ethanol can be reincorporated into the system and directed into the TCA cycle by converting ethanol back to acetaldehyde. Acetaldehyde can then be converted into acetate by aldehyde dehydrogenase (Ald) and acetate is converted into acetyl-coenzyme A by acetyl-coenzyme A synthase. The conversion between acetaldehyde and acetyl-coenzyme A requires an input of energy so will still produce less ATP overall than if only respiration was utilised.

In cellular energy metabolism Adenosine triphosphate (ATP) is a key compound which all free-energy dependent processes are reliant on. Yeasts with anaerobic growth capabilities can call upon two different pathways to process carbohydrates into ATP, respiration and fermentation as shown in Figure 1.1. Sugars such as glucose are converted into pyruvate through glycolysis, a metabolic pathway employed with deviations in nearly all organisms (Berg et al. 2002). Pyruvate can then be processed via the high yield respiratory pathway (in *Saccharomyces cerevisiae* approximately 18 ATP per glucose) using oxygen or, in the absence of oxygen, via the fermentation pathway, that has a much lower yield (2 ATP per glucose) (Verduyn et al. 1991; Berg et al. 2002). The respiratory pathway transforms pyruvate into acetyl-coenzyme A using pyruvate

dehydrogenase. This is then oxidised in the tricarboxylic acid (TCA) cycle, producing compounds such as GTP and NADH which can be further oxidised in the mitochondrion to produce more ATP. The fermentative pathway however transforms pyruvate into ethanol using pyruvate decarboxylase and alcohol dehydrogenase. While the fermentative pathway results in a lower energy yield than respiration, the produced ethanol can be readmitted into the system and directed into the TCA cycle once oxygen is available. However, this conversion comes at a cost of ATP.

Whilst the degradation of sugar via the fermentation pathway allows for some yeasts to adapt to anoxic conditions, at high levels of sugar and oxygen some yeasts can produce ATP via respiration, fermentation, or a concurrent use of both pathways (Boer et al. 2003; van Hoek & Hogeweg 2009; De Deken 1966). Utilising fermentation in the presence of oxygen is a trait most associated to *Saccharomyces cerevisiae*, although the *Dekkera bruxellensis* lineage also exhibits such a trait (Rozpędowska, Hellborg, et al. 2011). This ability to ferment in the presence of oxygen is known as the Crabtree effect (Crabtree 1929). Those yeasts that exhibit the Crabtree effect are known as Crabtree-positive while those lacking the ability are Crabtree-negative, examples of which are studied in research on yeast life strategies (Hagman & Piskur 2015; Dashko et al. 2014).

1.3 The Crabtree Effect

The Crabtree effect has been demonstrated in both chemostat (Postma & Verduyn 1989) and batch culture (Verduyn et al. 1984). These experiments will be reviewed here due to the key role they have played in the understanding of the Crabtree Effect.

For clarity, it is important to briefly discuss the experimental designs of chemostats and batch culture (although these will be further discussed in Chapter Two). In a chemostat, cells are grown at a specified volume of media which is continuously diluted by the simultaneous addition of fresh medium and removal of old medium and cells (Ziv et al. 2013). When the dilution rate increases, the population is required to replicate faster to maintain a steady state population size in the chemostat. This implies an increase in the residual glucose concentration. In contrast, in batch

culture cells are grown in a specified volume of media that does not get diluted, but entirely replaced with fresh medium at select time intervals (Gresham & Dunham 2014). This produces a glucose concentration that decreases over time and determines the population size but not the growth rate.

As mentioned, both these experimental methods have been utilised to demonstrate the Crabtree Effect. During the investigation of the physiology of *Saccharomyces cerevisiae* in glucose limited chemostat (Postma & Verduyn 1989) it was observed that at low dilution rates carbon dioxide (CO_2) production equalled oxygen (O_2) uptake. They found that biomass yield was high at around 0.5 g/g while the residual glucose concentration was low. With increasing dilution rate, both CO_2 production and O_2 uptake increased and remain coupled until a critical point was reached. After this critical point, at a dilution rate of 0.38 h^{-1}, fermentation was activated. This could be seen in the uncoupling of CO_2 and O_2 with the rapid increase in CO_2 production while O_2 uptake remained relatively stable or declined (Van Hoek et al. 1998). At the same point the biomass yield sharply decreased to below 0.2 g/g. In batch culture, aerobic fermentation in *S. cerevisiae* was recorded above glucose concentrations of 150 mg. 1^{-1} (Verduyn et al. 1984).

These observations are consistent with the view that respiration is confined to a maximal rate, that is, if more sugar is available than can be respired then the fermentation pathway is activated in response. Although there is debate on the cause of this mechanism, many suggest that a limited capacity for membrane proteins and enzymes results in this overflow fermentation metabolism (Hagman & Piskur 2015; Vemuri et al. 2007; Zhuang et al. 2011). However, some suggest that if this were the case progress towards an advantageous mutation which increases the intermediates might be expected, as opposed to the evolution of an entirely new pathway (Molenaar et al. 2009).

The Crabtree effect was also investigated in batch culture, which allows for the control of the glucose concentration to establish what concentration the fermentation pathway is activated in

S. cerevisiae (Verduyn et al. 1984). This experiment demonstrated the Crabtree effect at a glucose concentration of about 150 mg/l.

Of the many questions that result from these experiments, it is of particular interest to this study how this adaptation occurred and when in evolutionary history it arose. This will be discussed in the next section.

1.4 Phylogenetic History

The emergence of the fermentative metabolism in the phylogenetic history of yeast has been associated with the evolutionary event of the whole genome duplication (WGD) that occurred in the clade containing *Saccharomyces cerevisiae*. This has been correlated with the emergence of fruiting angiosperms around 125 million years ago (Conant & Wolfe 2007; Kellis et al. 2004; Merico et al. 2007; Bremer 2000; Magadum et al. 2013). It is proposed that any selective advantage that the WGD provided may have been linked to the presence of excess glucose in the form of fruit produced by the angiosperms (Conant & Wolfe 2007). This provided a new niche to adapt and compete for. A whole genome duplication however is a costly event (Wagner 2005) that would have resulted in reproductive isolation and thus a short-term detriment to the organism (Greig et al. 2002). While in the long run the WGD was succeeded by gene loss that returned most loci to single copies, many loci remained duplicated. These deletions were not simultaneous with the WGD, thus a period of lower viability occurred during which the ploidy of the yeast was higher than that of its ancestor. This suggests there is a cost effective advantageous gain in this investment to justify temporary reproductive isolation. In fact, many organisms have exhibited WGD suggesting it is a very beneficial evolutionary event (Ohno 1970; Taylor & Raes 2004; Conant & Wolfe 2008; Makino & McLysaght 2012). For *Saccharomyces cerevisiae,* many of the fermentative abilities of this yeast can be linked to this event, or were enhanced in the subsequent refining events (Liti & Louis 2005). In a study to reconstruct the steps between *Saccharomyces cerevisiae* and its ancestor using current yeast species (Gordon et al. 2009) 124 genes were identified that had been acquired since the WGD, many of which were associated

with fermentation and nutrient uptake. This suggests that the WGD is not wholly responsible for the development of the fermentative abilities of *S. cerevisiae* but likely played a key role in its development.

There are other genomic events that might be linked to the development of the Crabtree effect (Conant & Wolfe 2007; Hagman & Breunig 2013; Merico et al. 2007). Duplication usually precedes adaptation towards a novel feature, but it does not necessarily require entire chromosome duplication to result in such (Llorente et al. 2000). Indeed, some have linked the Crabtree Effect to the successive events, further duplications, deletions and general streamlining after the WGD, which were the real mechanistic origin of the pathway (Langkjaer et al. 2000; Hagman & Piskur 2015). The importance of deletions on developing higher glycolytic flux (Conant & Wolfe 2007), streamlining (Merico et al. 2007), and a number of other events on the development on the genome of present day post-WGD yeasts (Dujon et al. 2004), has been highlighted. It is widely believed that genome duplication provides the genetic raw material for new functions to develop, from mutation, allowing for large scale adaptation, such as would be required for exploiting a novel resource. It has been established that pre-WGD yeasts appear to have the traits of the progenitor, dependent on oxygen and poor ability to accumulate ethanol, while post-WGD yeasts are fermentative, accumulate ethanol and are less dependent on oxygen (Rozpędowska, Galafassi, et al. 2011).

1.5 Mechanistic Origin

Of the many studies devoted to discovering the causes of the Crabtree Effect, some suggest that at high levels of glucose a regulatory glucose repression pathway is activated (Raghevendran et al. 2005; Otterstedt et al. 2004; Vemuri et al. 2007; Kurtzman & Piškur 2005). Other theories suggest it is due to an overflow of the respiratory metabolism (Vemuri et al. 2007; Postma & Verduyn 1989), or that the mechanism develops as the result of enzymatic limitations (Smallbone et al. 2013) or oxygen level limitations (Holm Hansen et al. 2001; Merico & Galafassi 2009; Aceituno et al. 2012; Visser 1990). It has been proposed that while glucose levels may regulate

the expression of the Crabtree effect (Teusink et al. 1998), there is stronger support for the idea that another trigger is responsible for prompting respiratory suppression. For example, studies have shown that mitochondrial proteins, such as ribosomes, may limit the respiratory pathway and therefore cause a bottlenecking effect that results in activation of the fermentation pathway (Poole et al. 2012; Chatenay-Lapointe & Shadel 2011; Rintala et al. 2011; Wardrop et al. 2004).

There have been numerous experiments with the aim to delve deeper into yeast metabolism. In one such experiment using an upshift chemostat, where bursts of glucose increase over a set period of time, Crabtree-positive and Crabtree-negative yeasts were both studied in order to gain a better understanding of yeast metabolism. The findings indicate that Crabtree-positive yeast consume 50% of the extra resource, whereas Crabtree-negative yeasts only consumed 10%

(Wardrop et al. 2004). This suggests a potential evolutionary advantage in yeasts utilising the Crabtree effect in a sugar rich environment, which could potentially explain its origin.

1.6 Theories Behind the Evolution of the Crabtree Effect

The most fundamental evolutionary advantage of using the fermentation pathway, is the ability of anaerobic growth. However, it is not clear what benefit Crabtree-positive yeasts reap from fermenting in the presence of oxygen. It is apparent that there is a disadvantage in the form of reduced growth yield.

There have been suggestions that the benefit lies in the capacity to bypass bottlenecking in enzymes (Frick & Wittmann 2005a; Smallbone et al. 2013) and specifically in membrane proteins (Rintala et al. 2011; Zhuang et al. 2011), or the limited availability of intermediates (Molenaar et al. 2009; Goel et al. 2012; Pfeiffer & Bonhoeffer 2004). The aerobic fermentative metabolism thus might act as an overflow metabolism when the respiratory pathway is at maximum capacity (Hagman & Piskur 2015; Vemuri et al. 2007). The use of fermentation to bypass a bottleneck is consistent with the rate-yield trade-off hypothesis, which is outlined further below in section 1.6.2. However, it has also been proposed that ethanol is produced to act as a toxin (Goddard

2008b; Hagman et al. 2013; Piskur et al. 2006), which forms the core of the make-accumulate-consume hypotheses discussed in further detail in section 1.6.1.

It is also plausible that the benefit of the fermentative pathway is not in its ability to produce a toxin but in the act of doing so it can alter its environment through heat production (Goddard 2008b) or in its ability to relieve stress from the reactive oxygen species burden in respiration (Slavov et al. 2014). In the following sections, the most popular theories are presented, namely the make-accumulate-consume theory, and the rate/yield trade-off theory. This latter theory will be the basis of this thesis.

1.6.1 Make-Accumulate-Consume Theory

From the genetic work carried out investigating the Crabtree effect, one event in particular gained attention in the field. This was the duplication of alcohol dehydrogenase (Adh) resulting in two distinct enzymes in the present-day Crabtree-positive yeasts. These two Adh enzymes have differences in both expression and functionality. Adh1 has greater proficiency in ethanol excretion while Adh2 has greater inclination to uptake and convert ethanol into acetaldehyde. Thomson et al (2005) investigated the role of the hypothetical ancestor alcohol dehydrogenase of these two homologs (Thomson et al. 2005). The kinetics of the resurrected ancestral Adh was shown to resemble the kinetics of Adh1 rather than Adh2. This study argued that the role of the ancestral Adh therefore was to excrete ethanol anaerobically and had no purpose for reincorporating the ethanol back into the system. Before the Adh duplication, a separate event to the WGD, it is assumed that the ancestral yeast did not have the same qualities as the aerobic fermentative present-day yeasts. That is, it did not consume ethanol and therefore also did not accumulate ethanol. This trait of the present-day yeasts has likely occurred in response to competition with other organisms.

This study led to the development of the make-accumulate-consume theory (MAC) as an explanation for the emergence of respiro-fermentative metabolism (Piškur et al. 2006). This theory suggests that the Crabtree effect allows yeasts to ferment glucose in order to defend a

carbon source, such as sugar-rich fruit, from competitors utilising the toxicity of ethanol at the same time. The production of ethanol using these Adh enzymes allows for the accumulation of a separate resource to later be consumed once the original carbon source is depleted. This resource would be useless to the competitors. Due to the low ATP production of the fermentative pathway and the loss in biomass yield, the Crabtree effect is seen as wasteful and energetically costly even when the ethanol is reintroduced into the system (Thomson et al. 2005) as there is still a loss of ATP due to the conversion of ethanol requiring one ATP.

Other evidence given to support the MAC theory comes from the believed parallel evolution of *S. cerevisiae* (Piškur et al. 2006) and *Dekkera bruxellensis* (Rozpędowska, Hellborg, et al. 2011). This research concluded that the development of ethanol as a toxin is a valuable strategy that has occurred more than once, separate from the whole genome duplication, and therefore must be the overall advantage the Crabtree effect supplies.

The MAC theory, however, does not explain why new yeast variants with ethanol production have benefits over ancestors without. The ancestor and new variant would likely have similar resistance to ethanol; therefore, an explanation that a new variant can out-compete the ancestor might fall too short if the toxic effect is the only benefit of fermentation. In other words, utilising a toxin that all the surrounding competition is resistant to will not be an effective strategy.

1.6.2 Rate/Yield Trade-off

An alternative theory on the origin of the Crabtree effect is the rate/yield trade-off (RYT) as outlined by Pfeiffer et al (Pfeiffer et al. 2001). This theory suggests that trade-offs can emerge between the rate of ATP production and the yield of production, and subsequently the growth rate and growth yield of the organism. For clarity, ATP yield is defined as the amount of ATP produced per unit of substrate while the rate of ATP production is the amount of ATP produced per unit of time. A trade-off would produce a bias towards one trait at the cost of the other. Between ATP rate and yield a trade-off can produce ATP in either a fast and inefficient or a slow and efficient way. A fast and inefficient metabolism would result in a high growth rate and a low

growth yield, while a slow and efficient metabolism would result in a low growth rate and high growth yield. If considered in terms of a carbon source, when a larger amount of sugar is available than can be processed through respiration there are two strategies that can be employed. The yeast may either process the excess sugar through the fermentation pathway or continue using only the respiration pathway. These two strategies correspond to the difference between Crabtree-positive and Crabtree-negative yeasts, the former employs the fermentation pathway during aerobic growth, the latter does not. The RYT proposes that for many microorganisms there is a greater fitness advantage in rate of growth than in yield over a wide variety of environmental conditions (Pfeiffer & Morley 2014).

A trade-off like this could arise for various reasons, for example as a result of the thermodynamic constraints of ATP production or mechanistic constraints of metabolic pathways such as intermediate costs (Pfeiffer & Bonhoeffer 2002; Heinrich & Schuster 1996; Pfeiffer & Bonhoeffer 2004). Evidence in *Escherichia coli* exhibited trade-offs not between populations but in fact between clones in a population. This suggests that rate/yield trade-off is on a clonal level rather than a population level (Novak et al. 2006). Experimental evidence has also demonstrated that engineered yeast strains exhibit trade-offs between rate and yield, depending on the spatial and temporal structure of the environment (Maclean & Gudelj 2006).

RYT and the toxic effect of ethanol are not mutually exclusive. Both can contribute to the selective advantage of aerobic fermentation. The difference between the two theories is the focus of what specifically provides the selective advantage, an ability to kill off competitors through ethanol production or the increased rate of ATP production to outcompete more closely related competitors. The benefit of the toxic effects of ethanol are viewed as a secondary, but consequently unnecessary, side effect of a metabolic pathway that allows for a greater competitive advantage.

1.6.3 Evaluation of Theories

Evidence in support of MAC comes from the combination of comparative analyses of yeasts and the reconstruction of the hypothetical ancestral Adh (Thomson et al. 2005; Piškur et al. 2006; Rozpędowska, Hellborg, et al. 2011; Merico et al. 2007; Hagman et al. 2013). While the toxic effect of ethanol is apparent, there is no direct supporting evidence that the selective advantage of the Crabtree effect correlates with this specific trait. One particular criticism of this theory is that isolates, such as laboratory samples which are devoid of competition from other microbial species, do not lose the ability to ferment aerobically (Jasmin et al. 2012; Ferea et al. 1999). No study has come to light in the review of this thesis that demonstrates a loss of fermentation in isolation. Under the MAC theory, a loss of fermentation would be expected in isolation because it suggests that fermentation occurs only in the presence of microbial competitors and is energetically costly. Therefore, in an environment without such competition, the fermentation pathway would be redundant and unnecessary. This would not appear to be an especially complicated loss when it has been shown that the copy number of hexose transporter (HXT) genes and the expression of key HXT genes can affect the level of aerobic fermentation and success of growth on particular sugars (Reifenberger & Freidel 1995; Lin & Li 2011; Otterstedt et al. 2004).

Another observation in contradiction of MAC is the production of ethanol at low glucose concentrations (< 1g/l). Ethanol toxicity levels usually occurs at several grams per litre, at sugar concentrations below 1g/l there is not enough ethanol to produce a toxic effect (Casey & Ingledew 1986). Ethanol tolerance of bacteria has been reviewed, and toxicity has been recorded between 6-20% (v/v) depending on the species (Ingram & Buttke 1985). Similarly, if ethanol toxicity was the benefit of this pathway it would not explain why metabolic shifts in energy pathways occur in other systems not producing ethanol, for example the Warburg effect in tumour cells (Warburg 1956) and in some bacterial systems (Molenaar et al. 2009).

Criticism may also be directed towards RYT, however. Direct evidence in the yeast metabolism has yet to be presented. Many studies have tried and failed to find evidence of a rate/yield trade-

off (Fitzsimmons et al. 2010; Velicer & Lenski 1999). It has been suggested that this is due to the preference for suspension culture and an unstructured environment, when an emulsion-based propagation may more successfully demonstrate this trade-off, albeit for the selection of yield rather than rate (Bachmann et al. 2013). There are also other explanations for the selective advantage of the Crabtree effect, such as heat production causing a modification of the environment, or other complex factors such as osmotic constraints, intermediate availability and reactive oxygen species to name but a few (Goddard 2008a; Molenaar et al. 2009; Zhuang et al. 2011; Heinrich & Schuster 1996; Goel et al. 2012; Pfeiffer & Bonhoeffer 2004; Slavov et al. 2014). Due to the lack of experimental evidence it would be of interest to explore the rate/yield trade-off in yeast. While exploring the loss of fermentation from a Crabtree-positive yeast in a selection for yield experiment would prove enlightening, these experiments are more difficult to execute than an experiment focusing on rate. Therefore, exploring the selection for rate in Crabtree-negative yeasts would be the next ideal scenario.

1.7 Game Theoretical Considerations

Natural selection is often depicted through the use of fitness landscapes (Nowak 2004) in which an evolving population shows a steady climb to optimal fitness. Optimisation theory is a common tool for studying the trajectory towards an optimum. However, this framework does not take into account that as an organism adapts to its surrounding environment, these adaptations can change the environment, causing a dynamic fitness landscape. In a dynamic fitness landscape, optimum fitness cannot necessarily be achieved due to a constantly shifting and changing landscape resulting in there possibly being no optimum fitness.

The mathematical framework to account for such a situation is found in game theory (Pfeiffer & Schuster 2005). Originally developed in the context of human and economic decisions (Kemeny et al. 1956; Neumann 1928), game theory was applied to biological scenarios to investigate evolutionary populations (Nowak & Sigmund 2004; Pfeiffer & Schuster 2005) including the behaviour of macroorganisms (Axelrod & Hamilton 1981) and microorganisms (Pfeiffer et al.

2001; West & Buckling 2003; West et al. 2006). Rather than looking at fitness reaching an optimum, game theory views fitness as dependent on the evolving population itself, thereby creating a dynamic fitness landscape (Ebner et al. 2000; Pfeiffer & Schuster 2005). An example of this is found in host-parasite interactions, where a parasite adapts to infest the most abundant host, making it an advantage to be a rare mutant of the host. However, this mutation will become more common due to its advantage against parasite infection. Once this mutant becomes the majority, the parasite adapts to infest it and it is no longer an advantageous mutation. Here evolution constantly favours rare variants, which is clearly not compatible with the population approaching optimal fitness in a static fitness landscape (Barrick & Lenski 2013).

With the potential for systems to involve such complex or cyclic behaviour, researchers develop alternatives to optimisation to characterise long term evolutionary outcomes. One such method is to look for evolutionary stable strategies (ESS), as defined by J. Maynard Smith and G. R. Price, where upon adopting said strategy no mutant could potentially have higher fitness than the individual or population using an ESS. This means the ESS population would be at the highest it can be on its dynamic fitness landscape at that time (Maynard Smith & Price 1973; Nowak & Sigmund 2004). There is no requirement however that there be an ESS in a system.

Application of the game theory to the MAC and RYT theories, results in two very different characteristics. MAC resembles a rock-paper-scissors game. This game employs three strategies as demonstrated in Figure 1.2 and has been modelled and experimentally analysed in a number of systems, ranging from plants and microbes to lizards (Lankau & Strauss 2007; Károlyi et al. 2005; Nahum et al. 2011; Kerr et al. 2002; Sinervo 2001). In particular the production of a toxin has been demonstrated to follow the rules of this game (Kerr et al. 2002; Nahum et al. 2011). As this strategy would be seen as producing a public good then it is plausible that the strategy could be invaded by “cheaters” that do not produce the public good (the toxin). The invasion of cheaters, such as a resistant strain that does not produce the toxin, would result in an eventual taking over of the system. Naturally this strain would also be susceptible to cheaters that didn’t have resistance or produce a toxin.

The RYT strategy however does not view ethanol as a public good, but more as a destruction of a public good, the carbon source. The inefficient fast metabolic trait is not beneficial to the overall population but to the individual. This resembles the Tragedy of the Commons (Hardin 1968) or Prisoner Dilemma (Axelrod & Hamilton 1981). The players have a choice between cooperating and defecting (cheating). When an individual cooperates, it pays the cost of cooperating, however also gains the benefit only if the other player cooperates too. If an individual cheats, it will not pay the cost but still might benefit from the cooperation of the other player. Thus, regardless of the other player's choice, the most beneficial choice is to always defect, as it avoids the overall cost of cooperating. This has been demonstrated in models (Schuster et al. 2011; Schuster et al. 2008; MacLean 2008) and experimentally (Greig & Travisano 2004).

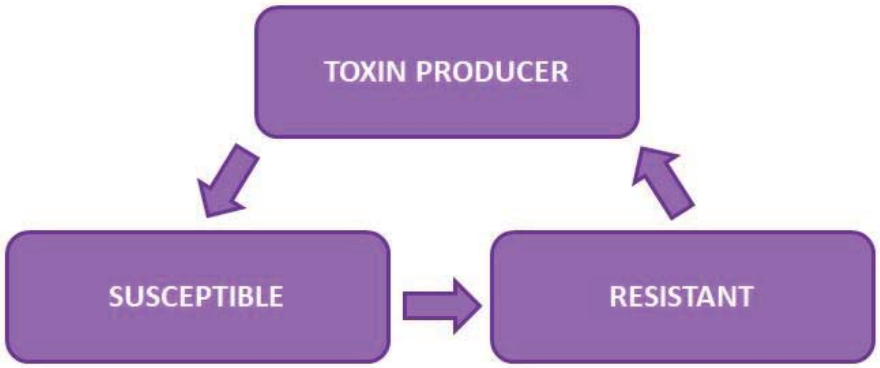


Figure 1.2: Rock-Paper-Scissors Game. The figure demonstrates the rock-paper-scissors dynamic of toxin production. Toxin producer beats the susceptible strain because the susceptible strain cannot resist the toxin, the susceptible strain beats the resistant strain due to not having the cost of the resistant trait. While lastly the resistant strain beats the toxin producer due to not being susceptible to the toxin and not paying the cost of the production of said toxin. No one strategy can beat both the other strategies and no ESS is present (Kerr et al. 2002; Biernaskie et al. 2013; Nowak 2004).

Cooperation has been demonstrated as a common strategy in social communities; however, the fundamental point behind natural selection would be of an overall selfish nature unless an underlying system was directing the situation otherwise. Defectors are a prospective issue in a social system (Velicer et al. 2000) as they take advantage of the cooperative nature of others to

benefit without cost. Taking the inherently selfish nature of the prisoner's dilemma into account, it is difficult to understand how altruistic behaviour would evolve if it is costly to maintain (Nahum et al. 2011). However, cheaters are a common occurrence (Velicer et al. 2000) and it has been shown that there may be some benefit to the group in mixed cultures (Maclean et al. 2010).

1.8 Implications

Discovering the benefit of a trait that could be interpreted as costly to the overall population is a promising area of research. This key metabolic pathway in yeasts are of paramount importance to such industries as wine-making and beer brewing, and thus a deeper understanding of its origin could have a crucial impact on how yeasts adapted for use in future. Of further importance is the strong link of the Crabtree effect with the Warburg effect. This linkage with the metabolic strategies of tumour cells could allow for the future development of strategies against tumour growth by further understanding how this metabolism occurred and developed (Blount et al. 2008; Diaz-Ruiz et al. 2011). A greater understanding of the strategies employed by yeasts may be gained from the application of game theory. This is turn can allow for the adaptation of novel experimental techniques to investigate an area of research that has remained elusive.

1.9 Aims

Previous work on engineered yeast strains has highlighted the potential interest in the field to determine if the trade-offs between rate and yield demonstrated in the study (Maclean & Gudelj 2006) would also occur in non-engineered, natural yeast strains. There is also interest in whether this will be achieved through the development of increased fermentative ability to result in a higher growth rate. Therefore, the primary aim of this thesis will be to investigate whether the Crabtree effect is an evolutionary trade-off response to resource competition by providing a faster metabolic route rather than a means to produce a toxin to kill off competitors. This will be investigated using experimental evolution study, employing serial transfer batch cultures at a high glucose concentration, on a selection of Crabtree-negative or weakly respiro-fermentative, non-

engineered, natural yeasts. These yeasts are expected to evolve, over the course of approximately 1500 generations, towards an increase in their growth rate at the cost of growth yield to outcompete the ancestor strain.

Chapter Two presents the data from the long term experimental evolution study investigating whether in a high glucose environment Crabtree-negative yeasts increase their growth rate at the cost of yield. Chapter Three presents data investigating the ethanol production of the evolved lines compared to the ancestor lines. Chapter Four aims to support the data from Chapter Two, by investigating the changes in cell size and cell counts using flow cytometry and CFUs. Chapter Five presents exploratory data into the changes in DNA quantity in the evolved lines compared to the ancestor and whether any changes in fitness could be detected. Finally, Chapter Six is the discussion and synthesis chapter, looking to collate the data from the previous chapters and present the overall conclusions and limitations of the thesis as a whole.

CHAPTER TWO

Long Term Experimental Evolution of Yeasts in a High Glucose Environment

''The light that burns twice as bright burns half as long – and you have burned so very very brightly, Roy.''

-Tyrell, Blade Runner.

2.1 Introduction

The Crabtree effect, as highlighted in the Introduction, is a trait present in some yeast species that allows for fermentation of sugars in the presence of oxygen. The evolutionary advantages of this trait are the subject of debate. The aim of this thesis is to investigate whether the Crabtree effect evolves as a consequence of resource competition in a high sugar environment. Experimental evolution is a popular and well-suited technique to address such a question. This chapter will briefly introduce and review experimental evolution as a technique and justify its use for this study. The chapter will then go on to present the specific methods used for a long-term evolution experiment conducted with a selection of Crabtree-negative yeast isolates from natural populations. The aim of this experiment is to examine how growth rate and growth yield change as the evolving yeast populations adapt to a high sugar environment.

2.2 Experimental Evolution

Experimental evolution provides a unique method of studying natural selection and the key changes in genotype and phenotype in response to a manipulatable environment over a large number of generations (Barrick & Lenski 2013; Kussell 2013; Ratcliff et al. 2013). The technique allows an experimenter to observe the occurrence and consequence of novel mutations in a controlled, repeatable and catalogued ecosystem, monitored over time (Lenski & Travisano 1994). This is particularly useful when attempting to simulate an event of interest in an organism's evolutionary history and develop a model of an organisms fitness in response to such (Kussell 2013). Studies investigating traits relevant to adaptation often exploit *de novo* generated variation via random mutation allowing for a study of real-time evolution in response to a given stressor or selection pressure (Lenski & Travisano 1994; Kawecki et al. 2012). There are several experimental designs for long-term evolution experiments that allow for testing of different types of hypotheses, which will now be discussed.

2.3 Experimental Designs

Experimental evolution has been carried out on multiple organisms including *Drosophila melanogaster* (Burke & Rose 2009)*, Escherichia coli* (Lenski et al. 1991)*, Arabidopsis thaliana* (Porcher et al. 2006), *Myxococcus xanthus* (Manhes & Velicer 2011), *Psuedomonas fluorescens* (Rainey & Rainey 2003), *Saccharomyces cerevisiae* (Ratcliff et al. 2012; Goddard et al. 2005) and field studies have been conducted on *Daphnia magna* (Ebert et al. 2002) and *Poecilia reticulata* (Reznick et al. 1990).

Microorganisms are ideal organisms for experimental evolution for several reasons. Populations of *E. coli* or *S. cerevisiae* are easily propagated, as they reproduce quickly and thus allow for a large number of generations to be tested over the course of the experiment. Large populations can be stored in a small space, increasing the number of populations that can be sampled in an experiment, especially compared to macroorganisms (Elena & Lenski 2003; van Ditmarsch & Xavier 2014) . A very useful aspect of microorganisms is the ability to survive suspended animation or cryopreservation therefore allowing for storable, revivable "fossil records" throughout the whole experiment. By freezing a time point, whole new avenues of research are revealed, which are unavailable when monitoring field populations or larger organisms. Microorganisms are also generally clonal and thus new mutations can be easily tracked throughout an evolution experiment, making them useful model organisms for studying genetic and genomic changes in experimental evolution (Elena & Lenski 2003; Barrick & Lenski 2013; Kawecki et al. 2012).

Several methods are employed for experimental evolution with microorganisms, centring around mutation accumulation or adaptive evolution.

2.3.1 Mutation Accumulation and Adaptive Evolution

Mutation accumulation, through the use of bottlenecking, amplifies the effect of random drift and reduces the impact of selection by severely decreasing the population size (Elena & Lenski 2003; Lynch et al. 2008). The selection of one or a few cells removes genetic diversity and results

in the fixation of random mutations (Barrick & Lenski 2013; Denver et al. 2004; Jasmin & Zeyl 2014). Mutation accumulation experiments are thus suited to the study of random mutations, their interaction, and their impact on phenotypic traits. However, the mutation rate provoked by this technique in a laboratory environment may not be representative of the mutation rate of natural populations, due to increased stress and nutritional differences between the two situations.

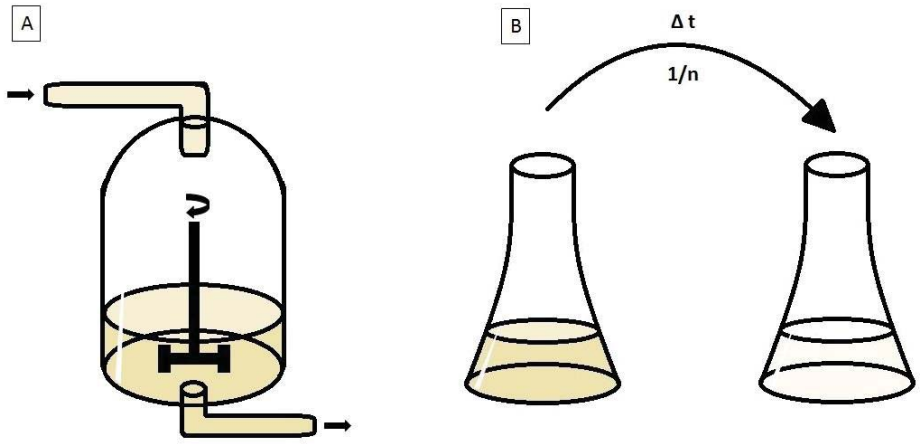


Figure 2.1: Adaptive Evolution Experiments (Barrick & Lenski 2013)**: A)** Continuous culturing usually takes place in a chemostat, a bioreactor where nutrients are continuously delivered into the system with waste and a random fraction of the population is removed to maintain a constant steady state **B)** Serial transfer sees a dilution of a proportion of the population (1/n) transferred into fresh medium after a set amount of time (Δt).

Adaptive evolution predominates when larger populations are propagated in environments of either constant or fluctuating selection pressure (Barrick & Lenski 2013). These pressures can be achieved by continuous culture (Figure 2.1A) or serial transfer (Figure 2.1B). Continuous culture usually takes place in a chemostat, where nutrients have a continuous influx, while waste and a random fraction of the population are continuously removed. Typically, chemostat culture implies a low steady state resource concentration (Boer et al. 2003). The second adaptive evolution method, serial transfer entails the renewal of resources for an inoculated portion of the saturated population (Dhar et al. 2011). This means that the population, for a substantial period of time after inoculation, has high levels of resource available. Another advantage of serial

transfer is that it more effectively withstands contamination when compared with the chemostat method, a major concern if dealing with a long term experimental evolution experiment. Since the Crabtree effect occurs at relatively high resource concentrations and is regarded as an adaptive trait, serial transfer in batch culture is an appropriate choice to study the evolution of the Crabtree effect.

2.3.2 Experimental Evolution Disadvantages

While experimental evolution is a fascinating technique to study evolution in action, and several studies have been conducted with yeasts (Boer et al. 2003; Ratcliff et al. 2013; Jasmin & Zeyl 2014; Jasmin & Zeyl 2012; van Dijken et al. 1993; Dunham et al. 2002; Dhar et al. 2011; Goddard et al. 2005), it is important to also consider the limitations of this technique, and be aware of the bias it may introduce. Studying yeast evolution in a laboratory setting can be argued to be an over-simplification of the environment, resulting in an evolution habitat that would never occur in a natural setting. This is why field experiments are an important complementary approach to laboratory experiments. A laboratory setting eliminates the effects of some extraneous factors, which may also influence yeast growth, but are not being measured in an individual experiment. However, it would be an unfathomable task to monitor every possible effector in the organism's environment and deduce what is the responsible stressor(s) inducing evolutionary change. Consequently, laboratory experiments tend to be favoured.

When testing a specific hypothesis, it is important to recognise that other relevant processes may be overlooked, leading to biased conclusions. For example, in a laboratory context it is theoretically possible to artificially increase mutations beyond the level at which they naturally occur, leading to irrelevant or biased results. It is also difficult to know the evolutionary history of the lineage that is used to start a long-term evolution experiment, and thus it is unclear what selection pressures the starting organism has been subjected to. This will have an impact on how much an organism will adapt to a specific force used in an experiment. Suppose, for example, an organism comes from an environment where it has been subject to strong selection for size, as a

response to predation for instance. When such an organism is brought into a laboratory environment to study adaptation to high sugar concentrations, not all observed adaptive changes might be attributed to the high sugar concentrations. A considerable fraction of the observed changes might instead be attributed to the different selective forces on the organism's size.

Moreover, the predominant use of model organisms such as *E. coli* or *S. cerevisiae* might be problematic because it is unclear whether these organisms represent typical bacteria, or yeast. While the use of model organisms is of advantage when studying genetic and genomic changes, it is also of importance to study and compare the responses of a larger samples of species subjected to the same selective forces. Simulating evolutionary change in yeast respiro-fermentative metabolism is useful for gaining insight into the similar pathways in tumour cells in humans but is clearly a different system.

2.3.3 Optical Density and Growth Rate and Yield

Optical density is a way of measuring the estimated biomass of a culture over time (Davey & Kell 1996; Julou et al. 2012). Spectrophotometers allow for the measurement of multiple samples simultaneously over a long period of time, providing a convenient way to acquire growth curve data. From these growth curves we can get the exponential growth rate, a quantification of by what factor biomass grows per unit of time, and the maximal growth yield, the final biomass quantity a sample reaches over a specific period of time (Jasmin et al. 2012). Growth rate is often used as a measure of fitness and is therefore of particular use in evolution studies (Spor et al. 2008; Spor et al. 2009a; Jasmin & Zeyl 2012). While using OD as a measure of biomass entails assumptions, it is a frequently used technique. The relation between biomass and OD can, however, be affected by cell size i.e. the maximal optical density could be higher due to more cells or bigger cells, but the rate at which it got to that maximal yield should be unaffected.

2.4 Aims

The aim of this thesis is to investigate whether the Crabtree effect could be an evolutionary response to resource competition in an environment with a high glucose concentration. To address this question, this chapter will focus on the following questions:

- Do natural populations of Crabtree-negative yeasts increase or decrease their growth rate in response to a high glucose environment?
- Do natural populations of Crabtree-negative yeasts increase or decrease their cell yield in response to a high glucose environment?

Starting with seven Crabtree-negative yeast isolates and one *Saccharomyces cerevisiae* for comparison, experimental evolution will be used to address these questions.

2.4.1 Predictions

With reference to the aims, it might be expected that, yeasts in batch culture are largely selected to increase their exponential growth rate. If there is a trade-off between rate and yield, and the Crabtree effect indeed represents a high rate/low yield strategy, the yield is expected to decrease. We therefore test if changes in rate and yield are observed in lineages of Crabtree-negative yeasts after 1500 generations of evolution in a high sugar environment. For the Crabtree-positive reference yeast, *S. cerevisiae,* it would be expected that the changes in rate and yield are small compared to the other species used in the experiment.

An important question to consider is whether the switch from Crabtree-negative to Crabtree-positive is plausible. Most yeasts can ferment in anaerobic conditions; therefore, it is reasonable to assume that they possess the enzymes to ferment. In addition to respiration only two additional enzymes are required to ferment, Pdc and Adh (see Figure 1.1). The main hurdle therefore is regulatory. Firstly, the regulation of which enzyme converts pyruvate, the down regulation of Pdh and upregulation of Pdc must occur to increase the fermentation rate, while the upregulation of Adh must occur to prevent accumulation of toxic acetaldehyde (Pfeiffer &

Morley 2014). Secondly, the regulation of the expression of glucose transporters would be a key factor in the move towards Crabtree-positive metabolism. Engineered *Saccharomyces cerevisiae* that had all glucose transporters save one removed, exhibited fully respiratory metabolism at high glucose levels and only switches to fermentation in limited oxygen environments (Otterstedt et al. 2004; Maclean & Gudelj 2006). This highlights that the regulation of glucose transporter expression is a key element to yeast exhibiting Crabtree-positive metabolism.

2.5 Material and Methods

2.5.1 Starting Isolates

Prior to discussing the results of the experimental evolution study, a brief review of the isolate-derived yeast populations (from here these will simply be referred to as populations) is given to highlight the existing metabolic and physiological traits of each yeast. The yeast isolates were sourced originally from Mat Goddard's collection of natural yeast isolates at Auckland University. The populations are not laboratory yeasts propagated for many generations in isolation but were only recently sourced from natural populations and have had very few generations in a laboratory environment. Those that were isolated from ferment in wine-making were sampled 75% of the way through fermentation and would have originated from the skin of the grapes used not introduced separately (Gayevskiy & Goddard 2012). The cultures obtained from the Goddard lab were single clonal isolates. The yeast species were identified through sequencing the ITS and/or the 26S rDNA region after collection from a number of areas around New Zealand (Goddard 2008b). This section will review the current knowledge of each yeast species, their natural habitat outside of the laboratory, their growth requirements, fermentative ability and appearance. Finally, at the end of this section a compiled table of all known metabolic tests for each yeast species is presented.

2.5.1.1 *Kodamaea* sp.

Kodamaea ohmeri also known as *Yamadazyma ohmeri or Pichia ohmeri* was assigned to *Kodamaea* due to its phylogenetic distance from both *Yamadazyma* and *Pichia* when comparing

partial sequences of 18s and 26s Ribosomal RNAs (Yamada et al. 1995). It was first isolated from cucumber brine and has been associated with honey bee colonies, and as an emerging pathogen usually in immunocompromised individuals (Biswal et al. 2015; Graham et al. 2011; Piredda & Gaillardin 1994; Etchells & Bell 1950). It has the ability to form pseudohyphae but cannot assimilate nitrate. Appendix A.1 covers more detail on its metabolic capabilities. Cells are roughly 3-6µm in size and generally globose, ellipsoid, ovoid to cylindrical in shape. The species exhibits fermentative abilities, requires biotin for growth and can grow above 30°C (Fiol & Claisse 1991). Our *Kodamaea* are of unknown species however due to their association to bees (see Table 2.2) it is assumed they are most likely *Kodamaea ohmeri* and due to the Biolog results (discussed later) the metabolic similarities would suggest they are most likely the same species but have become vicariant.

2.5.1.2 *Issatchenkia* sp.

Issatchenkia orientalis Kudryavtsev was isolated in fruit juice or berries in Russia by Kudravtsev in 1960 (Kurtzman et al. 2011). This yeast is also referred to by the names *Candida krusei* and *Pichia kudriavzevii* but has a large number of synonyms which makes collecting a comprehensive record of information on this yeast difficult (Kurtzman et al. 2011). *Candida krusei* has been isolated from bronchomycosis specimens and human excrement suggesting a pathogenic or opportunistic nature. It has the ability to produce both pseudohyphae but lacks the ability to assimilate potassium nitrate. Appendix A.1 summarises the metabolic traits of this yeast. Cells are roughly 5µm, spheroidal, ellipsoidal or elongate and pseudohyphae present. *I. orientalis* exhibits fermentative ability and can grow in a vitamin free medium above 30°C (Kurtzman et al. 2011). One of our strains is definitely identified as *I. orientalis* being sourced from ferment (see Table 2.2 species naming conventions), while our second *Issatchenkia* strain is of unknown species and origin, so therefore could be slightly different in its metabolic capabilities and indeed its fermentative ones as there is no record for this either.

2.5.1.3 *Candida railenensis*

Candida railenensis was isolated in decaying wood in Chile (Ramirez & Gonzalez 1984). It was also found to be abundant on *Nothofagus* New Zealand (Serjeant et al. 2008) and in acorns of *Quercus robur* (Isaeva et al. 2009). It was assigned to the *Candida* genus due to its ability to produce both pseudo and true mycelium and assimilate potassium nitrate, and its lack of ability to produce ascospores (Ramirez & Gonzalez 1984). Appendix A.1 summarises the metabolic traits of this yeast. Cells are roughly 5µm, generally globose, ellipsoidal or elongate, occasionally ogival, triangular or lunate and the cell wall is ascomycetous and two layered. *C. railenensis* exhibits very limited fermentative ability and cannot grow in vitamin free medium or above 30°C (Kurtzman et al. 2011; Ramirez & Gonzalez 1984). Our strain was sourced from ferment (see Table 2.2).

2.5.1.4 *Pichia kluyveri*

Pichia kluyveri was isolated from rotting cacti (Lachance et al. 1988; Starmer et al. 1987). However it has been found to span cactophilic and non-cactophilic habitats and has been isolated from olives, fruit and coffee beans (Kurtzman et al. 2011; Ganter et al. 2000). It was split into three varieties due to differences in physiological abilities, infertile crosses and a lower degree of genetic similarity than expected (Ganter et al. 2000; Kurtzman et al. 2011; Phaff et al. 1987). Pseudohyphae are formed but true hyphae are not while assimilation of nitrate is negative. Appendix A.1 summarises the metabolic traits of this yeast. Cells are roughly 4-11µm, generally spheroidal, ellipsoidal or elongate and occasionally may be tapered but they are not ogival in shape. *P. kluyveri* exhibits varying fermentative ability depending on the variety of *P.kluyveri* it is, for example *P.kluyveri var. kluyveri* ferments glucose strongly while *P. kluyveri var. cephalocereana* is comparatively slow at fermenting. Some shared characteristics include the lack of ability to grow in vitamin-free medium but they can grow above 30°C. The strain we used was most likely *P. kluyveri var. kluyveri* as the isolate information provided by the Goddard lab records it with fermentative abilities in an anaerobic environment (Anfang et al. 2009).

2.5.1.5 *Kluyveromyces nonfermentans*

Kluyveromyces nonfermentans was isolated from deep sea mud in Suruga Bay and Sagami Bay, Japan (Nagahama et al. 1999). It is hypothesised that *K. nonfermentans* is derived from a *Kluyveromyces aestuarii* that evolved in deeper regions of the marine environment (Nagahama et al. 1999). Pseudohyphae and true hyphae are not formed and nitrate is not assimilated (Nagahama et al. 1999; Kurtzman et al. 2011). Cells are around 2-7.5µm in size and spheroidal to ellipsoidal in shape. *K. nonfermentans* lacks the strong fermentative abilities of the other *Kluyveromyces* and has the differentiating lack of ability to assimilate sucrose, lactic acid and succinic acid like other *Kluyveromyces*. Growth requires vitamins biotin, niacin and thiamin, while growth above 30°C is variable. For full metabolic capabilities see Appendix A.1. Our strain was imported from the Netherlands to the Goddard Laboratory, NZ (under permit 105, July 2006).

2.5.1.6 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has been used for decades in the brewing of beer and the making of bread. It is one of the most widely used model organisms in biology, with yeast research having resulted in several Nobel Prizes (Suh et al. 2006) including, the Nobel Prize in Physiology or Medicine 2016 by Yoshinori Ohsumi, and the Nobel Prize in Physiology or Medicine 2001 by Leland Hartwell.

Pseudohyphae are formed but septate true hyphae are not and assimilation of nitrate is negative. Appendix A.1 summarises the metabolic traits of this yeast. Cells are roughly 3-10µm, generally globose, ellipsoidal or cylindroidal in shape. *S. cerevisiae* exhibits very strong fermentative ability, lacks the ability to grow in vitamin-free medium while growth above 30°C can be variable (Kurtzman et al. 2011). The strain we used was isolated from ferment. It is hypothesised that the *Saccharomyces* fermenting ability is adaptive as it modifies the environment to its advantage even though it is energetically inefficient to do so, not just by the production of ethanol but by heat also (Goddard 2008b). It is also theorised that due to lack of evidence of a specific adaptation

to fruit, *S. cerevisiae* may have evolved a more general ability to inhabit and persevere in numerous different environments (Goddard & Greig 2015).

2.5.1.7 Summary

From the compiled research on the yeast population it can be supposed that each have metabolically different traits, that all the populations save *Kluyveromyces nonfermentans* have the ability to utilise the fermentative pathway, albeit in an anaerobic setting, and all species have the ability to assimilate glucose and survive the environmental conditions employed for the experimental evolution study. We expect *S. cerevisiae* to display the most pronounced, and *K. nonfermentans* the least pronounced Crabtree effect, with the other species being placed between these two extremes; and as shown in Chapter Three, this is confirmed by ethanol assays. It is also apparent that there is a large amount of information left to be discovered about these yeasts, which highlights potential areas for future study. Table 2.2 presents the names of all the yeasts used in this study.

Abbreviated Name	Species	Goddard Lab Origins
NSC A1	*Kodamaea sp.*	Wax isolate of beehive in Mangere Bridge
NSC A8	*Kodamaea sp.*	Beesonline isolate
NSC A11	*Issatchenkia sp.*	Chardonnay juice, Kumeu River, Auckland
NSC B3	*Candida railenensis*	Chardonnay ferment, Kumeu River, Auckland
NSC B5	*Pichia kluyveri*	Chardonnay juice, Kumeu River, Auckland
NSC C10	*Issatchenkia orientalis Kudryavtsev*	Sauvignon blanc juice, Marlborough
NSC F8	*Kluyveromyces nonfermentans*	Netherlands
SC E2	*Saccharomyces cerevisiae*	Sauvignon blanc ferment, Hawkes Bay

Table 2.2: Species Abbreviated Names and Origins. The table provides the species names of all yeast populations, their allocated abbreviations and the origin of the strains from around New Zealand, save *Kluyveromyces nonfermentans* which was sourced from the Netherlands.

2.5.2 Methods for Experimental Evolution

The yeast used throughout this thesis were sourced from our collaborator Mat Goddard. Cultures were grown in 1g/l glucose synthetic minimal medium with 1.7g/l ammonium phosphate and 0.006g/l uracil mix, components bought from Formedium™. Environmental conditions were set at 30°C and shaking set to 150 rpm in an incubator. Eight yeasts were grown from freezer stocks in 1g/l glucose synthetic minimal medium for 48 hours in 96-well plates (TCP000-096 from Interlab Ltd.) set out as shown in Appendix A.2 and duplicated to 6 replicates per species (2 plates in total). These were then inoculated into fresh medium and saturated for 24 hours and the next round the experiment commenced. Serial transfer into fresh medium was carried out every 24 hours using a 1/100 dilution in 200µl of culture. This corresponds to 6.7 generations per growth cycle.

The experiment was carried out for 1500 consecutive generations, except for B3 (*Candida railenensis*) lines 2 and 6 which had persistent contamination at the beginning of the experiment and required a full restart from starting culture over a fossil record. For contamination checks 100µl of culture from each line was streaked onto yeast extract peptone dextrose agar (YPDA) petri dishes every two weeks and incubated for 48 hours, then manually checked for contamination. Frozen samples were taken every two weeks of every line as a fossil record, stored in 15% glycerol (30% glycerol made up in YPD and diluted half and half with culture to 15%). Ethanol was also monitored every month sampling at hour 0 (T0), hour 10 (T10) and hour 13 (T13) taking optical density (OD) measurements of the duplicated plate, filtering samples straight away and freezing. Enzymatic assays were conducted on these samples, methods and results are described in Chapter Three.

In the event of the loss of a line, the sample was monitored for three consecutive transfers and if no OD change was detected the line was reinoculated from the last fossil record. Contamination was treated similarly. Contamination was controlled for using the plate layout shown in Appendix A.2, containing 72 blanks and 24 inoculated samples per plate. For blank contamination, the event

was recorded and prevented from carrying forward. If more than three blanks were contaminated at once the plate was started fresh from the last fossil record. Naming conventions for the lines are derived from their original stock allocations in the Goddard Lab (A1, A8, A11, B3, B5, C10, F8, SCE2) this was based on their storage location on a 96-well plate. Table 2.2 shows the species and its abbreviated corresponding name. Lines were allocated a 1 to 6 number based on the replicate across plate one and two (A1.1, A1.2 etc.). Frozen samples were then named A for ancestor (A1.1 A) and for the evolved, Fn, for the sequence of fossil record, A1.1 F1 for freeze one, A1.1 F2 for freeze two etc. and the date of freezing was added to the side of the tube. These were then stored in a -80°C freezer.

These experimental conditions were designed to establish a large population size (between 10^6 to 10^8) and therefore should not be limiting. Although it cannot be entirely ruled out, recombination should not be occurring due to the avoidance of nitrogen starvation and having a single mating type. While mutation rate and selective strength are unknown, similar conditions have seen glucose-adapted *Saccharomyces cerevisiae* adapt to a galactose-limited environment in 120 generations with growth rate increasing and growth yield decreasing (Jasmin & Zeyl 2012).

2.5.3 Growth Curves

Growth was measured in duplicated 96-well plates over 24 hours every two weeks in a SpectraMax® M2 with the Softmax Pro Software. The lids were treated with a mild detergent to prevent condensation, a regular issue when plates were grown over 24-hour periods in the plate reader.

There are a number of factors that need to be considered before attempting to interpret data like this. The growth curves were affected by varying levels of noise. This could be due to yeast cells clumping, air bubbles, especially affecting the beginning of the curves, and condensation which could not be completely eliminated. Smoothing of the curves was used to estimate how noisy a curve was, while data was log transformed as in log transformed curve, exponential growth is converted into a line. This allows for the use of linear regressions for estimating the growth rate.

Firstly, back to back measurements were taken with the ancestor strain on the same plate as all the evolved lines at 500, 1000 and 1500 generations. This approach allows to control for plate-to-plate variance. These were then repeated to get more precise estimates of growth parameters. At the same time five blank plates of medium were measured in the plate reader. These were used as reference blanks to subtract from the values of each measurement and compared to the back to back plate blanks. If equivalent this would equal zero when subtracted, but if there was specific plate variance this would provide an average higher or lower value to apply to that specific plate, hopefully eliminating most plate effects from the data. On top of this, data was filtered with an initial inoculation threshold, that if, after the removal of the position dependent blanks, the initial optical density was greater than 0.05 then the curve was excluded as this would indicate that the inoculate could be contaminated, there was something wrong with the plate or that bubbles or clumping had occurred. A robust linear model (RLM) was then applied to the data to reduce the effect of outliers. The RLM is applied at every point of the growth curve, taking moving averages across 12 points i.e. four hours of data, at a time to construct the slope of the growth curve. The rate is determined by taking the highest slope value. The yield is obtained from the Huber average optical density, taking the highest value as the maximal yield. The Huber average diminishes the impact of outliers on averages, in a similar way to robust linear models.

For each growth curve, we obtain an estimate for the maximal growth rate and growth yield, unless the noise on the growth curve exceeded a pre-defined value. The resulting values are statistically analysed to test for difference between ancestor and evolved populations as discussed in the following sections. Results are presented in the subsequent section.

2.5.4 Statistical Analysis of Rates and Yields

To test if rates and yields differ between ancestor and evolved populations, we use a linear mixed effect model. The plate effect is treated as random effect, the yeast line at generation 1500 (i.e. line A1 at generation 1500) was used as independent variable, and rate and yield as dependent variables. T statistics were used to test for statistically significant differences between ancestor

and line generations. Models were applied to each species separately to evaluate the differences between ancestor and evolved lines.

Additionally, binomial tests were used on the results of the evolutionary experiment to test for general trends over the yeasts. These statistically test the significance of success of a trend out of a given number of events and allows for the statistical testing of whether a majority increase or decrease in rate and increase or decrease in yield. This is applied to a) all rate and yield changes as estimated by the linear model, and b) only statistically significant results. Binomial tests are such that no change or a same value is not counted as a result.

2.6 Results

In the experimental evolution study the main features of interest were whether the yeast populations growth rate and yield altered in response to the high sugar environment over the course of 1500 generations in serial transfer batch cultures. During the experiment, the growth curves of each yeast were taken every two weeks, a practice not only good for contamination monitoring but also for real-time tracking of the changes in yield and rate.

2.6.1 Events

During the experiment, there were several points that required replacement of lines: one full plate contamination occurred at day 22 losing all yeast population lines 4-6 resulting in a full plate restart, and then A1.5, all C10 and B5 lines, and lines 4-6 of F8 were lost at day 30 and were restored from a previous freeze. A8 1 was lost on day 224 and restored from freezer stock. B3 reached the 1500 generations in lines 1, 3, 4 and 5, while lines 2 and 6 only reached 1000 generations due to persistent contaminations. Initially line 6 was replaced on day 17 followed by replacement of lines 4, 5 and 6 due to the full plate contamination event 22 days in Then line 6 and line 2 were replaced on day 30, line 6 on day 43 then both lines returned to ancestor on day 52. Line 1 was replaced on day 61, line 5 on day 144 and lastly line 3 on day 199. Finally, a mass low growth of all yeast populations occurred due to a power failure at day 224 and persisted for a period of time in some lines. For a full visualisation of these events refer to Appendix A3.

2.6.2 Yeast Specific Results

2.6.2.1 *Kodamaea* A1

	Rate			Yield		
Line	**Estimate**	**T-value**	**P-value**	**Estimate**	**T-value**	**P-value**
A1.0	0.594			0.429		
A1.1	-0.012	-0.371	0.711	-0.013	-1.084	0.281
A1.2	0.041	1.302	0.196	-0.001	-0.096	0.923
A1.3	0.058	1.688	0.094	**-0.111**	**-8.822**	**<0.001**
A1.4	0.059	1.710	0.090	0.004	0.295	0.769
A1.5	0.024	0.752	0.454	**-0.068**	**-5.843**	**<0.001**
A1.6	0.000	-0.001	0.999	**-0.112**	**-8.861**	**<0.001**

Table 2.3: A1 Mixed Effect Model Output. See section 2.5.4 for further information on this model. The data here presents the rate and yield for the ancestor line A1.0 and then the estimated change from this rate for each evolved line at 1500 generations. The t-value and p -value are presented here also. Significant changes between ancestor and evolved lines are highlighted.

For the first *Kodamaea* yeast population A1, Figure 2.4 shows the progression of the rate and yield for each line across evolutionary time throughout 1500 generations. The lines do not exhibit a continuous increase in rate across the generations. None of the lines exhibit statistically significant change by 1500 generations as shown by the p-values in Table 2.3. There appears to be a trend towards increased rate when considering the t-values of lines A1.2-5 and looking at the average trendline on Figure 2.4, however this is not at a statistically significant level.

In terms of growth yield, at 500 generations three lines have had a decrease in yield with three remaining relatively unchanged. By 1500 the three lines have maintained this decrease at a statistically significant level of change, while the other three lines have more or less maintained the same level of yield. The overall trend line from Figure 2.4 suggests an overall species decrease in yield, however it must be noted that only half the lines demonstrate a statistically significant decrease.

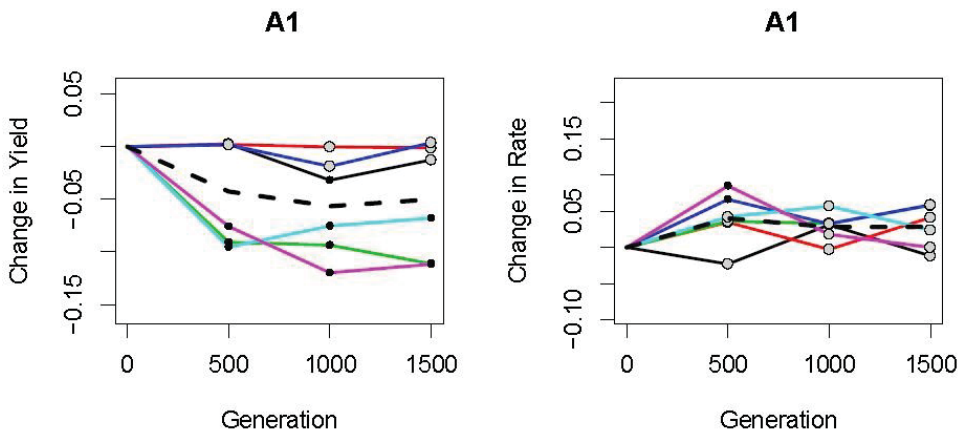


Figure 2.4: A1 Evolutionary Changes Across 1500 Generations. The estimates for both rate and yield are presented here from ancestor through 500 generations, 1000 generations to 1500 generations. Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple.

2.6.2.2 *Kodamaea* A8

In the second *Kodamaea* yeast population A8, five out of six lines show an increase in rate by 500 generations and by 1500 generations have a statistically significant increase in rate compared to the ancestor (see Table 2.5). Line 1 however maintains no change from the ancestor until 1000 generations and by 1500 generations develops a statistically significant decrease in rate.

	Rate			Yield		
Line	**Estimate**	**T-value**	**P-value**	**Estimate**	**T-value**	**P-value**
A8.0	0.558			0.410		
A8.1	**-0.066**	**-2.750**	**0.007**	**-0.024**	**-2.380**	**0.019**
A8.2	**0.068**	**3.080**	**0.003**	**-0.061**	**-6.488**	**<0.001**
A8.3	**0.090**	**4.090**	**<0.001**	**-0.055**	**-5.884**	**<0.001**
A8.4	**0.051**	**2.309**	**0.023**	-0.004	-0.386	0.700
A8.5	**0.050**	**2.273**	**0.025**	**-0.108**	**-11.546**	**<0.001**
A8.6	**0.047**	**2.142**	**0.034**	**-0.060**	**-6.383**	**<0.001**

Table 2.5: A8 Mixed Effect Model Output. See section 2.5.4 for further information on this model. The data here presents the rate and yield for the ancestor line A8.0 and then the estimated change from this rate for each evolved line at 1500 generations. The t-value and p-value are presented here also. Significant changes between ancestor and evolved lines are highlighted.

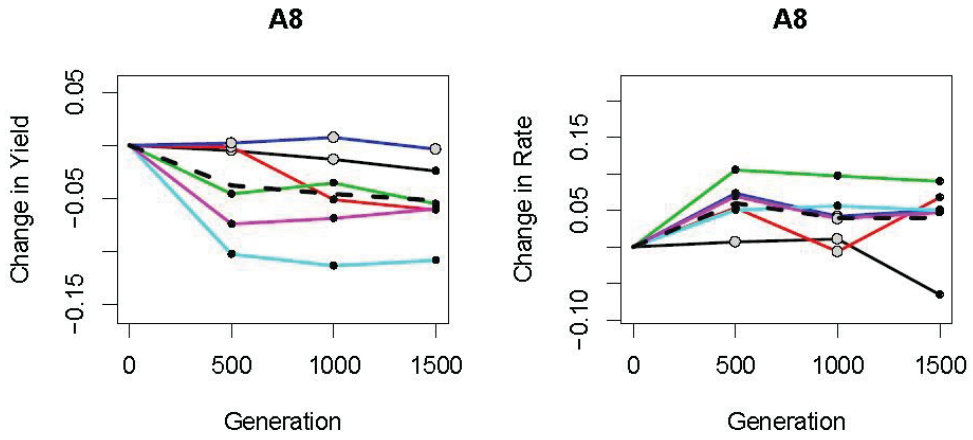


Figure 2.6: A8 Evolutionary Changes Across 1500 Generations. The estimates for both rate and yield are presented here from ancestor through 500 generations, 1000 generations to 1500 generations. Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple.

At 500 generations three lines have exhibited a decrease in growth yield, while the other three remain unchanged. By 1500 generation all lines save line A8.4 developed a statistically significant decrease in yield, line A8.4 having remained virtually static. It is noteworthy to mention that lines A8.4, A8.5 and A8.6 have extremely similar evolutionary growth rate patterns, with an increase in rate at generation 500 a dip at 1000 and then a levelling off between these points at 1500. Line A8.3 may also reflect this same pattern at a higher rate and is the line with the most statistically significant increase in rate while even line A8.2 may follow the same pattern to a more amplified degree. In terms of yield line, A8.5 exhibits the most dramatic decrease in yield; a consistent trait from 500 generations onwards. Across the entire yeast population, the general trend is that this yeast increased its rate and decreased its yield over 1500 generations as seen in Figure 2.6.

2.6.2.3 *Candida railenensis* B3

In the *Candida railenensis* yeast population B3, four lines demonstrate an increase in rate by 1500 generations, a statistically significant increase compared to the ancestor (see Table 2.7). Three of the lines developed this increase by 500 generations.

	Rate			Yield		
Line	**Estimate**	**T-value**	**P-value**	**Estimate**	**T-value**	**P-value**
B3.0	0.373			0.374		
B3.1	0.031	1.664	0.099	**-0.048**	**-4.925**	**<0.001**
B3.2	**0.048**	**2.586**	**0.011**	**-0.061**	**-6.305**	**<0.001**
B3.3	**0.108**	**5.803**	**<0.001**	**-0.051**	**-5.254**	**<0.001**
B3.4	**0.064**	**3.414**	**0.001**	**-0.061**	**-6.283**	**<0.001**
B3.5	-0.022	-1.152	0.252	**-0.067**	**-6.921**	**<0.001**
B3.6	**0.068**	**3.639**	**<0.001**	**-0.050**	**-5.122**	**<0.001**

Table 2.7: B3 Mixed Effect Model Output. See section 2.5.4 for further information on this model. The data here presents the rate and yield for the ancestor line B3.0 and then the estimated change from this rate for each evolved line at 1500 generations. Save for line 2 and 6 which are at 1000 generations. The t-value and p-value are presented here also. Significant changes between ancestor and evolved lines are highlighted.

This includes lines B3.2 and B3.6 which only reached 1000 generations but still achieved a statistically significant rate increase in that time (Figure 2.8). Lines B3.1 and B3.3 were slower in developing a rate increase, however both achieved this in the last 500 generations of the experiment. Only line B3.5 did not show an increase in rate. Over all lines, yield has decreased statistically significantly, and all decreased within the first 500 generations.

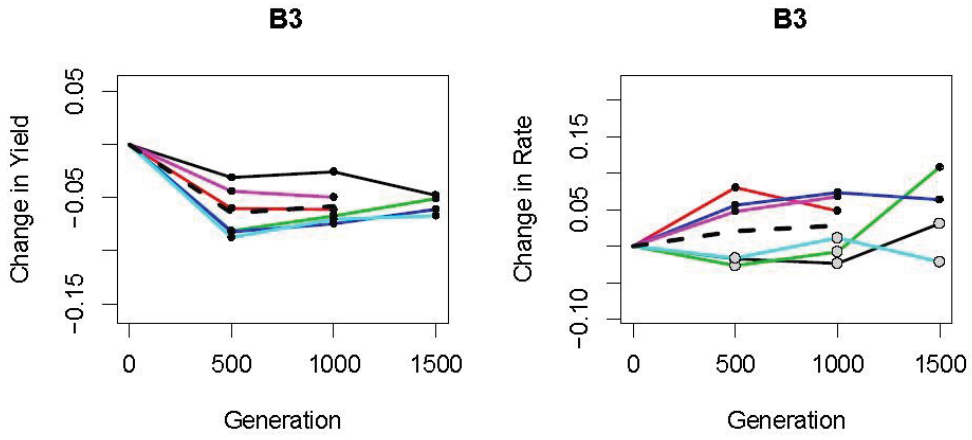


Figure 2.8: B3 Evolutionary Changes Across 1500 Generations. The estimates for both rate and yield are presented here from ancestor through 500 generations, 1000 generations to 1500 generations. Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple.

2.6.2.4 *Pichia kluyveri* B5

	Rate			Yield		
Line	**Estimate**	**T-value**	**P-value**	**Estimate**	**T-value**	**P-value**
B5.0	0.62			0.427		
B5.1	-0.037	-1.244	0.216	**-0.101**	**-7.762**	**<0.001**
B5.2	**0.071**	**2.380**	**0.019**	**-0.147**	**-11.243**	**<0.001**
B5.3	**0.072**	**2.414**	**0.018**	**-0.069**	**-5.285**	**<0.001**
B5.4	-0.027	-0.923	0.358	**-0.064**	**-4.870**	**<0.001**
B5.5	**0.103**	**3.460**	**0.001**	**-0.159**	**-12.188**	**<0.001**
B5.6	-0.031	-1.049	0.297	**-0.050**	**-3.800**	**<0.001**

Table 2.9: B5 Mixed Effect Model Output. See section 2.5.4 for further information on this model. The data here presents the rate and yield for the ancestor line B5.0 and then the estimated change from this rate for each evolved line at 1500 generations. The t-value and p-value are presented here also. Significant changes between ancestor and evolved lines are highlighted.

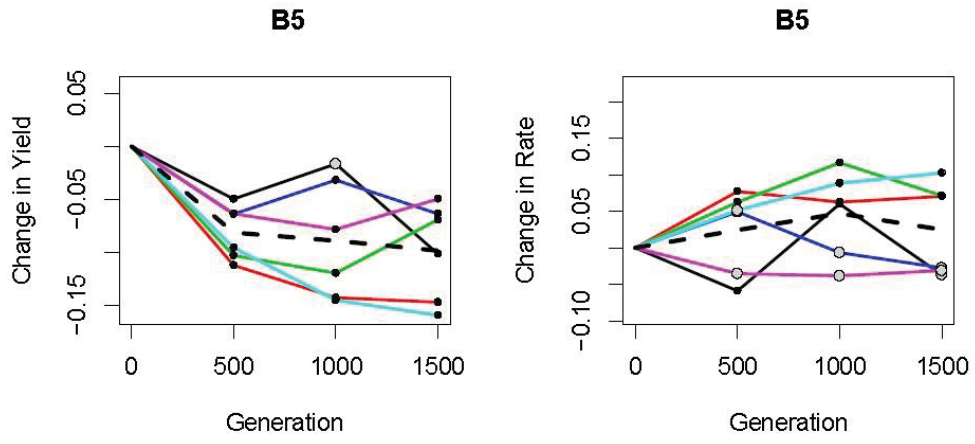


Figure 2.10: B5 Evolutionary Changes Across 1500 Generations. The estimates for both rate and yield are presented here from ancestor through 500 generations, 1000 generations to 1500 generations. Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple.

The *Pichia kluyveri* yeast population B5, three lines demonstrate an increase in rate by 1500 generations at a statistically significant increase compared to the ancestor while the other three decrease but not by a statistically significant degree (see Table 2.9). Four of the lines developed an increase by 500 generations with lines B5.1 and B5.6 staying static (Figure 2.10). Line 4 however dropped in rate after 500 generations to return to more or less the same rate as the

ancestor. Over all lines, yield decreased statistically significantly, and all decreased within the first 500 generations.

2.6.2.5 *Issatchenkia orientalis* C10

	Rate			Yield		
Line	**Estimate**	**T-value**	**P-value**	**Estimate**	**T-value**	**P-value**
C10.0	0.579			0.375		
C10.1	0.017	0.529	0.599	0.026	1.755	0.083
C10.2	**0.090**	**2.869**	**0.005**	**0.057**	**3.855**	**<0.001**
C10.3	**0.114**	**3.641**	**0.001**	0.012	0.840	0.404
C10.4	**0.104**	**2.908**	**0.005**	**0.034**	**2.051**	**0.044**
C10.5	**0.092**	**2.939**	**0.004**	**-0.031**	**-2.083**	**0.041**
C10.6	**0.120**	**3.829**	**<0.001**	-0.006	-0.425	0.672

Table 2.11: **C10 Mixed Effect Model Output.** See section 2.5.4 for further information on this model. The data here presents the rate and yield for the ancestor line C10.0 and then the estimated change from this rate for each evolved line at 1500 generations. The t-value and p-value are presented here also. Significant changes between ancestor and evolved lines are highlighted.

In the *Issatchenkia* yeast population C10, five of the six lines demonstrate an increase in rate by 1500 generations at a statistically significant increase compared to the ancestor (see Table 2.11) and developed the increase by 500 generations. Line C10.1 remains static throughout most of the experiment with an increase in rate between 500 and 1000 generations but then a return to the ancestor's level of growth rate at 1500 (Figure 2.12). In terms of yield only lines C10.2, C10.4 and C10.5 change statistically significantly from the ancestor. The lines C10.2 and C10.4 increase in yield rather than decrease as the other populations have done. Line C10.5 and C10.6 are the only lines to decrease in yield but only line C10.5 does so statistically significantly. Overall it would appear that there is a general trend towards increased rate and increased to static yield. Those that both increase in rate and yield are of particular interest due to the difference in trend compared to the other populations.

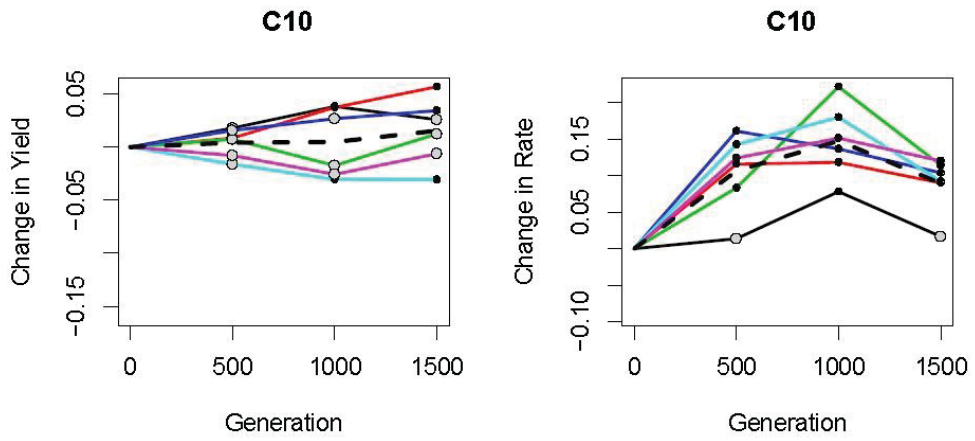


Figure 2.12: C10 Evolutionary Changes Across 1500 Generations. The estimates for both rate and yield are presented here from ancestor through 500 generations, 1000 generations to 1500 generations. Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple.

2.6.2.6 *Kluyveromyces nonfermentans* F8

In the *Kluyveromyces nonfermentans* yeast population F8, all six lines demonstrate an increase in rate by 1500 generations at a statistically significant level compared to the ancestor (see Table 2.13) and developed the increase by 500 generations with only line F8.1 demonstrating a slower progression towards increased rate.

	Rate			Yield		
Line	**Estimate**	**T-value**	**P-value**	**Estimate**	**T-value**	**P-value**
F8.0	0.491			0.359		
F8.1	**0.065**	**2.763**	**<0.001**	**-0.032**	**-4.966**	**<0.001**
F8.2	**0.119**	**5.023**	**<0.001**	0.002	0.293	0.770
F8.3	**0.159**	**7.131**	**<0.001**	**-0.038**	**-6.219**	**<0.001**
F8.4	**0.156**	**6.977**	**<0.001**	**-0.053**	**-8.827**	**<0.001**
F8.5	**0.108**	**4.547**	**<0.001**	-0.002	-0.364	0.716
F8.6	**0.140**	**6.296**	**<0.001**	**-0.028**	**-4.666**	**<0.001**

Table 2.13: F8 Mixed Effect Model Output. See section 2.5.4 for further information on this model. The data here presents the rate and yield for the ancestor line F8.0 and then the estimated change from this rate for each evolved line at 1500 generations. The t-value and p-value are presented here also. Significant changes between ancestor and evolved lines are highlighted.

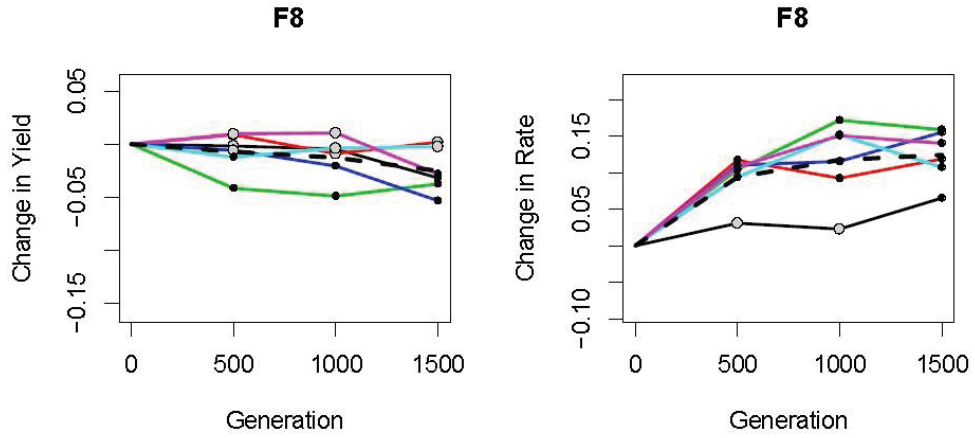


Figure 2.14: F8 Evolutionary Changes Across 1500 Generations. The estimates for both rate and yield are presented here from ancestor through 500 generations, 1000 generations to 1500 generations. Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple.

In terms of yield four lines, specifically, F8.1, F8.3, F8.4 and F8.6 exhibited a decrease in yield while lines F8.2 and F8.5 were of no statistically significant change. From Figure 2.14 it can be seen that overall there is a species trend towards increased rate and decreased yield, at least towards the final 500 generations.

2.6.2.7 *Saccharomyces cerevisiae* SCE2

	Rate			Yield		
Line	**Estimate**	**T-value**	**P-value**	**Estimate**	**T-value**	**P-value**
SCE2.0	0.585			0.377		
SCE2.1	**-0.084**	**-3.196**	**0.002**	**-0.033**	**-3.586**	**0.001**
SCE2.2	-0.039	-1.415	0.160	**-0.022**	**-2.298**	**0.023**
SCE2.3	0.002	0.075	0.941	**-0.078**	**-8.096**	**<0.001**
SCE2.4	-0.045	-1.616	0.109	**-0.053**	**-5.498**	**<0.001**
SCE2.5	-0.040	-1.467	0.145	0.007	0.729	0.468
SCE2.6	-0.032	-1.075	0.285	**-0.034**	**-3.260**	**0.001**

Table 2.15: SCE2 Mixed Effect Model Output. See section 2.5.4 for further information on this model. The data here presents the rate and yield for the ancestor line SCE2.0 and then the estimated change from this rate for each evolved line at 1500 generations. The t-value and p-value are presented here also. Significant changes between ancestor and evolved lines are highlighted.

In the *Saccharomyces cerevisiae* yeast population SCE2, only line SCE2.1 demonstrates a statistically significant change in rate by 1500 generations, decreasing compared to the ancestor

(see Table 2.15). Across evolutionary time the lines appear to initially increase in rate in the first 500 generations but decrease even past the ancestor rate between 500 and 1000 generations and return to the ancestor range of growth rate in the last 500 generations (Figure 2.16). In terms of yield, five out of six lines exhibited a decrease in yield to a statistically significant level while line SCE2.5 remained static. The observed changes in rate set the *S. cerevisiae* lines apart from the other lines.

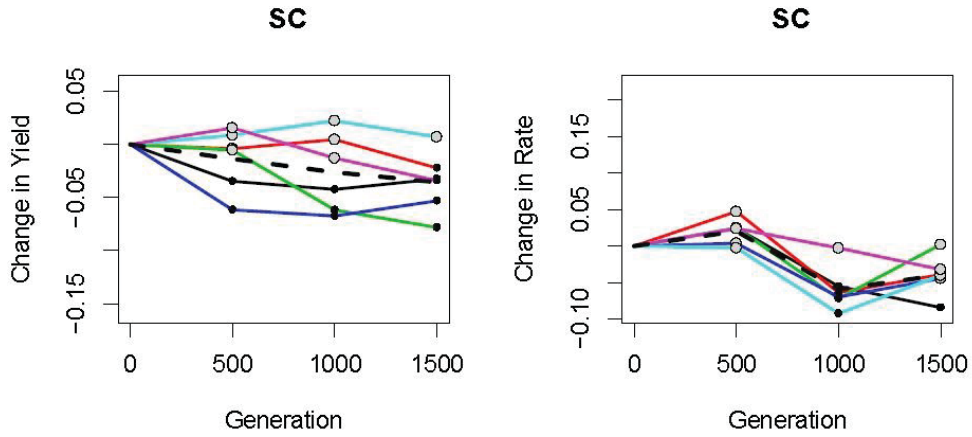


Figure 2.16: SCE2 Evolutionary Changes Across 1500 Generations. The estimates for both rate and yield are presented here from ancestor through 500 generations, 1000 generations to 1500 generations. Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple.

2.6.2.8 *Issatchenkia* A11

For the *Issatchenkia* yeast population A11, rate and yield data could not reliably be estimated due to the level of noise displayed by the data. As is shown in Figure 2.17 the ancestors of A11 and the other *Issatchenkia* yeast population C10 start relatively noisy in comparison to other yeasts save *Saccharomyces cerevisiae* SCE2. It would appear for A11 that the level of noise increases over the course of the 1500 generations of the experiment. Compared to C10 there is a distinct difference in level of noise across the generations, with C10 demonstrating very little noise change save a gradual overall increase across evolutionary time.

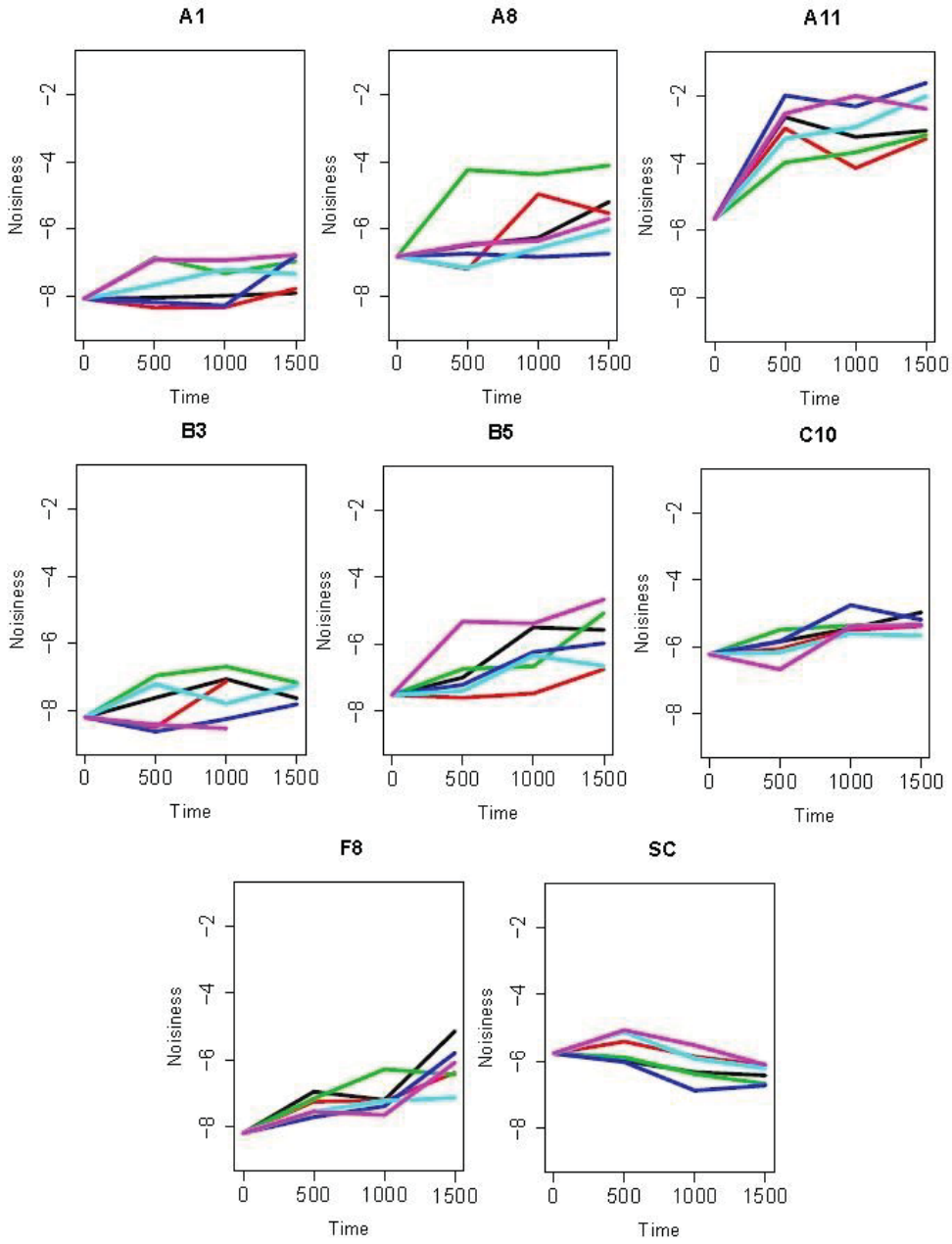


Figure 2.17: Noise Effects Over the Course of the Evolutionary Experiment for all Yeast Lines. Noise is defined as the sum over the residuals between original growth data and smoothed data**.** Each line is represented by a single colour consistent across all yeasts. Line 1 = black, Line 2 = red, Line 3 = green, Line 4 = dark blue, Line 5 = light blue and Line 6 = purple. The data is log transformed. The data is presented from the ancestor, 500 generations, 1000 generations and 1500 generations.

A11 however demonstrates erratic changes throughout and a steep increase over evolutionary time. Taking into consideration the other yeast populations, the variation and level of increase does not seem to be a universally shared trait. *Kluyveromyces nonfermentans* F8, appears to only

increase in noise after 1000 generations but starts initially with a lot lower level of noise, while SCE2 seems to overall decrease in noise from 500 generations onwards.

Candida railenensis B3 seems to remain at a steady level of low noise, while *Kodamaea* A8 has one particular line that appears uncharacteristically noisy compared to the other lines that show a small increase in noise gradually over the 1500 generations. *Pichia kluyveri* B5 and *Kodamaea* A1 both show a gradual increase with B5 being slightly more variable across lines than A1 but once again the variation and increase is not nearly as dramatic as A11.

2.6.3 Summary

The aim of this chapter is to determine whether growth rate and yield increased or decreased over evolution in a high sugar environment. If, for example the Crabtree effect evolved in a yeast population, we would expect to observe growth rate to increase at the cost of yield. This would be due to the fact the Crabtree effect is beneficial due to its ability to provide a faster metabolic pathway. This in turn allows the yeast to outcompete its ancestor which lacks this metabolic trait by exploiting the carbon source earlier.

In these yeast populations, changes do appear across lines in response to such an environment. The maximal rates and yields are shown in the tables. Figure 2.18 presents the collective changes in rate and yield for each yeast. From the data, we observed that over all yeasts (save A11 as mentioned earlier), the majority of lines evolve towards an increase in growth rate.

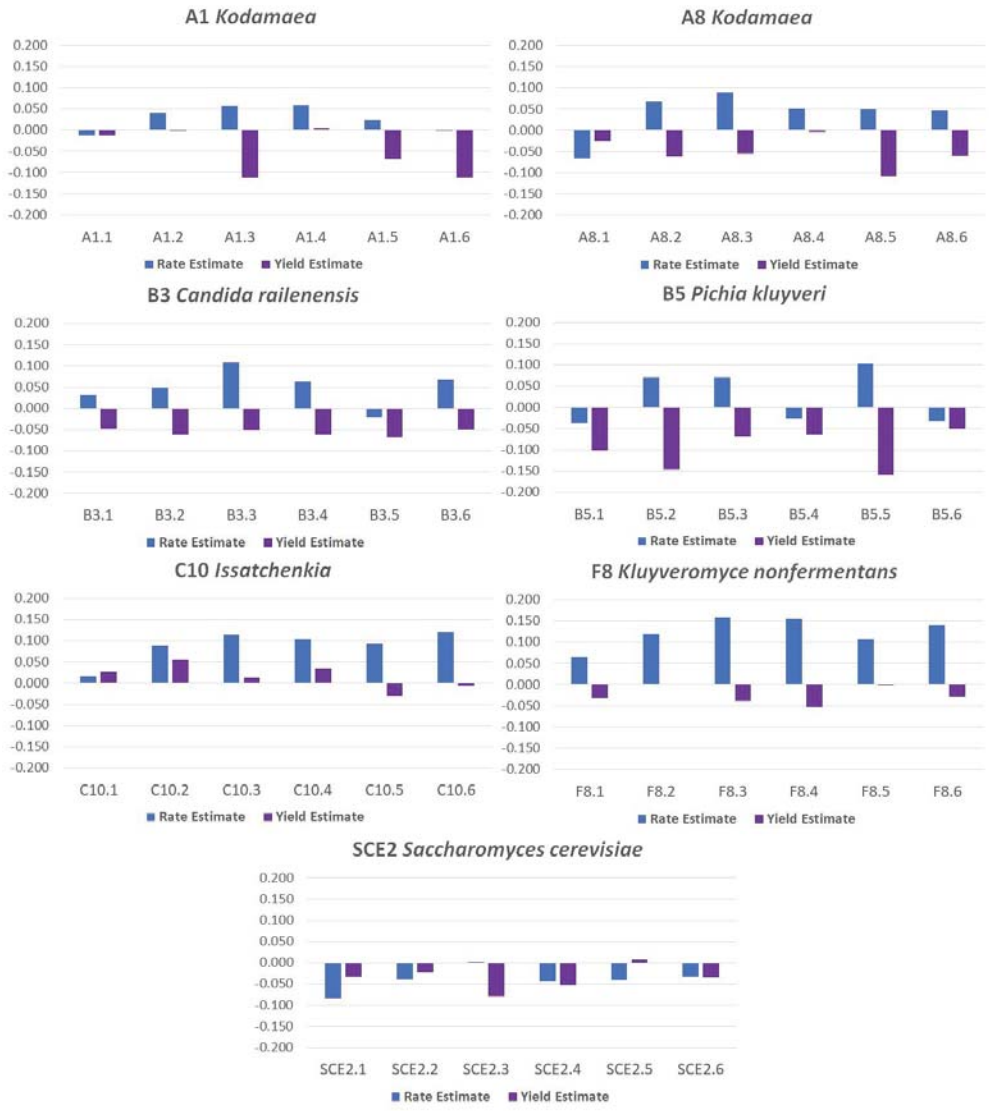


Figure 2.18: Changes of Rate and Yield from the Ancestor Across all Lines and Yeasts. Each yeast is represented graphically in the figure above, demonstrating the changes in each evolved line from the ancestor in rate (blue) and yield (purple).

The only exceptions are A1 and SCE2, the latter of which developed a single decrease in rate. In terms of significance however A1 and SCE2 do not have statistically significant changes despite the trend exhibited in the Figure 2.18. C10 exhibits an increase in rate, even though its yield also increased. All yeasts save *Issatchenkia* C10, exhibit a decrease in yield across at least three lines after 1500 generations. C10 appears to show an overall increase in yield although when compared to the other *Issatchenkia* species, A11, it can be noted this was the only yeast to have invalid

measurements due to the high level of noise that could not be improved even through the RLM. This noise was likely due to the yeast clumping. While A11 was not included in this data analysis of growth rate and yield, it is still included in further investigations in the following chapters. It is striking to see the common trend of yield decrease across all other yeast as demonstrated in Figure 2.18. Even *Saccharomyces* exhibits this trend with a statistically significant change from the ancestor in five of the six lines.

Variable	Fraction that change	P-value
All rates	35/42	**1.5e-5**
All yields	30/42	**0.008**
Significant rates	30/32	**2.5e-7**
Significant yields	23/25	**1.9e-5**

Table 2.19: Binomial Test Results. Testing the significance of the changes throughout the yeast lines collectively.

When counting the changes across the yeasts, 35/42 times yield decreases, while 30/42 times rate increases when including all estimates. If only counting the statistically significant changes 30/32 samples decrease in yield, and 23/25 increase in rate. If a binomial test is applied to this to test the significance of the trend the p-values of Table 2.19 are produced. These p-values demonstrate a statistically significant change in every eventuality. This leads to the conclusion that the yeast populations overall are more likely to evolve a decrease in yield rather than an increase, whilst being more likely to increase in rate than decrease.

Conclusions are less clear when looking on a species level. In Table 2.20 binomial tests were run on each yeast, testing the significance of change across all lines. Due to the fact that six samples are the minimum number of tests that can produce a statistically significant result for a binomial test, only those yeasts with all lines changing the same way are statistically significant results. This implies that this test has limited power to detect species wide changes. From this analysis, the conclusion can be drawn that, for C10 and F8 rate is more likely to increase than decrease, and for A8, B3 and B5 yield is more likely to decrease than increase.

	Rate			Yield		
Yeast	**Increased**	**Decreased**	**P-value**	**Increased**	**Decreased**	**P-value**
A1	4/6	1/6	0.688	1/6	5/6	0.2188
A8	5/6	1/6	0.2188	**0/6**	**6/6**	**0.0313**
B3	5/6	1/6	0.2188	**0/6**	**6/6**	**0.0313**
B5	3/6	3/6	1.3125	**0/6**	**6/6**	**0.0313**
C10	**6/6**	**0/6**	**0.0313**	4/6	2/6	0.688
F8	**6/6**	**0/6**	**0.0313**	1/6	5/6	0.2188
SCE2	1/6	5/6	0.2188	1/6	5/6	0.2188

Table 2.20: Species Specific Binomial Tests. Testing the significance of species specific changes in rate and yield.

2.7 Analysis and Conclusions

This section will review the results and analyse whether they can provide insight into the overall aim to investigate whether the development of the Crabtree effect could be an evolutionary trade-off response to resource competition.

This chapter asked if natural populations of Crabtree-negative yeasts increased or decreased their growth rate and yield in adaptation to a high glucose environment. From subjecting seven yeast populations and one Crabtree-positive reference yeast to a long term experimental evolution study of 1500 generations in a high sugar environment, the progression of these populations could be monitored from ancestor to evolved lines and the growth compared. From this data, it was apparent that each yeast developed individually over the time course but that 5 out of 8 populations developed a higher growth rate than the ancestor in 3 or more lines to a statistically significant level. In terms of yield, 6 out of 8 populations developed a lower cell yield than the ancestor in 3 or more lines. One yeast, *Issatchenkia* A11 could not be analysed for rate and yield due to the level of noise that developed and increased over the experiment. C10 increased in both rate and yield and therefore did not share the trend of the other yeast population. This might possibly be an effect of there being greater noise in the *Issatchenkia* yeast populations. However, with the significance of the change and the treatment of the data, there is evidence this is a true change towards increased yield and therefore a noteworthy factor that could be

researched in future studies. *Saccharomyces cerevisiae* SCE2, showed a decrease in both rate and yield.

The data collected from this study would suggest that there is indeed a change in rate and yield in response to a high sugar environment. If the development of the Crabtree effect is the result of an evolutionary trade-off it would be expected that an increase in growth rate achieved by utilising the fermentation pathway would result in a cost to the yield due to the redirecting of the carbon source into the production of ethanol over the production of biomass. The results of this study would support this hypothesis in that yeasts that exhibited an increase in rate generally experienced a decrease in yield, save C10 which had an increase in yield also. However, yield decrease does not appear to be proportional to the rate increase in this study. While the yield does decrease when selecting for rate, the two are not correlated which is further discussed and presented in Chapter Six. This is of interest because if the rate increase was at the cost of yield the changes between the two would theoretically be expected to be proportional, however for a gain in rate there may be a disproportional demand in loss of yield. There might be beneficial mutations that do not relate to metabolism and affect rate and yield independently. The presence of such mutations may make it difficult to observe a direct relation between rate and yield.

There appears to be consistency in many lines in the form of increasing growth rate and the loss of yield, reflected over the majority of the lines. This highlights that there is likely a strong selective pressure enacting upon the yeasts to evolve towards a faster growth rate and that loss of yield is a cost worth paying for the advantage gained. This may provide insight into the evolution of the Crabtree effect. This data supports the idea that competition on a selfish level may drive Crabtree-negative yeasts towards respiro-fermentative pathways (and thus becoming Crabtree-positive) to gain the advantage of a faster metabolic route to compete for a resource, rather than solely for the antibacterial benefits of the ethanol production itself.

As discussed in more detail in Chapter Six some of the measurement variability could potentially be counteracted with more repetitions. While in the current structure more repetitions would

have proven difficult, a reduction in yeasts used and an increase in overall lines of each yeast population may increase the robustness of this experimental data. Other factors could also play a part in the clarity of the results. For example, while methods were implemented to avoid condensation, the quality of the 96 well plate utilised in the growth curve readings or even the way in which the cells clump in the wells, could have an impact. Further limitations, as mentioned, are discussed in Chapter Six.

With these results and taking in to account the nature of optical density measurements, further investigations into the cell size and cell counting to support this chapter is presented in Chapter Four. It would also be of interest to further analyse the role of the respiro-fermentative pathway in the development of increased growth rate in the yeast population. The next chapter will address this by investigating the fermentative abilities of the yeasts in comparison to the ancestors using spectrophotometric assays.

CHAPTER THREE

Tracking Metabolic Changes in Yeasts Using Spectrophotometric Assays

''I have something to say! It's better to burn out than to fade away!''

-Kurgan, Highlander

3.1 Introduction

This chapter will investigate the ethanol production of the yeast populations throughout the 1500 generations using colorimetric assays. It would be expected, in terms of the RYT theory, that the shift to a lower yield and higher growth rate as observed in Chapter Two is accompanied by an increased amount of ethanol production. This chapter will also present data from a colorimetric assay to determine protein quantities to further analyse the change in growth yield presented in Chapter Two. This chapter will conclude with a smaller experiment using Biolog Inc. YT MicroPlate™ (GEN II for Yeasts) identification panels to investigate any changes in the metabolic capabilities of the evolved lines compared to the ancestor of one yeast of particular interest.

3.2 Understanding the Respiro-Fermentative Metabolism

Saccharomyces cerevisiae is renowned for its use in the production of beer, wine and other alcoholic beverages through its production of ethanol. However, yeasts species are heterogenous in their metabolic properties. Some species are primarily respiratory in aerobic environments and grow poorly in anaerobic conditions (Holm Hansen et al. 2001; Visser et al. 1990). Others are facultative anaerobes, having the ability to utilise fermentative metabolism in an absence of oxygen. There are also Crabtree-positive species like *S. cerevisiae* that are able to use both pathways in aerobic conditions (Hagman & Piskur 2015; Gonzalez Siso et al. 1996).

As outlined in Chapter Two, many explanations have been put forward for the emergence of the Crabtree effect. This includes the effect of heat on the organisms environment (Goddard 2008b), the cost of synthesizing the enzymes for the pathway and the space they occupy when membrane bound (Zhuang et al. 2011; Molenaar et al. 2009), the constraints of protein synthesis and ribosome availability (Scott et al. 2010; Kussell 2013), the damaging side effects of reactive oxygen species produced by respiration (Slavov et al. 2014), to the antibacterial effects of the ethanol (Piškur et al. 2006) and the increase in metabolic rate that the pathway allows (Pfeiffer et al. 2001). As outline earlier, the MAC theory supports the hypothesis that the production of ethanol is in response to competition with bacteria and production of the toxin is beneficial in

dealing with competitors. In an experimental scenario, where a yeast grows in isolation in a high sugar environment, the production of ethanol would have no use as a toxin and would be costly to maintain. Such a trait would therefore be selected against and less ethanol production would occur, if not resulting in the complete elimination of the respiro-fermentative abilities of the yeast. Mechanistically, this is a plausible scenario as the manipulation of only a single step in the metabolism of yeast, the glucose uptake, can result in a switch in respiro-fermentative metabolism in *S. cerevisiae* to only utilise fermentation in anaerobic conditions (Otterstedt et al. 2004). In the RYT theory, the hypothesis is based on the trade-offs between rate and yield of ATP production and therefore growth rate and yield (Pfeiffer & Morley 2014). In the same experimental conditions, it would be expected that if respiro-fermentative ability was due to the advantage a yeast gains from a metabolically faster, therefore faster ATP producing pathway, ethanol production would increase from an already low-level ability, or in the long run develop in Crabtree-negative yeasts.

3.3 Experimental Designs

Like many other metabolites (An & Ough. 1993), ethanol is not stored in the cell but excreted into the environment. Ethanol is a small polar but uncharged molecule that passes easily through membranes by diffusion making storage of all ethanol impossible, which is beneficial, as high levels of intracellular ethanol are toxic. Experimental techniques to estimate ethanol concentrations from the environment, while not directly measuring the intracellular ethanol, will provide insight into the ethanol production level of the yeasts. A popular method is to use high performance liquid chromatography (HPLC) to analyse all metabolites present in a sample (Hagman et al. 2013; Frick & Wittmann 2005b; Ramon-Portugal et al. 2004). The spectrophotometric assay opted for in this study allows for a more cost-effective method that can process a large number of samples whilst also allowing for the processing of small sample volumes, a feature HPLC typically lacks.

The spectrophotometric assay is based on the reaction of alcohol dehydrogenase (ADH) (Walker 1992). The ADH uses NAD+ to dehydrogenate ethanol resulting in the reduction of NAD+, a reaction that is detectable in a spectrophotometer at 340nm. The method is easily adapted to 96-well microplates as reviewed in the methods section below.

3.4 Aims

This chapter will investigate the following questions:

- Do the evolved yeast lines increase or decrease in ethanol production after 1500 generations?
- Is the change in growth yield in the evolved lines loss reflected in protein quantity?
- Is there any exhibitable change in metabolic ability according to a commercial yeast identification panel in a selected yeast of interest?

These questions will be addressed through the use of assays to investigate whether over 1500 generations 7 yeast populations derived from isolates, and a *Saccharomyces cerevisiae* for comparison. Ethanol concentrations are measured during a 24-hour growth cycle in two different experiments. The first experiment covers all ancestors and evolved lines, but at a very coarse temporal resolution; while the second experiment focuses on four yeasts of particular interest and uses a higher temporal resolution.

Protein content of each line will be measured as a supplementary method of determining the growth yield. Finally, a few populations metabolic function will be further investigated using Biolog Inc. YT MicroPlate™ (GEN II for Yeasts) identification panels, discussed later in the chapter.

3.4.1 Predictions

The study carried out in Chapter Two on the growth properties of the evolved yeast lines shows that in many lines the growth rate increases while the growth yield decreases. If the observed changes were the result of a metabolic shift towards the Crabtree effect, ethanol concentration

would be expected to increase in the evolved lines in comparison to the Crabtree-negative ancestors'.

In terms of protein concentration, it would be expected that protein quantity would reduce in the evolved lines with reduced growth yield, reflecting the reduced amount of biomass produced. In respect to the yeast identification panel (Biolog) it would be expected that most metabolic capabilities remain consistent through the ancestor and evolved lines. Small variations in metabolic profile between ancestors and evolved lines as detect by the Biolog might be possible and be useful in the development of selective markers in the form of growth differences on specific media. Big changes would however point to potential contaminations of the evolving lines by different yeast species with different metabolic capabilities.

3.5 Ethanol

3.5.1 Sampling

The first experiment on ethanol production used samples taken monthly during the experimental evolution. The running culture plates were cloned for each time point measured, hour 0, hour 10 and hour 13 and grown using the same environmental conditions as the experimental evolution as specified in Chapter Two. Sampling consisted of measuring the optical density of the plate allocated to the time point, then taking 200µl of culture of each replicate and using 1ml syringes attached to 0.22µm PES 13mm diameter syringe filters, filtering the cells out to retain only the metabolites and media components. These samples were then frozen and run through a colorimetric assay at a later point. Assays of the samples of each ancestor and the derived lines at generation 1500 were run on a dedicated multi-well plate for each yeast at the end of the experiment.

For the second experiment, more extensive measurements were taken on four of the yeast populations, A8 *Kodamaea*, B5 *Pichia kluyveri*, C10 *Issatchenkia orientalis* and SCE2 *Saccharomyces cerevisiae.* For this a plate per yeast was set up with three ancestor samples and

one sample per evolved line. The yeasts were measured at inoculation, left to incubate for six hours and then sampled in two-hour intervals between hour six and hour fourteen, with one final measurement taken at saturation after 24 hours. Two plates were run per yeast for replication purposes. Samples were treated as during the experimental evolution with the same environmental conditions, with filtering and optical density measured at each time point. A blank was taken per plate per time point. Samples were frozen and assays run alongside each other at a later point.

3.5.2 Assay

The assay requires several components. Prior to set up a Tris/Lysine buffer must be made using 0.6M Tris and 0.4M Lysine in MilliQ water and PH adjusted to 9.7 using 1M KOH. This mixture was autoclaved prior to use.

The ADH assay converts ethanol into NADH which absorbs UV at 340nm. The concentration of NADH detected is a direct representation of ethanol concentration. For measurement of a large quantity of smaller samples (the experimental sample volume was 200µl) measurements were carried out in 96-well microplates. For this a batch of reaction mix was made for each plate. This consisted of 8.0mg NAD at 0.72mM per sample, 9ml H20 and 9ml Tris/Lysine buffer. 20µl of sample was placed in each well, along with a number of blank media samples. The reaction mix was vigorously mixed and 160µl of mixture pipetted into each well with a multichannel pipette. Prior to measuring a batch of ADH is prepared. Stock of 5mg was prepared prior to experimentation, as this was the smallest amount accurately measurable and this amount covered two entire microplate tests. Therefore 2.5mg of ADH was suspended in 2.25ml. 20µl of the ADH was added to each well immediately before the plate was placed in the photometer, which gave about 0.01mg per sample. A selection of test wells were devoid of sample with half containing the buffer mix and ADH and the rest without ADH. The plates were then measured every minute for 20 minutes at 340nm in the photometer.

Standards were constructed to run alongside all microplates in the form of a serial dilution of ethanol. Standards were composed of 1ml ethanol inoculated into 2ml H_2O and then serial transfer of 1ml the dilution into 2ml of H_2O carried out 11 times. The last 5 of these dilutions were utilised as the standards giving the concentrations 0.346 g/L, 0.115 g/L, 0.0384 g/L, 0.0128 g/L, 0.00427 g/L, and 0 g/L.

3.5.3 Analysis

While the assay gives an optical density measurement, this does not directly correlate to concentration in a sample. Linearity between absorption at 340nm and ethanol concentrations was observed for low ethanol concentrations, but absorption saturated at concentrations higher than 0.115g/L. A non-linear least-square regression was used to fit absorption data from the standards, and subsequently translate the absorption of the samples into ethanol concentrations. Upon the acquisition of these converted values unpaired, unequal variance t-tests were applied to determine the changes between the 6 evolved lines and 3 repeats of the ancestor line. These were done over all time points and for all populations.

3.5.4 Experimental Evolution Ethanol

During the experimental evolution study the ethanol content was measured every month. Due to plate to plate variance a selection of generations was assayed back to back on the same plate. This allows for the comparison of the evolved lines at 1500 generations to be compared with the ancestor, sampled after stability was achieved early on in the experiment, analysing six ancestor isolates against six evolved lines. The experiment provides data across three time points at inoculation, and at hour 10 and 13 which was selected because during pilot studies these times corresponded to exponential growth and the endpoint of exponential growth respectively for most yeast populations. Some patterns can be observed from the data presented in Figure 3.1. As would be expected SCE2 *Saccharomyces cerevisiae,* is the highest producer of ethanol between at time points T10 and T13.

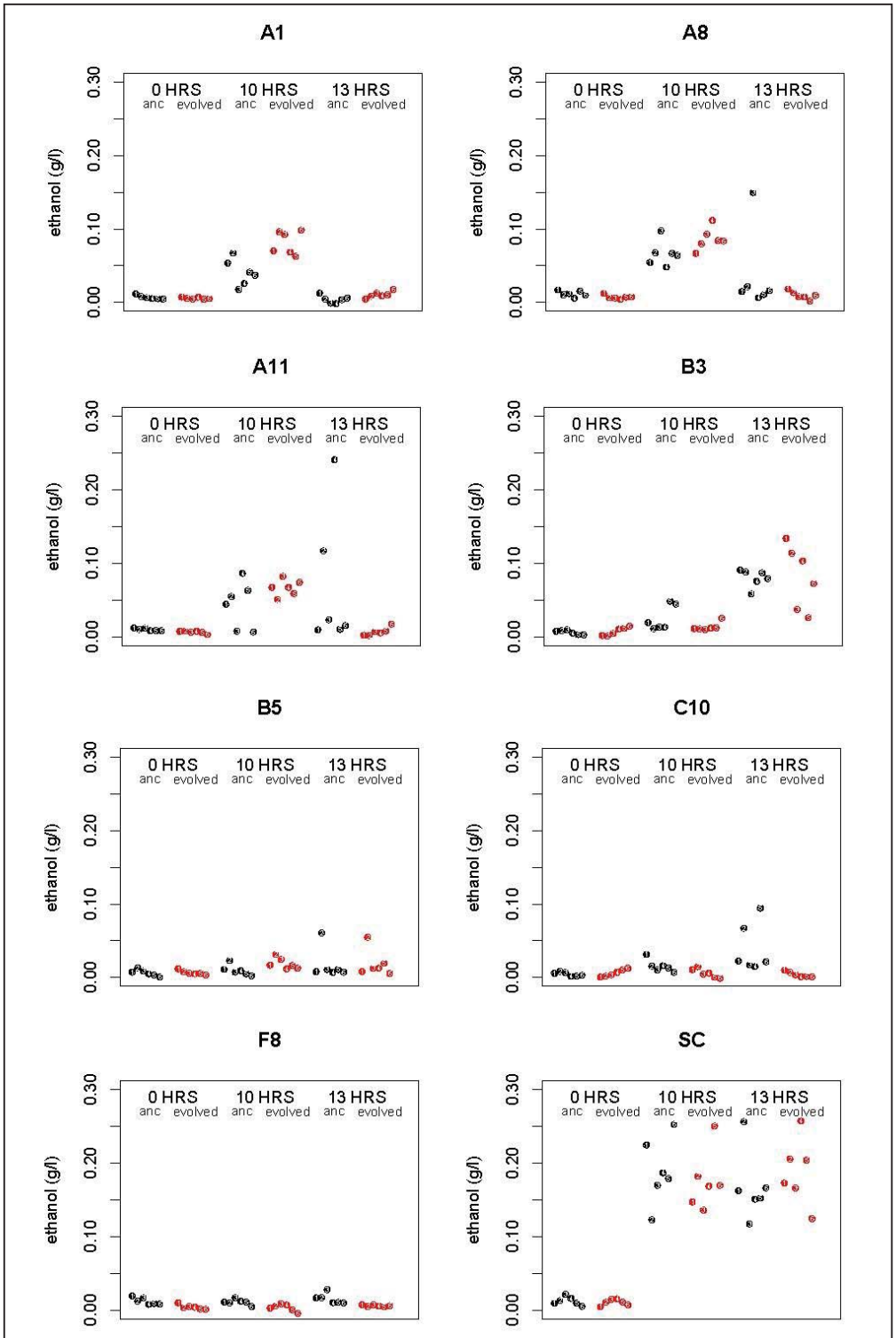


Figure 3.1: Graphs of each Populations Ethanol Concentration. Comparing early evolved lines (ancestors) with late evolved lines. Black represents the January evolved lines (ancestor) sampling at the start of the experiment while red represents the August evolved sampling at the end. Each time point is signified at the top of each graph, lines are numbered.

There is little variation detected between the ancestor and the evolved lines. The lowest producer of ethanol appears to be yeast population F8 *Kluyveromyces nonfermentans* with no change detected between ancestor and evolved lines. Other populations also show very low levels of ethanol in the ancestor, namely B5 *Pichia Kluyveri* and C10 *Issatchenkia orientalis Kudryavtsev,* with no substantial change in ethanol production in the evolved lines being observed. The remaining populations, as expected show low to no ethanol production in the ancestor. Some intermediate increases in ethanol production is shown, at T10 for A1 and A8 both

Kodamaea sp., and smaller increases in A11 *Issatchenkia sp..* Moderate increases in ethanol production in some of the evolved lines can be seen at T13 in yeast population B3 *Candida railenensis*. However, there are no consistent changes that systematically apply to all lines in these four populations but some small systematic changes are possibly shown in populations A1, A8 and B3.

3.5.5 In Depth Ethanol

Due to the small sampling window and the potential for a skewed impression to be gained from this limited window, a selection of populations were further investigated over 24 hours with two hourly intervals, during mid-late exponential growth (between 6 and 14 hours after inoculation). The strength of the rate yield changes observed and reviewed in Chapter Two resulted in selection of the populations A8, B5 and C10 for further investigation. SCE2 *S. cerevisiae* was measured alongside as a Crabtree-positive reference.

To establish ethanol production of each yeast population the ethanol over time was plotted. Replicates, both separate plates and the three samples per plate for the ancestor, were pooled. Then for added statistical robustness the evolved lines data were also pooled and statistical tests carried out of all evolved lines versus the ancestor for each of the four populations subjected to this experimental condition. This allows for the testing of systematic changes in all evolved lines compared to the ancestor. However, the statistical power from this experimental design did not allow for the detection of changes in single lines.

Figure 3.2 and Table 3.3 present the ethanol concentration changes demonstrated over a 24-hour period. Yeast population A8 demonstrates a significant decrease in ethanol concentration overall from hour 8 through to hour 24. This is surprising as the previous experiment suggested that there was an increase in most lines. It is possible that the higher ethanol concentrations in experiment 1 are observed because faster growth in the evolved lines means these lines were already at a higher population size.

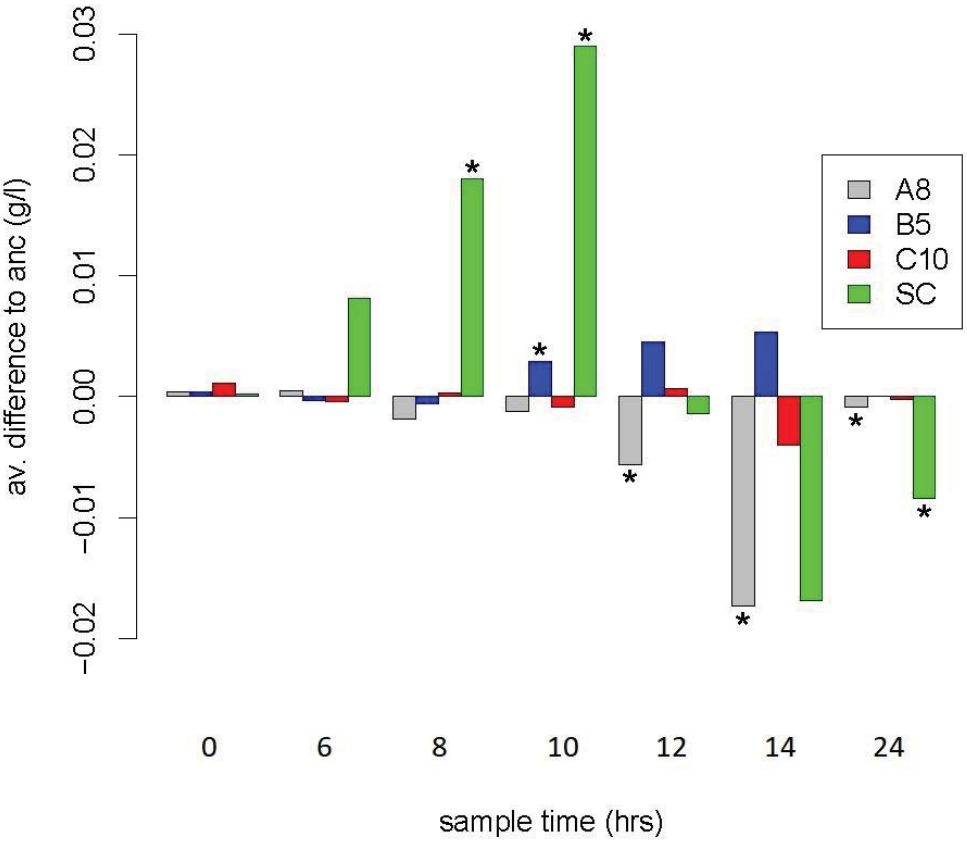


Figure 3.2: Average Change in Ethanol Production (g/L) per Population. Comparing evolved lines with the ancestor. Over 24 hours each of the four yeasts was sampled along with 3 repeats of the ancestor across two repeat plates. Each yeast is represented by a specific colour as shown in the key. Significance in marked with an asterisk.

Yeast population B5 demonstrates only one significant change over the 24 hours. At hour 6 there is a significant increase in ethanol concentration in comparison to the ancestor. While the trend appears to continue towards increasing, no significant change from the ancestor is exhibited.

Species	Sample Time (hrs)	D-value	T-value	P-value
A8	**0**	0.000	-0.908	0.397
	6	0.000	-0.898	0.424
	8	-0.002	1.612	0.159
	10	-0.001	0.629	0.553
	12	**-0.006**	**2.948**	**0.028**
	14	**-0.017**	**2.943**	**0.022**
	24	**-0.001**	**4.427**	**0.004**
B5	**0**	0.000	-0.814	0.446
	6	0.000	1.087	0.315
	8	-0.001	1.446	0.193
	10	**0.003**	**-3.545**	**0.010**
	12	0.005	-1.611	0.167
	14	0.005	-1.659	0.150
	24	0.000	-0.356	0.735
C10	**0**	0.001	-1.311	0.235
	6	0.000	1.508	0.177
	8	0.000	-0.319	0.772
	10	-0.001	0.466	0.660
	12	0.001	-0.217	0.836
	14	-0.004	0.907	0.395
	24	0.000	1.421	0.198
SC	**0**	0.000	-0.615	0.558
	6	0.008	-2.239	0.062
	8	**0.018**	**-2.751**	**0.031**
	10	**0.029**	**-2.948**	**0.035**
	12	-0.001	0.292	0.779
	14	-0.017	2.269	0.066
	24	**-0.008**	**2.555**	**0.040**

Table 3.3 Statistical Significance of Changes of Ethanol Concentration over 24 hours. Unpaired, unequal variance t-tests run on all the evolved lines versus the ancestor of four of the yeast populations. Statistically significant results are highlighted in bold.

With yeast population C10 there is no apparent change in ethanol concentration over the entire 24-hour period. Lastly, SCE2 demonstrates a different trend over the 24-hour sample period. Initially at hour 4 through to hour 6, there is a significant increase in ethanol concentration

compared to the ancestor. After this, the concentration decreases compared to the ancestor to result in a significant decrease at hour 24. This suggests that rather than a change in ethanol production the change is in the shift in timing of the peak ethanol point, i.e. the growth time has shifted to be faster than the ancestor and therefore reaches the peak ethanol earlier in the experiment than the ancestor. This is somewhat surprising because the growth data from Chapter Two do not suggest the evolved lines grow faster.

3.5.6 Summary

The experiments discussed above aimed to investigate the ethanol concentration change between the evolved and ancestor lines. Two experiments were carried out, firstly ethanol was monitored through the entire course of the experimental evolution to track any changes in real-time and then analysed back to back to eliminate plate variance. Secondly four populations were selected for more intensive study over a longer period of experimental monitoring. From the first experiment, no clear picture could be determined due to a small window of time being sampled. Therefore, A8 *Kodamaea*, B5 *Pichia kluyveri*, C10 *Issatchenkia* and SCE2 *S. cerevisiae* were further tested with more intense sampling carried out. The lack of statistical power in these experiments means that a conclusion cannot be made on the differences between ancestors and individual lines. In terms of systematic changes that affected all evolved lines of a given ancestor, only B5 seems to show a small increase in ethanol production, while A8 appears to decrease. From this it is concluded that without further investigation no notable results were observed. To achieve this, stronger investigation into optical density and glucose concentration may help in gaining further insight into the changes in ethanol across the evolution experiment and to clarify if the results here are in fact real observed change. Additionally, it would be of interest to look into other metabolites that could potentially have been excreted into the medium.

3.6 Protein Assay

Spectrophotometry is an objective way of measuring absorbance of light of a sample as an expression of wavelength. In a spectrophotometer, the light source is directed into the sample

and, upon hitting the sample, is absorbed and the transmitted fraction is measured as the optical density. Using optical density to measure highly concentrated samples is difficult as the observations become non-linear with increasing solution concentration. Moreover, when tracking populations of cells, the absorption behaviour of evolving populations could in principle change, for example, because of differences in cell composition, size and shape. Due to the indirect nature of optical density as measurements for growth yield, it is of interest to validate OD-based results with alternative methods. One alternative is to measure the dry mass of samples taken during growth. However, this technique requires larger samples, and is labour intensive. Optical density measurements can also be validated using colony forming unit (CFUs) counting, a method that will be tested further in Chapter Four. Another alternative is to measure the protein concentration of a culture. Therefore, the method opted for was a colorimetric assay, the Thermo Scientific™ Pierce™ 660nm Protein Assay.

3.6.1 Methods

3.6.1.1 Sample Preparation

To determine the protein concentration of the cultures, the culture had to be isolated from the medium and the cells broken down as the assay cannot identify protein as part of a complex structure such as cell walls and organelles. To prepare the samples, 1000µl of culture was saturated for 24 hours, then harvested into 1.5ml micro-centrifuge tubes. These were centrifuged at 4000rpm for 5 minutes at 4°C, and the supernatant discarded. 100µl of 0.5mm beads and 100µl of lysis buffer stock that consists of 100mM Tris-HCl, 5M NaCl, 2% SDS and 0.8% Triton X-100. The samples were then vortexed in 30 second bursts with 30 seconds on ice for roughly 10 rounds until a white film forms at the rim of the suspension. These were then centrifuged at low speed at 3500rpm for 10 minutes and the supernatant containing the protein isolation was transferred to fresh micro-centrifuge tubes and stored in at least a -20°C for short term storage or -80°C for long term storage.

3.6.1.2 Pierce™ 660nm Protein Assay

The kit from Thermo Scientific™ provides a selection of standards of Bovine Serum Albumin (BSA) at pre-set concentrations of 2000µg/ml, 1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml and one self-diluted from 1000µg/ml, 50µg/ml standard. Standard curves are constructed from standards run on every plate. For 96-well Microplate testing 10ul of sample is needed with 150µl of Protein Assay Reagent for each well. The plate is covered and mixed for 1 minute, then incubated at room temperature for 5 minutes. The absorbance is then measured using a spectrophotometer at 660nm. The standard curve is then used to determine the protein concentration of the samples.

3.6.2 Results

Two repeats of each line and ancestor were carried out due to the number of samples this entailed (56 samples per repeat). To produce a statistically robust result the values for all ancestors were pooled and compared with the pooled results of all lines. The data for individual lines were also statistically analysed but the reliability of such an analysis is minimal.

yeast	line	Av. Protein	Std. Error	T-value	P-value
A1	**ancestor**	32.465	9.280		
	evolved	40.831	10.020	0.840	0.420
A8	**ancestor**	30.625	6.700		
	evolved	29.665	7.240	-0.130	0.900
A11	**ancestor**	18.17	5.200		
	evolved	15.478	5.610	-0.480	0.640
B3	**ancestor**	13.73	6.530		
	evolved	19.681	7.060	0.840	0.420
B5	**ancestor**	17.515	7.420		
	evolved	29.768	8.020	1.530	0.150
C10	**ancestor**	16.205	7.610		
	evolved	23.244	8.220	0.860	0.410
F8	**ancestor**	22.32	6.220		
	evolved	25.557	6.720	0.480	0.640
SCE2	**ancestor**	43.76	5.080		
	evolved	33.016	5.490	-1.960	0.070

Table 3.4: Protein Content for Ancestor and Evolved in µg/ml. Statistically significant results are highlighted in bold.

The assay was intended to act as a supporting measurement of growth yield by measuring the biomass of a select quantity of culture. This method quantifies protein content via reference to a standard. The results are not statistically rigorous due to only two repeats conducted for each line, having been limited by size of experimental sampling possible. Despite this some insight can be gained from the results. While no statistically significant changes were detected in the assay when pooling the line data to compare to the ancestor in a statistically rigorous analysis, all yeast save A8, SC and A11 seem to increase in protein content, while these three seem to decrease. However, the protein levels of the samples measured were mostly below the lowest point of the standard, there was quite a large amount of variation between repeats. Moreover, this was despite the fact that the samples were already concentrated by a factor of 10 from 1000µl culture, as the experimental evolution culture size was unmeasurable. The data thus suggests that other methods should be utilised to confirm the OD-based growth yield data.

3.7 YT MicroPlates GEN II for Yeasts

The differences between ancestor and evolved lines in both rate and yield led to the development of the idea to examine natural markers that could be used to track each line during experimental procedures to determine fitness against the ancestor. A yeast identification panel, usually used to identify yeast species, but that contains a large number of nutrient based tests, seems the ideal technique for finding such markers. In carrying out this test it could also be useful in reaffirming no contamination has occurred and corroborating the yeast populations correlate to the species information.

3.7.1 Methods

Methods were provided upon purchase of the YT MicroPlates GEN II for Yeasts (Cat No: 1005) from Biolog Inc. which will be summarised here. The turbidity range of the turbidimeter or photometer should be established for use in the test. The desired transmittance level as stated by the protocol is 47% with a 20mm diameter tube in a turbidimeter. This translates to an absorbance of 0.148 at 590nm in a 1cm cuvette in a photometer. Firstly, a pure culture for testing

is isolated on fresh YPA plates and grown over 24 hours. Cells should be sampled from the agar plate using a sterile cotton swab so as not to carry over nutrients. This sample should be inoculated to 0.148 OD into sterile water, this must be done with precision since it establishes the oxygen concentration for the cells and for the redox chemistry. Enough suspension should be made to inoculate 100µl into each well of one MicroPlate. When inoculating the samples into water any clumps should be broken up to produce homogenous suspension. Once a homogenous culture is produced, inoculation of the culture should be conducted immediately due to the chance of some yeasts losing metabolic capabilities if suspended in water for too long. Once the plate is inoculated the plate should be covered with the provided lid and incubated at 26°C for 24, 48 and 72 hours measuring the turbidity/OD at each point. The decision was made to incubate at the experimental temperature of 30°C to simulate the same conditions as the experimental evolution. Providing a source of moisture in the incubator helped to minimize dehydration of the outer wells.

3.7.2 Results

Biolog Inc. recommends scoring each well against the negative controls at positions A1 and D1. Anything greater than 0.4 OD would classify as positive and anything below 0.1 OD would be negative. Positive and negative scores are used to identify yeasts in their database. Identification via the database was not the main aim of this test, thus the results for this experiment were plotted against each other in R to compare the correlation of results between replicates, populations, ancestor and evolved, and genus. This would highlight any obvious variations between the two plates and thus any potential metabolic differences would be framed as outliers on the plot. Figure 3.5 shows some examples of interest from these experiments while the full collection of graphs can be found in Appendix B.

Firstly, on a genus level, comparisons were taken of the two *Kodamaea* versus *Saccharomyces cerevisiae* and *Kluyvermyces marxianus*, two yeasts already recorded in the Biolog Inc. database with known metabolic panel results. These two yeasts were run initially as a pilot study of the

plates to test that the batch received was functional. The results however give us a rough comparison of genus. Figure 3.5 A demonstrates *Kodamaea* A8 ancestor versus *S. cerevisiae*, Figure 3.5 B A8 ancestor versus *K. marxianus* and Figure 3.5 C, an evolved line (5) with *K. marxianus*. These demonstrate how there is very little similarity between the Biolog results for these genera. A1 results can be found in Appendix B.1 and show similar conclusions. In a comparison between both A1 and A8 ancestor (Figure 3.5 D), they exhibit very similar results suggesting as expected that they are likely to be the same genus. There are a number of small variations such as at G9 (tween 80 assimilation) or E2 (gentibiose assimilation) which may suggest the two isolates are not necessarily the same species or variant. However, E2 at least here, is an example of a test that shows variation even between replicates in A8 (Appendix B.1) and therefore may not be a very reliable marker. Other outliers observed were E4 (maltriose assimilation) for A1 ancestor replicates, and E2 (gentibiose assimilation) and A12 (Inulin oxidation) for A8 ancestor.

Comparison of replicates of each of the evolved lines was carried out and once again although most tests show consistency (see Figure 3.5 E as an example), there were a few outliers which would affect any comparisons between ancestor and evolved lines (see Appendices B 1-3 for all replicate comparisons for A1 and A8). When comparing A1 evolved lines to the ancestor, test E2 in evolved line A1.6 was the only test with notable change that was consistent throughout the replicates of A1 ancestor and evolved (Appendix B.2). In terms of comparing A8 evolved lines with the ancestor (see Figure 3.5 F as an example), test E1 (d-cellobiose assimilation) in evolved line A8.2 was the only test of note that was also consistent throughout the replicates of A8 ancestor and evolved lines. All other changes could be attributed to possible variation in the replicates.

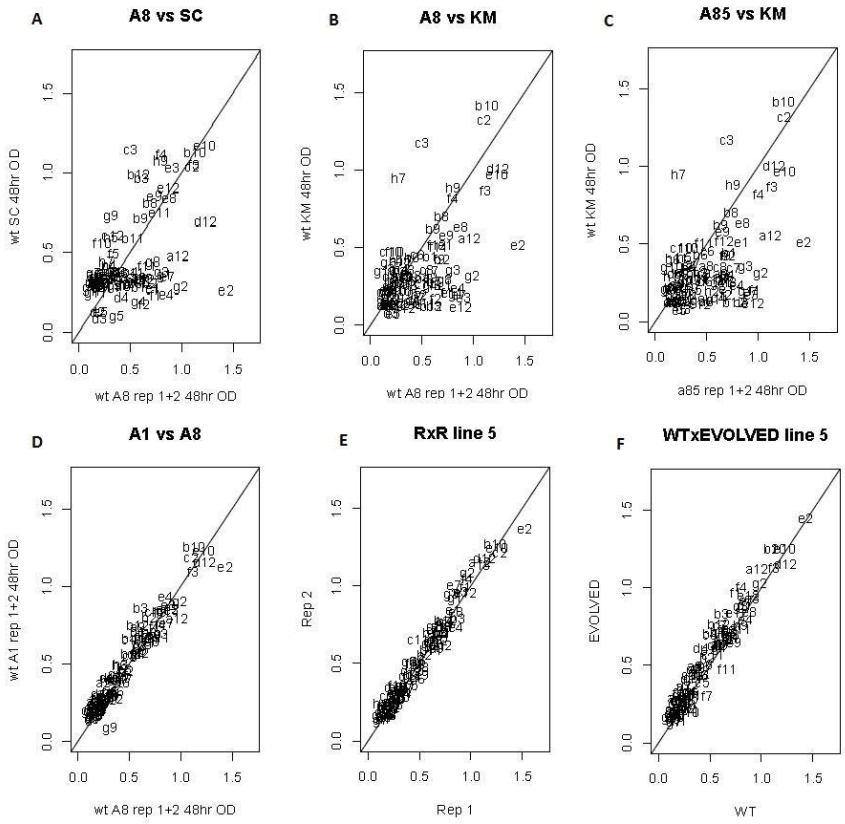


Figure 3.5: Biolog Results: A) *Kodamaea* A8 ancestor versus *Saccharomyces cerevisiae*, B) *Kodamaea* A8 ancestor versus *Kluyveromyces marxianus*, C) *Kodamaea* A8 evolved versus *Kluyveromyces marxianus*, D) *Kodamaea* A1 ancestor versus *Kodamaea* A8 ancestor, E) *Kodamaea* A8 line 5 repeat one versus repeat 2 and F) *Kodamaea* A8 ancestor versus *Kodamaea* A8 evolved line 5.

3.7.3 Summary

The Biolog Inc. plates allow for a wide range of metabolic tests to be carried out on a selected yeast. This can help with identification of yeast, contamination checks and possibly highlight markers for use in competition experiments. From this experiment, it can be confirmed that both A1 and A8 appear to both be the same genus, i.e. *Kodamaea,* due to a very strong correlation between the tests results with few variations. When looking at the evolved lines versus the ancestor it does not appear there is much in the way of variation that cannot be put down to replicate discrepancies. No contamination of the evolved lines with other yeast species is obvious from the test. Unfortunately, from this data there were no tests that strongly suggested they

could be used as markers for competition experiments between ancestor and evolved lines other than E2 in A1 line A1.6 and E1 in A8 line A8.2. In conclusion, this method showed no exhibitable change in metabolic ability according to the commercial yeast identification panel in both *Kodamaea*.

3.8 Analysis and Conclusions

This chapter aimed to further investigate the potential metabolic shift in populations due to the evolved lines being found to reflect a change towards a rate-yield trade-off through evolutionary history as reviewed in the last chapter. The use of a spectrophotometric assay based on the reaction of alcohol dehydrogenase was opted for. This technique measures extracellular ethanol and therefore is an indirect quantification of the overall ethanol production. It was predicted that if a metabolic shift towards respiro-fermentative metabolism occurred, the populations' ethanol production would increase in comparison to the low ethanol producing ancestors. The first experimental data obtained during the experimental evolutionary study presented an unclear image of the populations based on a small window of time for each yeast population and little correlation throughout the ancestors. To combat this, four populations were further studied over a 24-hour period at 2-hour intervals. Out of the populations, B5 appeared to increase while A8 appeared to decrease, compared to the ancestor lines. SCE2 did not appear to change but did seem to demonstrate a shift in when the ethanol production peak point occurred. This would reflect a change in the growth cycle over 24 hours in the yeast. While optical density and glucose were also measured for this, the data produced was unreliable due to standard inconsistencies and condensation on the optical density measurements skewing the measurements. Due to the number of samples being handled, there were only two replicates for each line, so individual differences could not be compared between evolved lines and the ancestor. This could explain the questionable nature of the results. The yeasts in this study are low in ethanol so there is no guarantee that the intra and extracellular ethanol quantities directly correlate and may in this case have affected the outcome of the results. While one line may have a major change and

others no change at all, this could skew the results in the pooled analysis toward an untrue reflection of change. Taking this into consideration it is concluded that no change could be reliably deduced from these experiments and further experiments would be required to make any definitive conclusion.

Secondly the chapter aimed to support the growth yield data presented in the Chapter Two. The protein content of each line was measured, however there was a lack of statistical robustness therefore pooled data of ancestor and evolved lines for each yeast population was used. It was predicted that this experiment would demonstrate a decrease in protein quantity in the evolved lines due to a reduction in growth yield and therefore the amount of biomass. However, there was no change of note detected. This could be the result of a lack of statistical robustness presenting inconsequential results, a genuine lack of change in protein content, or a change being undetected due to the small volume size of the samples resulting in protein content that was not realistically measurable in this assay. Despite sampling at a level for statistical validity (two replicates for each line) further testing would be required for to adequately derive robust measures of statistical significance. If a protein content change is occurring but was not detected due to volume size, this could be addressed in future experiments in a number of ways including: use of more refined techniques; analysis on a cellular level; or by running further tests on protein content at higher volumes. Due to the fact, the volume size had already been altered from the original experimental evolution study volume, it seemed unjustified to increase this volume again and further introduce distance between the relatability of the two experiment samples. Lastly, if there is legitimately no change occurring, this should be confirmed using another method of measuring growth yield to clarify that biomass has not changed. This would suggest cell size has changed, i.e. the cell count has increased but the cell size has decreased resulting in the same biomass as the ancestors but an actual increase in growth yield overall. This should be further investigated.

Lastly the chapter aimed to investigate if there were any trackable metabolic changes occurring through the use of Biolog Inc. YT MicroPlates GEN II identification panel for yeasts. This could

provide more in-depth detail on the evolutionary changes the yeasts were undertaking and result in trackable metabolic differences that could be used to compete ancestor with evolved lines. The *Kodamaea* populations were selected for further investigation. The results did not produce any strong tests for metabolic differences that could be utilised for markers in competition experiments, however they did confirm that the *Kodamaea* populations are most likely the same yeast and possibly the same species, but not so uniform that they are the same isolate. This provides evidence that at least the evolved lines are related to the ancestor lines and that both populations are likely to show common traits but have developed independently. Furthermore, consistencies seen in the data from the experimental evolution study are not due to identical isolates being used.

From the data presented in this chapter it is apparent that further experiments are required. It would be beneficial to find a further method of confirming growth yield as the protein assay was inconclusive, and therefore the next chapter will attempt to address this through the use of colony-forming unit counts, cell counts and size determination performed using flow cytometry.

CHAPTER FOUR

Using Flow Cytometry to Analyse the Physical Changes of the Population and Individual Cells

''Never give up, never surrender!''

-Commander Peter Quincy Taggert, Galaxy Quest.

4.1 Introduction

This chapter sets out to reaffirm the results from Chapter Two, in which growth yield was observed to decrease in six out of eight populations. Colony forming unit counts, and flow cytometry are used to count cells and measure cell size in both ancestor and evolved lines. These investigations are carried out due to the limitations of the use of optical density as a validation of population size, i.e. optical density can only demonstrate the population biomass as a whole, not the number of cells in a population directly. Furthermore, as size changes were observed in previous experimental evolution studies, flow cytometry will be used to acquire size estimates.

4.2 Measuring Populations

4.2.1 Optical Density

Optical density measurements are frequently used to determine the population size of a microorganism experimentally (Novak et al. 2009; Julou et al. 2012; Jasmin et al. 2012; Mo et al. 2001; Blount et al. 2008). While optical density is widely accepted as a measurement of a microorganism's overall population biomass, it is also understood that this measurement alone cannot be directly correlated to cell count and is often supported with other experimental means (Volkmer & Heinemann 2011). As optical density is a measurement of light absorption, anything that absorbs the light is read in the optical density measurement. This means that debris, live and dead cells and cells of variable size will be measured and can skew the results. For example, larger cells will absorb more light than smaller cells, creating the illusion of a higher population compared with a population of equal size with smaller cells. Furthermore, when given the same amount of space to occupy, smaller cells can maintain a higher population size than larger cells. Thus, the estimation of population size through the counting of colony forming units (CFU) and the counting of cells using flow cytometry, is used. In addition, flow cytometry is also used to measure the size of cells in a population.

4.2.2 Flow Cytometry

Flow cytometry is an very useful analytical tool to rapidly measure light scattering and fluorescence produced by cells (Álvarez-Barrientos et al. 2000). Flow cytometry can be utilised in cell counting, sorting, DNA quantification and marker detection. Cells are suspended in sheath fluid and individually passed through a laser beam. The light scattering during passage is measured by detectors together with fluorescence signals and sent to be interpreted by a computer. This method is valuable for microbiological sample analysis due to the ability to analyse a large number of single cells in a relatively short space of time and acquire multiparameter data sets (Davey & Kell 1996; Bruetschy et al. 1994). Flow cytometry is a useful tool that allows for the analysis of mixed populations, employing methods that can use fluorescence and markers to identify heterogeneity in samples. However, this technique is limited by the need for single cells suspended in fluid, meaning clumps and debris can interfere with readings and create noisy data. Vigorous mixing and utilising different voltages can prevent these interferences having a significant effect on data. Whole tissues and the spatial association of multiple cells cannot be investigated without breaking the structures into singular cells, which for this study was not an issue.

4.2.3 Colony-Forming Units

Counting colony-forming units (CFU) has been another popular method for quantifying population size (Sieuwerts et al. 2008) especially alongside optical density, and is especially useful for mixed cultures. The important factor to consider when employing this method is that CFUs are not cells, and there is no absolute surety one CFU was the result of one cell, many cells or overlapping CFUs. Therefore CFUs do not directly measure population size (Sutton 2011; Julou et al. 2012). However, when used collectively with optical density and flow cytometry, the significance of a given change may be realised.

4.2.4 Summary

The methods for population analysis all approach the issue of population quantification in different ways. Optical density is a popular measurement but it is proportional to the biomass of the population, including debris and dead cells, not the cell number. It also does not factor in the size of the cells which can introduce inaccuracies dependent on the time during the cycle a culture is sampled. Size changes can be conditional to what stage the cell is at in mitosis, post or pre-division (Akerlund et al. 1995).

Generally, CFUs are the next preferred method, but CFUs are only proportional to the observed viable colony forming cells that grow on agar plates, eliminating debris and dead cells, but also not factoring in the potential for colonies to be from multiple or overlapping cells (Bruetschy et al. 1994). Flow cytometry allows for a more accurate representation of cell number in a population, as each cell is counted individually. This method allows for the size of a cell to be determined, and in certain types of cell, other factors that can confound optical density can be measured, such as granulation. The down side is all elements that go through the laser will be measured, including debris and dead cells, but there are a number of methods that can address this. Flow cytometry cannot differentiate cells when clumping is a factor so results can be misinterpreted when this is not taken into consideration (Laplace-Builhé et al. 1993).

Taking this information into account, this chapter will address how to support the yield data obtained in the experimental evolution study using a combination of these methods. CFUs will be counted and flow cytometry samples for counts and size analysis with be run for all ancestors and evolved lines.

4.3 Aims

This chapter aims to investigate the following questions:

- Is there a change in cell count between ancestor and evolved lines?
- Is there a change in cell size between ancestor and evolved lines?

- Is there a change in colony number between ancestor and evolved lines?

These will be addressed through the use of flow cytometry and CFU counts. The ancestor and evolved lines of the seven populations and *Saccharomyces cerevisiae* will be investigated to determine whether the yield decrease exhibited in the optical density measurements in the experimental evolution study was accurate and a true decrease occurred in most lines over evolutionary time. This optical density decrease could however be the result of a cell size change and it is therefore worth verifying this.

4.3.1 Predictions

In this experiment, it would be expected that the decreased OD-based growth yield estimates are reflected through decreases in CFUs or cell counts or both. For cell size, there is no specific expectation. It is expected that OD reflects total biomass in the population and thus lower biomass would translate to fewer cells, smaller cells, or both. If one of these factors increase and the other decreases, it is unclear whether this would translate into more or less biomass. It is possible that indirect effects might change OD for the same biomass. If biomass is distributed in a different way, for example more cells but smaller sized cells, this might change OD despite the biomass remaining the same. It is expected, when considering the RYT theory, that most likely cell size has not changed and the growth yield decrease in most populations will be reflected in the CFUs and cell count.

4.4 Methods

4.4.1 Sample Preparation for Flow Cytometry

Two repeats of each ancestor and evolved line were inoculated to a 1/100 dilution in 200µl 1g/L SM medium for 24 hours in 96-well microplates at 30°C shaken at 150rpm in an incubator. These were then isolated into 15ml Falcon tubes and diluted with 1800µl of phosphate-buffered saline (PBS) stock buffer. The buffer was made up of 80g NaCl, 2g KCl, 14.4g NaHPO, 2.4g KHPO in 1 litre MilliQ water, adjusted to pH 7.4 and autoclaved. This is needed to be diluted prior to use 1:10 for

example 2ml of stock in 18ml MilliQ water. These samples were then vortexed and put on ice for sampling.

Size calibration beads were also prepared to provide a standard to compare the experimental samples to. Calibration beads of sizes 4µm, 6µm and 10µm were prepared for this experiment. Molecular Probes' Flow Cytometry Size Calibration Kit (F-13838) vials, were sonicated first to reduce clumping of the beads and one drop or 10µl of the microsphere mixtures were independently added to a Falcon tube containing 2ml PBS and vortexed to mix. Each size of bead was measured separately.

Samples were vortexed vigorously and run through a BD FACSCanto™ II cytometer. Forward scattered light (FSC) was used to determine cell size, and samples were run at low speed for 60 seconds with events at maximum capacity of recording, resulting in about 10µl of sample being analysed for each test. Samples were diluted with buffer prior to sampling 1/10, 200µl sample into 1800µl buffer. Debris could be a problem, confounding results, therefore voltage was set to 273 to only visualise the cells detected between 1 and 13 µm roughly. Calibration beads of sizes discussed above were run with each experiment.

4.4.2 Colony Forming Units

To plate out for CFU counting, serial dilutions of saturated cultures were used. 100µl of culture from 200µl of each yeast population ancestor and evolved lines was individually inoculated into 900µl of water in a 1.5ml micro-centrifuge tube. These were vortexed vigorously. From this dilution 100µl was taken and inoculated into another micro-centrifuge tube of 900µl of water and vortexed vigorously. This was done a total of four times. From the final dilution 100µl was inoculated on to YPD agar plates and grown at 30°C for 48 hours, checking at 24 hours to check for overgrowth. Population sizes were determined with 3 replicate plates.

Though the data is not presented here, the calculation of CFUs per the original 200µl volume of culture are shown in Appendix C.2.

4.4.3 Statistical Analysis of Size and Counts

For analysing the changes in these results, linear models were used to determine significance of changes between ancestor and evolved populations in both size and counts. The estimate changes, t-value and p-value, are presented below.

As a summary analysis, a binomial test was run on the results of the CFU counts, FACS counts and cell size. This test allows for the analysis of general trends over the yeasts. It statistically tests the significance of success of a trend out of a given number of events. This analysis was carried out on the collective data and then refined to only the statistically significant results and is presented in section 3.5.9. The CFUs, FACS counts and cell size are treated as dependent variables while the line of each yeast population is treated as the independent variable.

4.5 Results

Here the data from the colony-forming unit counts and the flow cytometry measurements are presented for each yeast population. Raw signal data for the size peaks can be found in Appendix C.1, while relative change from the ancestor will be discussed here. Any changes in FSC are interpreted as size changes.

4.5.1 *Kodamaea* A1

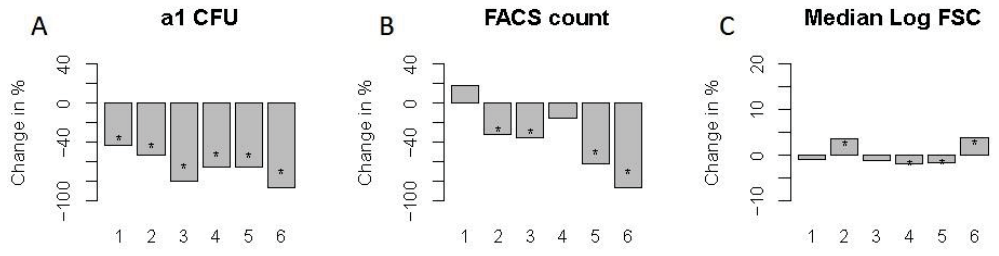


Figure 4.1: *Kodamaea* A1 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

From the data presented in Table 4.2 and Figure 4.1 A it would appear that, compared to the ancestor, all A1 evolved lines have statistically significant decreased numbers of CFUs for each

line. Table 4.2 and Figure 4.1 B depicts the cell counts collected from the flow cytometry experiment. From this only four of the six lines statistically significantly decrease in cell count compared to the ancestor. Lines A1.2, A1.3, A1.5 and A1.6 decrease, while lines A1.1 and A1.4 do not appear to change. Table 4.2 and Figure 4.1 C demonstrate the relative change in median log FSC of cells counted in the flow cytometer. Lines A1.4 and A1.5 show statistically significant reduction in cell size. Lines A1.2 and A1.6 show a statistically significant gain in size and lines A1.1 and A1.3 did not exhibit statistically significant change.

Test	Statistic	A1.0	A1.1	A1.2	A1.3	A1.4	A1.5	A1.6
CFU	**Estimate**	215.33	**-92.33**	**-115**	**-170.33**	**-139.67**	**-140**	**-186.67**
CFU	**Change**		**-42.9%**	**-53.4%**	**-79.1%**	**-64.9%**	**-65.0%**	**-86.7%**
CFU	**T-value**		**-6.76**	**-8.42**	**-12.47**	**-10.22**	**-10.25**	**-13.66**
CFU	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Count	**Estimate**	9620	1794	**-3068**	**-3360.5**	-1501.5	**-5895.5**	**-8333**
Count	**Change**		18.6%	**-31.9%**	**-34.9%**	-15.6%	**-61.3%**	**-86.6%**
Count	**T-value**		1.49	**-2.55**	**-2.79**	-1.25	**-4.90**	**-6.92**
Count	**P-value**		0.180	**0.038**	**0.027**	0.253	**0.002**	**<0.001**
FSC	**Estimate**	7.424	-0.065	**0.267**	-0.080	**-0.131**	**-0.113**	**0.285**
FSC	**Change**		-0.88%	**3.60%**	-1.08%	**-1.76%**	**-1.52%**	**3.84%**
FSC	**T-value**		-1.74	**7.11**	-2.14	**-3.47**	**-3.00**	**7.57**
FSC	**P-value**		0.126	**<0.001**	0.070	**0.010**	**0.020**	**<0.001**
Yield	**Estimate**	0.429	-0.013	-0.001	**-0.111**	0.004	**-0.068**	**-0.112**
Yield	**T-value**		-1.084	-0.096	**-8.822**	0.295	**-5.843**	**-8.861**
Yield	**P-value**		0.281	0.923	**<0.001**	0.769	**<0.001**	**<0.001**

Table 4.2: A1 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. Presented here are also the yield values measured in Chapter Two. Statistically significant results are highlighted in bold.

To summarise, it would appear that the evolved lines show a consistent decrease in CFUs ranging from between 42.9% to 86.7% in relative estimate change of population size. FACS appears to demonstrate the same trend of decrease in relative change in population size. However, there is less consistency in the FACS counts (A1.1 increases while A1.4 decreases but not significantly), and not the same level of change (decrease change ranges from 15% to 86.6%, a much broader spectrum of change). Cell size shows no consistent changes overall with two evolved lines

decreasing significantly, two increasing significantly and two with no statistically significant change. In terms of OD-based growth yield three of the evolved lines indicated decreases, which are reflected in both the CFUs and FACS count measurements.

4.5.2 *Kodamaea* A8

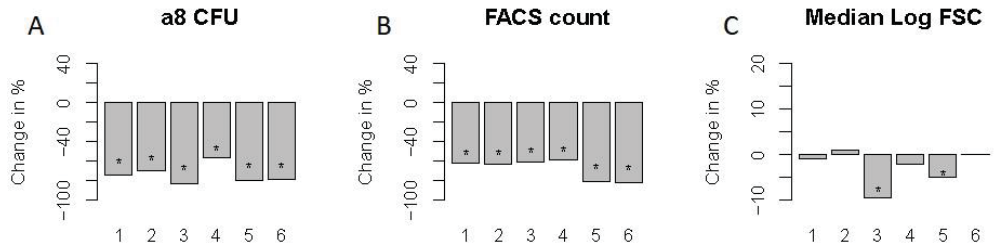


Figure 4.3: *Kodamaea* A8 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

The data presented in Table 4.4 and Figure 4.3 A demonstrates that, compared to the ancestor, all A8 evolved lines have statistically significant decreased numbers of CFUs for each line. All six evolved lines statistically significantly decrease in cell count compared to the ancestor, as shown in Table 4.4 and Figure 4.3.

Table 4.4 and Figure 4.3 C show that there are only two statistically significant changes in cell size of evolved lines A8.3 and A8.5, both showing a decrease in cell size. All other evolved lines do not demonstrate a statistically significant change in size compared to the ancestor.

Looking at the lines more specifically, in line A8.1, A8.2 and A8.6 we see CFU counts, cell count and growth yield, from Chapter Two, decrease while size does not change. The decrease in OD-based growth yield appears to be confirmed by the changes observed here; while cell size either stays the same or decreases, the FACS count and CFUs consistently decrease, indicating that the loss in yield translated into smaller population size and thus biomass in saturated culture.

Test	Statistic	A8.0	A8.1	A8.2	A8.3	A8.4	A8.5	A8.6
CFU	Estimate	305.33	**-227.33**	**-213.33**	**-252**	**-173.33**	**-242**	**-241**
CFU	Change		**-74.5%**	**-69.9%**	**-82.5%**	**-56.8%**	**-79.3%**	**-78.9%**
CFU	T-value		**-13.57**	**-12.74**	**-15.04**	**-10.35**	**-14.45**	**-14.39**
CFU	P-value		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Count	Estimate	10218	**-6363.5**	**-6428.5**	**-6207.5**	**-5980**	**-8287.5**	**-8359**
Count	Change		**-62.3%**	**-62.9%**	**-60.8%**	**-58.5%**	**-81.1%**	**-81.8%**
Count	T-value		**-6.39**	**-6.46**	**-6.23**	**-6.01**	**-8.32**	**-8.39**
Count	P-value		**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**
FSC	Estimate	7.105	-0.068	0.072	**-0.671**	-0.144	**-0.346**	-0.001
FSC	Change		-0.96%	1.01%	**-9.45%**	-2.02%	**-4.87%**	-0.01%
FSC	T-value		-0.71	0.74	**-6.99**	-1.49	**-3.60**	-0.01
FSC	P-value		0.502	0.481	**<0.001**	0.179	**0.009**	0.994
Yield	Estimate	0.410	**-0.024**	**-0.061**	**-0.055**	-0.004	**-0.108**	**-0.060**
Yield	T-value		**-2.380**	**-6.488**	**-5.884**	-0.386	**-11.546**	**-6.383**
Yield	P-value		**0.019**	**<0.001**	**<0.001**	0.700	**<0.001**	**<0.001**

Table 4.4: A8 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. Presented here are also the yield values measured in Chapter Two. Statistically significant results are highlighted in bold.

4.5.3 *Issatchenkia* A11

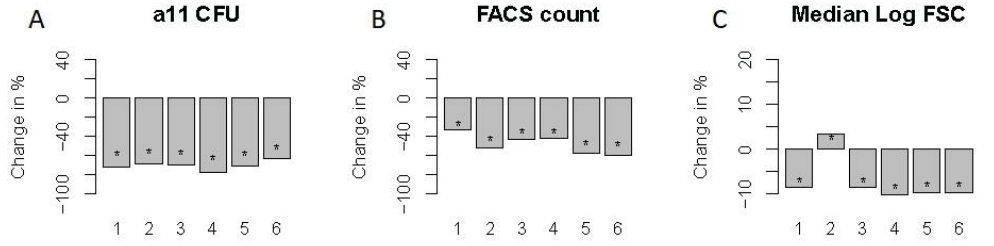


Figure 4.5: *Issatchenkia* A11 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

The A11 data seen in Table 4.6 and Figure 4.5 A suggest that, compared to the ancestor all evolved lines have statistically significant decreased numbers of CFUs for each line. Relative estimate change is shown in a range of between 63.3% and 76.8%.

Test	Statistic	A11.0	A11.1	A11.2	A11.3	A11.4	A11.5	A11.6
CFU	Estimate	113.67	**-81.67**	**-77.33**	**-79.67**	**-87.33**	**-81**	**-72**
CFU	Change		**-71.8%**	**-68.0%**	**-70.1%**	**-76.8%**	**-71.3%**	**-63.3%**
CFU	T-value		**-11.76**	**-11.13**	**-11.47**	**-12.57**	**-11.66**	**-10.36**
CFU	P-value		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Count	Estimate	10679.5	**-3471**	**-5531.5**	**-4530.5**	**-4446**	**-6090.5**	**-6337.5**
Count	Change		**-32.5%**	**-51.8%**	**-42.4%**	**-41.6%**	**-57.0%**	**-59.3%**
Count	T-value		**-5.22**	**-8.32**	**-6.82**	**-6.69**	**-9.16**	**-9.54**
Count	P-value		**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
FSC	Estimate	7.96	**-0.68**	**0.28**	**-0.67**	**-0.81**	**-0.77**	**-0.76**
FSC	Change		**-8.52%**	**3.48%**	**-8.40%**	**-10.13%**	**-9.69%**	**-9.60%**
FSC	T-value		**-10.37**	**4.23**	**-10.22**	**-12.32**	**-11.78**	**-11.67**
FSC	P-value		**<0.001**	**0.004**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table 4.6: A11 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. No yield values from Chapter Two are provided as the measurements were not possible due to noise. Statistically significant results are highlighted in bold.

Table 4.6 and Figure 4.5 B show that all evolved lines statistically significantly decrease in cell count compared to the ancestor, complimenting the results of the CFUs. There is a relative estimate change of between 32.5% to 59.3% across the lines.

All evolved lines show statistically significant changes in size (Table 4.6 and Figure 4.5 C). While in 5 of the lines, cells reduced in size, one line (A11.2) indicates statistically significant increase in size of 3.48%.

4.5.4 *Candida railenensis* B3

Table 4.8 and Figure 4.7 A present the data for all the B3 evolved lines. All lines save B3.4 have statistically significant changes in number of CFUs for each line compared to the ancestor. Line B3.6 appears to have increased, contrary to the trend of rest of the lines. The remaining lines show an estimate change range of 30.2% to 84.7%.

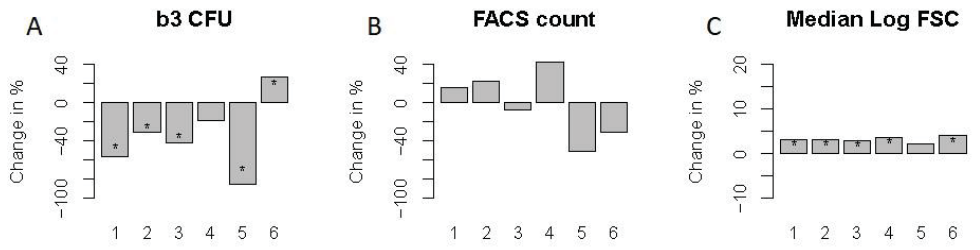


Figure 4.7: *C. railenensis* B3 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

No statistically significant changes in the evolved lines were observed in terms of FACS count compared to the ancestor. Table 4.8 and Figure 4.7 C depicts that of the evolved lines, 5 out of 6 statistically changed in size: lines B3.1, B3.2, B3.3, B3.4 and B3.6 all increased in size. Line B3.5 did not demonstrate any significant change in size.

Test	Statistic	B3.0	B3.1	B3.2	B3.3	B3.4	B3.5	B3.6
CFU	**Estimate**	96	**-53.67**	**-29**	**-40.33**	-18	**-81.33**	**26.33**
CFU	**Change**		**-55.9%**	**-30.2%**	**-42.0%**	-18.8%	**-84.7%**	**27.4%**
CFU	**T-value**		**-6.14**	**-3.32**	**-4.62**	-2.06	**-9.31**	**3.01**
CFU	**P-value**		**<0.001**	**0.005**	**<0.001**	0.058	**<0.001**	**0.009**
Count	**Estimate**	2483	403	559	-175.5	1053	**-1261**	-760.5
Count	**Change**		16.2%	22.5%	-7.1%	42.4%	**-50.8%**	-30.6%
Count	**T-value**		0.74	1.03	-0.32	1.94	**-2.32**	-1.40
Count	**P-value**		0.482	0.337	0.756	0.094	**0.053**	0.204
FSC	**Estimate**	7.27	**0.23**	**0.23**	**0.22**	**0.27**	0.16	**0.29**
FSC	**Change**		**3.14%**	**3.22%**	**3.02%**	**3.67%**	2.15%	**4.04%**
FSC	**T-value**		**3.04**	**3.12**	**2.92**	**3.55**	2.08	**3.91**
FSC	**P-value**		**0.019**	**0.017**	**0.022**	**0.009**	0.076	**0.006**
Yield	**Estimate**	0.374	**-0.048**	**-0.061**	**-0.051**	**-0.061**	**-0.067**	**-0.050**
Yield	**T-value**		**-4.925**	**-6.305**	**-5.254**	**-6.283**	**-6.921**	**-5.122**
Yield	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table 4.8: B3 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. Presented here are also the yield values measured in Chapter Two. Statistically significant results are highlighted in bold.

Overall the data suggests a reduction in cells (CFUs) but an increase in cell size. This reduction in cells reflects the OD-based growth yield but is not seen in the FACS counts. It is difficult to say whether the results are equivalent to more or less biomass in the population.

4.5.5 *Pichia kluyveri* B5

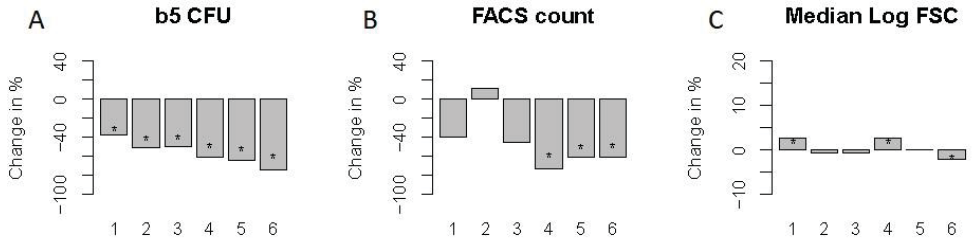


Figure 4.9: *P. kluyveri* B5 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

From the data presented in Table 4.10 and Figure 4.9 A all evolved lines have statistically significant decreased numbers of CFUs for each line compared to the ancestor. The observed estimate change ranges from 37.5% to73.9%. Three statistically significant changes were observed in lines B5.4, B5.5 and B5.6 in the form of a decrease in cell counts. All other evolved lines had no statistically significant changes in comparison to the ancestor. Of the evolved lines, as shown in Table 4.10 and Figure 4.9 C, three statistically changed in size, lines B5.1, B5.4 and B5.6. Lines B5.1 and B5.4 increased in size while line B5.6 decreased in size. All other lines showed no sign of statistically significant change.

Overall it seems that cell size stays constant, with no consistent change over all the evolved lines. CFUs indicates a decrease overall which in a less systematic way is reflected in the FACS counts. This would support the OD-based growth yield being a reduction in biomass in the population.

Test	Statistic	B5.0	B5.1	B5.2	B5.3	B5.4	B5.5	B5.6
CFU	**Estimate**	304.67	**-114.33**	**-155.33**	**-149.67**	**-184.67**	**-194**	**-225**
CFU	**Change**		**-37.5%**	**-51.0%**	**-49.1%**	**-60.6%**	**-63.7%**	**-73.9%**
CFU	**T-value**		**-4.25**	**-5.77**	**-5.56**	**-6.86**	**-7.21**	**-8.36**
CFU	**P-value**		**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Count	**Estimate**	19526	-7806.5	2203.5	-8898.5	**-14280.5**	**-11901.5**	**-11869**
Count	**Change**		-40.0%	11.3%	-45.6%	**-73.1%**	**-61.0%**	**-60.8%**
Count	**T-value**		-1.92	0.54	-2.19	**-3.51**	**-2.93**	**-2.92**
Count	**P-value**		0.096	0.605	0.065	**0.010**	**0.022**	**0.022**
FSC	**Estimate**	7.30	**0.19**	-0.05	-0.04	**0.20**	0.00	**-0.15**
FSC	**Change**		**2.59%**	-0.67%	-0.56%	**2.71%**	0.00%	**-2.01%**
FSC	**T-value**		**4.31**	-1.12	-0.93	**4.51**	0.00	**-3.34**
FSC	**P-value**		**0.004**	0.301	0.381	**0.003**	0.997	**0.012**
Yield	**Estimate**	0.427	**-0.101**	**-0.147**	**-0.069**	**-0.064**	**-0.159**	**-0.050**
Yield	**T-value**		**-7.762**	**-11.243**	**-5.285**	**-4.870**	**-12.188**	**-3.800**
Yield	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table 4.10: B5 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. Presented here are also the yield values measured in Chapter Two. Statistically significant results are highlighted in bold.

4.5.6 *Issatchenkia orientalis Kudravtsev* C10

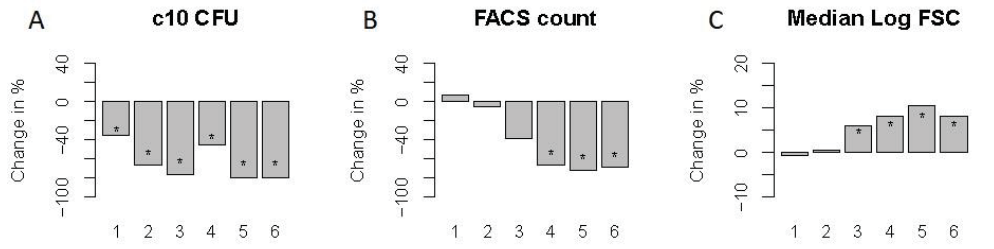


Figure 4.11: *Issatchenkia* C10 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

From the data presented in Table 4.12 and Figure 4.11 A, all evolved lines have statistically significant decreased numbers of CFUs for each line compared to the ancestor. Lines 5 and 6 appear to have equally had the largest reductions in CFU counts compared to the ancestor. Table 4.12 and Figure 4.11 B demonstrate that only three evolved lines have statistically significant changes, in the form of decreased cell counts, while lines C10.1, C10.2 and C10.3 remain

unchanged statistically. Table 4.12 and Figure 4.11 C shows that only four lines have statistically significant changes compared to the ancestor, in the form of increased cell size. Lines C10.1 and C10.2 remain unchanged statistically.

Test	line	C10.0	C10.1	C10.2	C10.3	C10.4	C10.5	C10.6
CFU	**Estimate**	170.33	**-59**	**-112.67**	**-129.67**	**-77.33**	**-135**	**-135**
CFU	**Change**		**-34.6%**	**-66.1%**	**-76.1%**	**-45.4%**	**-79.3%**	**-79.3%**
CFU	**T-value**		**-6.05**	**-11.56**	**-13.31**	**-7.94**	**-13.85**	**-13.85**
CFU	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Count	**Estimate**	9821.5	689	-500.5	-3750.5	**-6493.5**	**-7039.5**	**-6708**
Count	**Change**		7.0%	-5.1%	-38.2%	**-66.1%**	**-71.7%**	**-68.3%**
Count	**T-value**		0.42	-0.30	-2.28	**-3.95**	**-4.29**	**-4.08**
Count	**P-value**		0.687	0.769	0.056	**0.006**	**0.004**	**0.005**
FSC	**Estimate**	7.95	-0.04	0.04	**0.48**	**0.65**	**0.84**	**0.66**
FSC	**Change**		-0.53%	0.52%	**6.03%**	**8.12%**	**10.63%**	**8.29%**
FSC	**T-value**		-0.92	0.90	**10.35**	**13.94**	**18.24**	**14.22**
FSC	**P-value**		0.390	0.399	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Yield	**Estimate**	0.375	0.026	**0.057**	0.012	**0.034**	**-0.031**	-0.006
Yield	**T-value**		1.755	**3.855**	0.840	**2.051**	**-2.083**	-0.425
Yield	**P-value**		0.083	**<0.001**	0.404	**0.044**	**0.041**	0.672

Table 4.12: C10 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. Presented here are also the yield values measured in Chapter Two. Statistically significant results are highlighted in bold.

Overall it would appear that CFUs consistently decrease in relative estimate change from between 34.6% to 79.3%. This is somewhat reflected in the FACS counts to a lesser extent, with only half of the counts showing change. Cell size appears to show an overall increase in four out of six lines, including those that showed decreases in both CFUs and FACS counts. OD-based growth yield showed very little consistency over the lines with only three significant changes, two of which were increases in yield (C10.2 and C10.4). There is no clear consistent pattern in the cell size and FACS counts between these two to explain the increase in OD-based growth yield. Three evolved lines exhibited no change in OD-based growth yield, two of which indicated decreases in CFUs and an increase in cell size (C10.3 and C10.6). C10.5 is alone in its decrease across CFUs,

FACS count, OD-based growth yield and an increase in cell size, similar to other yeasts in this experiment, that results in an unclear effect on total biomass of the population.

4.5.7 *Kluyveromyces nonfermentans* F8

From the data presented in Table 4.14 and Figure 4.13 A all evolved lines have statistically significant changes in CFU counts, with line F8.5 having increased, whilst the remaining lines having decreased.

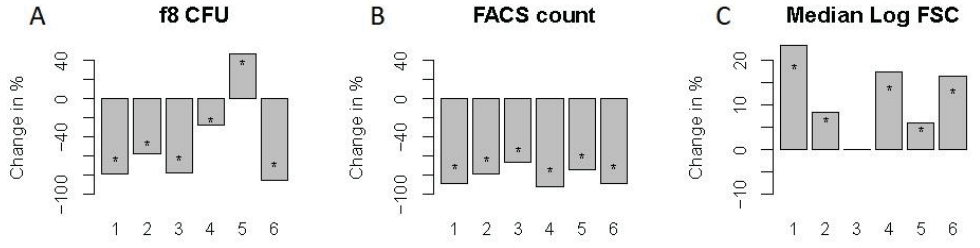


Figure 4.13: *K. nonfermentans* F8 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

All evolved lines show a statistically significant decrease in cell count compared to the ancestor. While in terms of cell size, 5 out of 6 statistically changed between a relative estimate change range of between 5.99% to 23.36%, all increased in cell size compared to the ancestor. Line F8.3 did not demonstrate any statistically significant change in size.

Overall it would appear that the evolved lines show a consistent decrease in CFUs and FACS counts. With only one deviation, there is also consistency in cell size with five evolved lines increasing in cell size. OD-based growth yield showed four statistically significant decreases in yield which, from these results, it is unclear what effect the increase in cell size would have on the overall biomass of the population. Three evolved lines (F8.1, F8.4 and F8.6) show changes in size that exceed those observed in other yeast.

Test	Statistic	F8.0	F8.1	F8.2	F8.3	F8.4	F8.5	F8.6
CFU	**Estimate**	249	**-195.67**	**-143.67**	**-192.33**	**-68**	**118.33**	**-211.33**
CFU	**Change**		**-78.6%**	**-57.7%**	**-77.2%**	**-27.3%**	**47.5%**	**-84.9%**
CFU	**T-value**		**-9.23**	**-6.78**	**-9.08**	**-3.21**	**5.58**	**-9.97**
CFU	**P-value**		**<0.001**	**<0.001**	**<0.001**	**0.006**	**<0.001**	**<0.001**
Count	**Estimate**	10120.5	**-8937.5**	**-7897.5**	**-6701.5**	**-9295**	**-7540**	**-8950.5**
Count	**Change**		**-88.3%**	**-78.0%**	**-66.2%**	**-91.8%**	**-74.5%**	**-88.4%**
Count	**T-value**		**-10.21**	**-9.02**	**-7.65**	**-10.62**	**-8.61**	**-10.22**
Count	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
FSC	**Estimate**	6.93	**1.62**	**0.58**	0.00	**1.21**	**0.41**	**1.15**
FSC	**Change**		**23.36%**	**8.42%**	0.07%	**17.47%**	**5.99%**	**16.59%**
FSC	**T-value**		**42.72**	**15.39**	0.13	**31.95**	**10.95**	**30.34**
FSC	**P-value**		**<0.001**	**<0.001**	0.904	**<0.001**	**<0.001**	**<0.001**
Yield	**Estimate**	0.359	**-0.032**	0.002	**-0.038**	**-0.053**	-0.002	**-0.028**
Yield	**T-value**		**-4.966**	0.293	**-6.219**	**-8.827**	-0.364	**-4.666**
Yield	**P-value**		**<0.001**	0.770	**<0.001**	**<0.001**	0.716	**<0.001**

Table 4.14: F8 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. Presented here are also the yield values measured in Chapter Two. Statistically significant results are highlighted in bold.

4.5.8 *Saccharomyces cerevisiae* SCE2

From the data presented in Table 4.16 and Figure 4.15 A, all evolved lines have statistically significant decreased numbers of CFUs for each line compared to the ancestor.

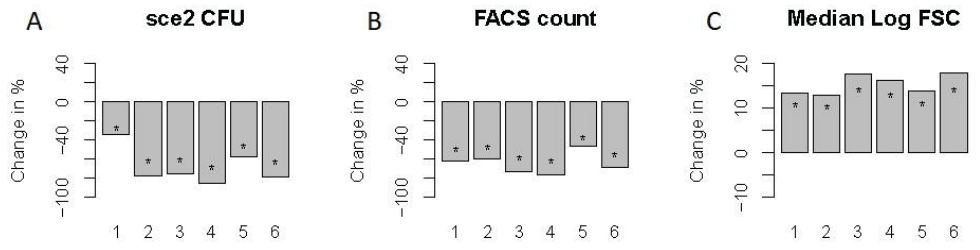


Figure 4.15: *S. cerevisiae* SCE2 Relative Changes in CFU's, FACS Counts and FSC. This figure presents the percentage change in each line in comparison to the ancestor. Significance is marked by an asterisk.

Table 4.16 and Figure 4.15 B show that all lines demonstrated a statistically significant decrease in cell count compared to the ancestor. The evolved lines exhibited statistically significant increase in cell size compared to the ancestor (Table 4.16 and Figure 4.15 C).

Test	line	SCE2.0	SCE2.1	SCE2.2	SCE2.3	SCE2.4	SCE2.5	SCE2.6
CFU	**Estimate**	113	**-38.33**	**-87**	**-85.33**	**-96.67**	**-65.33**	**-88.33**
CFU	**Change**		**-33.9%**	**-77.0%**	**-75.5%**	**-85.5%**	**-57.8%**	**-78.2%**
CFU	**T-value**		**-9.19**	**-20.86**	**-20.46**	**-23.18**	**-15.66**	**-21.18**
CFU	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Count	**Estimate**	7605	**-4719**	**-4491.5**	**-5525**	**-5830.5**	**-3529.5**	**-5239**
Count	**Change**		**-62.1%**	**-59.1%**	**-72.6%**	**-76.7%**	**-46.4%**	**-68.9%**
Count	**T-value**		**-8.66**	**-8.24**	**-10.13**	**-10.70**	**-6.47**	**-9.61**
Count	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
FSC	**Estimate**	7.93	**1.07**	**1.02**	**1.40**	**1.28**	**1.11**	**1.41**
FSC	**Change**		**13.49%**	**12.88%**	**17.67%**	**16.19%**	**13.96%**	**17.83%**
FSC	**T-value**		**13.62**	**13.01**	**17.85**	**16.35**	**14.10**	**18.01**
FSC	**P-value**		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Yield	**Estimate**	0.377	**-0.033**	**-0.022**	**-0.078**	**-0.053**	0.007	**-0.034**
Yield	**T-value**		**-3.586**	**-2.298**	**-8.096**	**-5.498**	0.729	**-3.260**
Yield	**P-value**		**0.001**	**0.023**	**<0.001**	**<0.001**	0.468	**0.001**

Table 4.16: SCE2 Statistical Analysis. Table presents the estimates, relative estimates, t-value and p-value for the CFU counts, the FACS counts and the FSC for each line and the ancestor. Presented here are also the yield values measured in Chapter Two. Statistically significant results are highlighted in bold.

Overall, it is important to note with what consistency all the lines change. All lines show a decrease in CFUs, FACS counts and increase in cell size. With the OD-based growth yield also decreasing for all lines save SCE2.5, which remains unchanged, it is unclear what effect the increase of cell size would have on total biomass of the population.

4.5.9 Summary

The experimental data provided by the flow cytometry and the CFU counts reveals some notable results. In terms of CFU counts and cell counts it would be expected that they would reflect the same changes in each line. Overall, if evolved lines decrease in CFU counts so do the cell counts, other than in B3 *C. railenensis* where cell counts generally do not statistically change overall while

CFU counts decrease. Four of the eight yeasts demonstrate an increase in size with a decrease in cell count and/or CFU counts. These were B3, C10, F8 and SCE2.

Result	Increased	Decreased	P-value
CFU	**2/48**	**46/48**	**<0.001**
of which significant	**2/47**	**45/47**	**<0.001**
FACS Count	**6/48**	**42/48**	**<0.001**
of which significant	**0/37**	**37/37**	**<0.001**
FSC	29/48	19/48	0.193
of which significant	**25/35**	**10/35**	**0.017**

Table 4.17: Binomial Analysis for Counts and Size Results. This test is carried out on both the total results and significant results only. Statistically significant results are highlighted in bold.

From the results of the binomial test presented in Table 4.17 it can be determined how likely our results are to be statistically significantly different from an equal likelihood of occurring. Firstly, the CFU counts overall present 46/48 decreases in cell count compared to the ancestor over all the species, leaving only 2/48 as increases. It is important to emphasise this statistically significant result at a p-value of <0.001. When only examining statistically significant changes, this reduces to 45/47 for decreases, resulting in the test still being highly statistically significant at a p-value of <0.001, due to only one loss in the sample size. Looking at the FACS counts, the results reflect a similar pattern, with 42/48 decreases in cell count across all species and 6/48 increasing, once again highly statistically significant with a p-value of <0.001. If only reviewing statistically significant changes the sample size reduces to 37/37 decreases in cell count across species and 0/37 increasing compared to the ancestor cell count, and still regarded as statistically significant at a p-value of <0.001. Finally, looking at the size of the counted cells in the FACS 29/48, an increase is observed across all species, and a decrease (19/48) is observed compared to the ancestors. This however is not seen as statistically significantly different from random chance. When looking at only statistically significant results however 25/35 increase while 10/35 decrease and this is shown to be statistically significant at a p-value of 0.017. This allows us to conclude that the yeast populations overall are more likely to evolve a decrease in CFU counts and FACS

counts over increasing compared to the ancestor, whilst being more likely to increase in size than decrease, compared to the ancestor, when the results are statistically significant.

Yeast	fraction increased	fraction decreased	P-value
A1	2/6	4/6	0.6875
A8	1/6	5/6	0.2188
A11	1/6	5/6	0.2188
B3	**6/6**	**0/6**	**0.0313**
B5	2/6	4/6	0.6875
C10	5/6	1/6	0.2188
F8	**6/6**	**0/6**	**0.0313**
SCE2	**6/6**	**0/6**	**0.0313**

Table 4.18: Binomial Analysis Individually for FSC. Due to the individual changes of each yeast the binomial analysis was applied to all candidates individually. Statistically significant results are highlighted in bold.

Due to the varying differences in size between populations, binomial analysis was applied individually (Table 4.18). From this it is established that out of all populations we can conclude that only B3, F8 and SCE2 have a statistically significant trend of increased size. The results of the size measures appear at first sight to be very small changes, ranging from the lowest changes at 1% change for some A1 lines, to a change of 17.8% for some SCE2 lines and even 23% in line 1 of F8.

While these percentage changes appear small, when translated into diameter this is comparatively more noteworthy, for example 1% change in median FSC is equivalent to a change of 1.21µm in A11 line 4 (see Appendix C.2 for a Table of diameters and how they were calculated). Similarly, a change of 17.8% equates to 9.4µm change between the ancestor and Line 6 SCE2, and 23% change in F8 equals 3.4 µm (see Appendix C.2 for size in µm). The range of size across each species is demonstrated in Figure 4.19. From this figure, it is also made apparent that all yeast ancestors at least started in the size range expected of yeast cells, roughly between 4-8 µm.

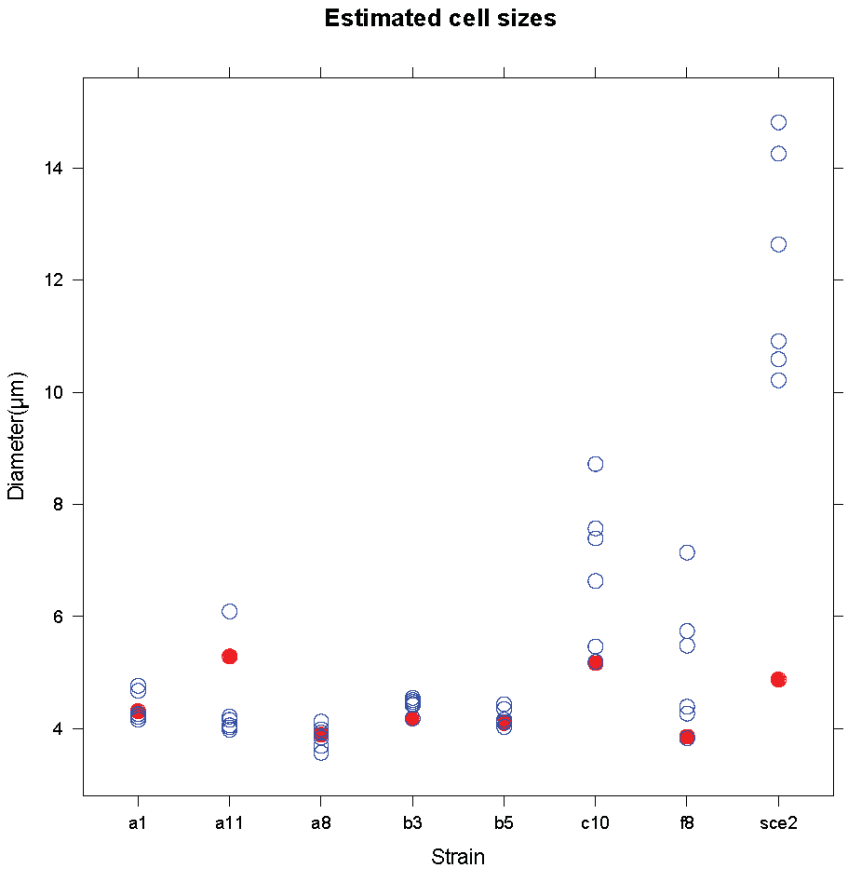


Figure 4.19: Estimated Cell Size Range for all Yeasts. Graph representing the average size range of the ancestor lines (red dots) and the evolved lines (blue dots) of all yeast populations.

Based on a line-by-line analysis there are some clear patterns through populations but there are also some patterns that are difficult to find explanations for. Overall, the decreased CFUs and FACS counts seem to support the observed reduction in stationary phase OD. However, the magnitude of change in population size tends to be larger than the changes in OD-based growth yield. Moreover, CFUS and FACS counts are not always consistent with each other. This would suggest that this could be the result of other factors such as cell counts measuring dead cells as well as live and thus providing a larger cell count than is actually viable. Another explanation could be that the differences observed between OD-based growth yield and the counts, might be the result of timing of sampling. OD was measured immediately at 24 hours early in saturation, while

the counts were prepared from 24 hours, sampled at 24 hours and typically measured sometime after and thus later in saturation. This could potentially mean mortality occurs in some cells reducing the overall cell count. This study has raised some notable questions to prompt further study and will be discussed in Chapter Six.

4.6 Analysis and Conclusions

The aims of this chapter were to investigate whether the OD-based growth yield changes detected in Chapter Two were corroborated by CFU counts and FACS cell counts. It was predicted that cell size had likely not changed and the growth yield changes would be, in most populations, reflected in the CFUs and FACS count. There could be potential for the cell size to have decreased and therefore be responsible for the OD-based growth yield decrease, which is why this experimental set was important to conduct.

From these results, it would appear that, overall, CFUs and FACs counts do show the trends to be expected if OD-based growth yield reflected total biomass in the population. Fewer cells would result in less biomass and a lower OD. 47/48 CFU measurements were statistically significant with 45/47 statistically significant results resulting in a decrease across all yeast evolved lines tested. Of the 48 evolved lines tests using FACS counts 37 were significant and 37/37 resulted in a decrease.

When evaluating cell size however the story becomes more variable than predicted. Three yeasts (A1, A8 and A11) demonstrated the decrease in cell size in four or more lines, that would be expected if the total biomass decrease was affected by cell size. However, what was unexpected was the change in cell size towards larger cells that was demonstrated in three yeasts (B3, F8 and SCE2) consistently across lines and in one yeast in five out of six lines (C10).

While for those yeasts that demonstrated the expected results, it can be concluded that OD growth yield is equivalent to a decrease in biomass in the population, this cannot be concluded for those yeasts where cell size increases but CFUs, FACS count and OD decrease. It is ultimately

unclear what effect an increase in cell size would have on the total biomass of the population when cell count decreases.

There are a number of questions raised by this experimental data. Firstly, one aspect that is noticeable in the data sets is that CFUs quite consistently decrease in all populations, to quite an extreme level, in comparison to the growth yield and sometimes do not correlate to the FACS counts, mainly in scenarios where the cell counts do not change from the ancestor but the CFUs still decrease. This could potentially be another factor separate from the biomass decreasing in the population and may be the result of a loss in viability on YPA. This might be explained by a metabolic change or environmental adaptation to liquid culture over solid agar. It is also possible that CFUs cannot be directly correlated to FACS counts. Potentially increased flocculation may result in overlapping CFUS or a requirement for more cells to form a single CFU.

Overall, this chapters presents strong statistically significant evidence that generally supports the changes in OD-based growth yield that were observed in Chapter Two. Both CFUs and FACS counts agreed with the change in growth yield of the evolved lines compared to the ancestor, save in B3 where no change was found and to a lesser extent C10 and B5 that did not systematically change as much as the CFUs. There was no expectation for cell size to change so dramatically, thus this was an unexpected observation. In terms of correlation between cell size, FACS count and OD-based growth yield, these are presented in the overall correlation analysis in Chapter Six.

Secondly the cell size increase is an important change in the evolved lines that warrants further consideration. There has been some research into cell size associated with growth yield in the literature (Jasmin et al. 2012) which associates decreases in yield with a decrease in cell size, which as we assumed, would occur if yield was not a true decrease and in fact a cell size effect. However, the cell size change most observed in this study was an increase not a decrease. This has also been observed previously and equally unexpectedly (Dhar et al. 2011) in *S. cerevisiae.* During a study into the effects of salt stress on *S. cerevisiae* in an evolution experiment, cell size

consistently increased and while it has been observed previously as mentioned in this paper (Lenski & Travisano 1994) in *Escherichia coli* it is not a very well investigated or understood adaptation, if that is what it is. It would appear that this change must be of some fitness advantage for it to occur in so many of the lines of the majority of the evolved lines. In this paper the cause is put down to genome size increase (Dhar et al. 2011), as larger cells are associated with higher levels of ploidy (Edgar & Orr-Weaver 2001; Mayfield-Jones et al. 2013; van Hoek & Hogeweg 2009). The general increase in cell size would suggest that increased size was selected for. Cell size has been mechanistically linked to growth rate in previous research, with lower nutrient concentration being connected to smaller cell size and increased cell size being observed when nutrients are plentiful (Pasciak & Gavis 1974; Smith et al. 2014; Raven 1998; Henrici & Henrici 1928). With the data from Chapter Two demonstrating a decrease in growth yield but with no correlation directly to the increase in growth rate, the biomass yield decrease may be a correlated to the selection for larger cell size in an abundance of nutrients. Correlations were carried out and are presented in Chapter Six, and no direct correlation was found.

Taking this information into account if the cell size change is due an increase in genome content this may be again in support of the theory that fermentative ability is a selective advantage. If fermentation developed from the whole genome duplication event then an increase in genome content would be expected in the shift towards such a metabolic change. Therefore, the next step would be to study if DNA content of the populations has changed and if any fitness advantage has developed in the evolved lines compared to the ancestor. The literature suggests that DAPI is a suitable way to measure only the viable cells in a culture and to quantify DNA at the same time (Yu et al. 1995; Dong-ju Kim, 2012). To support this, a secondary method of DNA quantification should be conducted, in this case SYTOX® was selected due to its strong cytometry record. Lastly it would be of interest to determine, if possible, whether any fitness advantage has developed in the evolved lines, so the next chapter will attempt to address this through the use of cell tracking dyes, in a competition experiment to determine whether the evolved lines have developed traits that can outcompete the ancestor. The next chapter will address these studies in further detail.

CHAPTER FIVE

Using Flow Cytometry to Analyse the Genetic Content and Fitness of Yeasts

Dr. Ian Malcom: ''God creates dinosaurs. God destroys dinosaurs. God creates man. Man destroys God. Man creates dinosaurs.''

Dr. Ellie Sattler: ''Dinosaurs eat man. Woman inherits the Earth.''

-Jurassic Park

5.1 Introduction

This chapter will investigate whether the cell size changes observed in the Chapter Four are the result of an increase in DNA content. Changes in cell size are frequently associated with changes in ploidy. It is of interest to quantify this as important changes in the metabolic capabilities of *Saccharomyces cerevisiae* occurred from the whole genome duplication (WGD) event and while no statistically significant changes in ethanol capabilities were observed in Chapter Three, the evolved lines may demonstrate genome content changes similar to the transition *S. cerevisiae* made. This chapter will also aim to address whether the changes observed have resulted in a fitness advantage in the evolved lines compared to the ancestor in four selected yeasts.

5.2 Cell Size and Ploidy

Chapter Four presented evidence that cell size increases consistently over many of the yeast populations. This has been associated with an increase in ploidy in a number of publications (Edgar & Orr-Weaver 2001; Mayfield-Jones et al. 2013; van Hoek & Hogeweg 2009; Fred et al. 1954). While there has been a strong connection observed between ploidy and cell size in a number of studies, it has also been demonstrated that cell size can be changed without ploidy increase in plant cells (Tsukaya 2008). It has been suggested that gene regulation has more of a role in ploidy dependent cell size than initially thought (Tsukaya 2013). The overall reason for cell size increase associated to ploidy is still unknown. Polyploidy occurs throughout eukaryotes and has played significant roles in the evolutionary history of plants, animals and fungi as can be detected in the current genomes of the species (Blanc & Wolfe 2004). Investigations in to a variety of yeasts both post and pre WGD, revealed that chromosome number increase only occurs through WGD (Gordon et al. 2011). The WGD appears to have been the most significant event in yeasts evolutionary history giving rise to the metabolic capabilities of *Saccharomyces cerevisiae* and a multitude of variations in other post WGD yeasts (van Hoek & Hogeweg 2009). It was observed that post WGD yeasts mainly appeared to be Crabtree-positive while pre WGD were Crabtree-negative (Merico et al. 2007). Therefore, it would be logical to predict that if a cell

increases in size it is due to an increase in chromosome number and this is the result of a WGD event.

5.3 Competitive Fitness

In the case of *Saccharomyces cerevisiae*, it was argued that WGD was responsible for important evolutionary change affecting significant metabolic functions in the yeast. However it is also initially a detrimental trait for an evolved yeast growing in the same environment as its ancestor, but is of particular benefit in new environments (van Hoek & Hogeweg 2009). Therefore, no initial fitness advantage would be demonstrated where the evolving yeast was growing in the same environment as its ancestor, but when switched to another environment it would exhibit an advantage. During an experimental evolution study of 1800 generations where diploid individuals arose in haploid populations the researchers investigated whether a fitness advantage had arisen from the change in ploidy (Gerstein & Otto 2011). The results of their experiments revealed no obvious fitness advantage in the change of ploidy. However, another study connects fitness increase with cell size specifically due to associated changes in transcription (Wu et al. 2010).

5.4 Experimental Designs

Most staining methods requires cell membranes to be compromised for access to the component being stained, be it a specific organelle or DNA. The staining of DNA can be achieved through several methods. A popular method is the use of propidium iodide however it has been highlighted to be less effective compared to SYTOX Green for flow cytometric analysis (Haase & Reed 2002). SYTOX Green has been compared to a number of other stains and it was concluded to be the best for cell cycle analysis and almost as reliable as SYBR Green for DNA content quantification (Delobel & Tesnière 2014). 4’.6-diamidino-2-phenylin- dole or DAPI has shown to be an effective DNA stain for both mitochondrial and nuclear DNA even in yeast cells where quantification of mitochondrial DNA has previously proven difficult (Kapuscinski 1995; Sazer &

Sherwood 1990). DAPI has also been shown to be effective for use in flow cytometry with very little loss in fluorescence over time (Yu et al. 1995; Hosny et al. 1998).

To measure competitive fitness, typically competitors are grown together in the same environment, and it is tested how the frequencies of the competitors change over time. Usually this would involve some form of marker. Markers generally involve creating or using differences between competitors. This can consist of metabolic differences resulting from mutations; which can be exploited on specific growth media. Markers can also be achieved through the manual addition of markers such as GFP, or deletion of such genes as URA3 in one competitor (Cubillos et al. 2009). Manual addition of genetic markers however can prove difficult and time consuming, particularly with uncharacterised yeast isolates from the wild, as used in this thesis. Metabolic differences were investigated for *Kodamaea* A8 using an identification panel analysing metabolic capabilities (see Chapter Three), but this proved fruitless due a lack of reliable metabolic differences between ancestor and evolved lines. The solution pursued here was inspired by a paper on quantum dot probes used to track strains in a mixed culture which highlighted the use of stains that were incorporated into the vacuoles of the cells (Gustafsson et al. 2014). This is important because vacuoles are passed down from mother to daughter cells for at least three days meaning the tracker signal would be maintained easily throughout the measurable growth cycle of the experimental evolution study conducted in Chapter Two. This paper highlighted such stains as the Cell Tracker™ range, that while not suitable for the amount of time required for the experiment in the literature, would suit the needs of this experiment well. The fluorescent tags that utilise the transport system in yeast cells to bypass the cell membrane permeability issue are available in several detectable wavelengths.

5.5 Aims

This chapter will aim to investigate the following questions:

- Is there a change in DNA content between ancestor and evolved lines?
- Is there a change in ploidy or a duplication event occurring?

- Is there consistency through different staining techniques?
- Is there a demonstrable fitness advantage in the evolved lines in comparison to the ancestors?

These will be addressed by two DNA stains on permeabilised cells on the ancestor and evolved lines of each yeast and measured through Flow Cytometry. Three populations and the Crabtree-positive reference will also be run through a fitness experiment using Cell Tracker dyes to stain the vacuoles of the ancestor and individual evolved lines separate colours that will be detected by flow cytometry and measured periodically through exponential growth.

5.5.1 Predictions

Due to the strong correlation observed in the literature between cell size and ploidy, it is predicted that the experiments will demonstrate an increase in DNA content reflecting an increase in ploidy such as from haploid to diploid or diploid to tetraploid. This is based on the assumption that, due to the previous evolutionary events of whole genome duplication this could occur in this experiment. In terms of the measuring of fitness, the aim is to validate whether fitness increases over evolutionary time. If the rate increase observed in a number of the evolved lines was the result of developing a fitness advantage, this would suggest that the evolved lines would outcompete the ancestor and demonstrate a fitness advantage in conjunction with the rate changes.

5.6 Methods

5.6.1 DNA Staining

Phosphate-buffered saline (PBS) stock buffer made up of 80g NaCl, 2g KCl, 14.4g NaHPO, 2.4g KHPO in 1 litre MilliQ water, adjusted to pH 7.4 and autoclaved is used to dilute the samples for sampling. DAPI stock is made up to 3mM using 5mg DAPI in 5ml MilliQ water, aliquoted out into 500µl aliquots and stored in the freezer until required. Two repeats for all ancestor and evolved lines were grown in 1g/l glucose SM medium for 24 hours to saturation. DAPI samples were grown

in 250µl in 96-well plates. 1.5ml micro-centrifuge tubes were filled with 25µl of 37% formaldehyde. Formaldehyde was used for fixation as pilot studies using Ethanol appeared to obscure flow cytometry readings. 250µl of sample is added to the formaldehyde and mixed by inversion of the tubes gently. These tubes are incubated at room temperature for 15-20 minutes. After incubation, the samples are spun down at 8000rpm for 5 minutes. The supernatant is discarded then the pellet is re-suspended in 250µl of PBS buffer and inoculated with 3µl of DAPI stock to make a 3µM solution and then incubated for 15 minutes. This is then finally diluted into 2250µl buffer in an Eppendorf 15ml tube and put on ice until sampling using flow cytometry. Results were gained from analysing the laser readings at 405nm excitation (with fluorescence occurring at excitation 358nm and emission 461nm).

SYTOX® Green was supplied in 5mM solution in DMSO in 250µl aliquots. Due to concentration difference, samples were grown in 1000µl 1g/l SM medium. 1000µl of sample was fixed in 110µl formaldehyde, incubated for 15-20 minutes and then spun down at 8000rpm for 5 minutes. This was then re-suspended in 1000µl of PBS and inoculated with 1µl of SYTOX and incubated for 20 minutes. 100µl of this was taken and inoculated into 15ml Eppendorf tubes filled with 900µl fresh PBS, these were mixed and put on ice until sampling using flow cytometry. Results were gained from analysing laser readings at 488nm excitation (resulting in a bright green fluorescence between excitation 450nm and emission 490nm).

Both DAPI and SYTOX F8, were not further diluted, due to low cell yield combined with loss through fixing.

5.6.2 Cell Tracker

Cell Tracker Colour	Molecular Weight	Mass (µg)	Volume of DMSO to add (µl)	Concentration of stock (mM)	Addition to 1ml culture for 10µM concentration
Violet	334.2	100	29.922	1	1µl
Green	464.9	50	10.755	1	1µl

Table 5.1 Cell Tracker Stock Solutions.

For populations A8, B5, C10 and SC 12 replicates of the ancestor and two replicates of each evolved lines were grown up in 1ml of 1g/l SM medium over 24 hours. 1ml of SM medium inoculated with dye was warmed to 37°C prior to the experiment. The ancestor samples were re-suspended in the warmed medium with 1µl of violet cell tracker added, while the evolved lines were re-suspended with 1µl of green cell tracker. These inoculated samples were mixed and then incubated at for 1.5 hours at 37°C. After incubation, the samples were spun down at 8000rpm for 5 minutes, then re-suspended in 1ml of 1g/l SM medium.

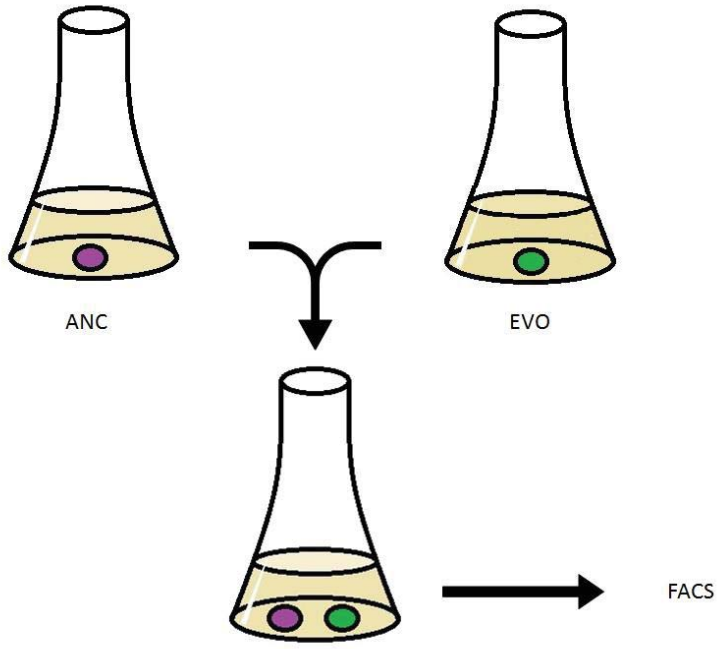


Figure 5.2: Cell Tracker Experiment. The ancestor and evolved lines are dyed different colours using a vacuole stain that can be detected in different channels by the flow cytometer. This allows for mixing of the two together for a competition experiment and the counting of cells over a period of exponential growth.

For the cell tracker experiment, 12 x 125ml conical flasks containing 8ml of medium were inoculated with 1 ancestor sample and one evolved line as demonstrated in Figure 5.2, there were two repeats per line. The flasks were incubated at 30°C for 15 minutes and then 1ml was removed for analysis as sample 1 and measured using flow cytometry. Another sample was taken at 90 minutes and a last one taken at 180 minutes. The dyes were detected at excitation 415nm and

emission 516nm for the violet dye and excitation 492nm and emission 517nm to detect the green dye. To differentiate between these two dyes, different excitations in the flow cytometer were used. To detect the violet dye, the best laser to use, as determined in pilot studies, was the violet laser (455nm, commonly used for Pacific Blue™) while to detect the green dye, the blue laser (525nm, commonly used for FITC) was used. The controls carried out for detection of these dyes can be found in Appendix D11.

5.7 Results

5.7.1 DNA Staining and Quantification

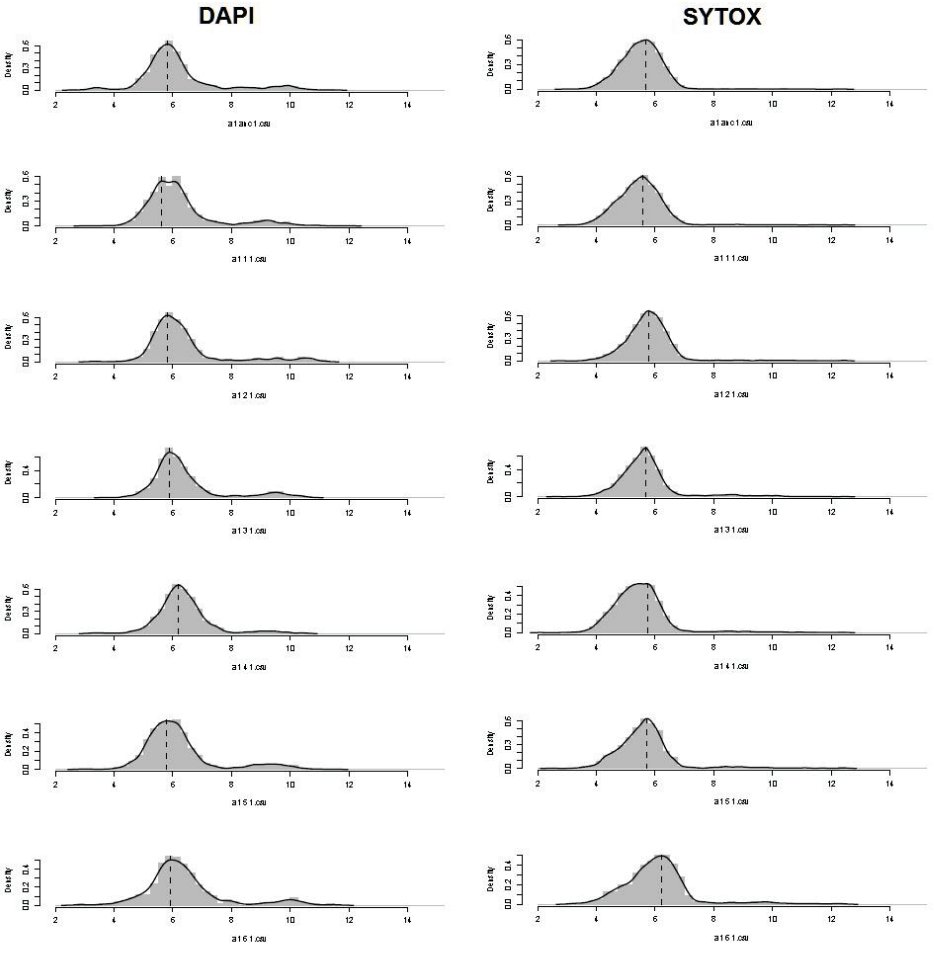


Figure 5.3 Candidate A1 *Kodamaea* DAPI and SYTOX Stain Density Graphs. An example graph of the flow cytometry measurements. The output and all candidate graphs for both dyes can be found in Appendix D.

Quantification of DNA content was attempted through the use of DNA stains DAPI and SYTOX and analysing the samples through flow cytometry. The raw data and graphical results presented in Figure 5.3 and Appendix D were obtained through the measurement of the fluorescence of the specific dye in question and plotted against cell count. This allowed for the visualisation of the peaks of DNA stain signal. To allow for the quantification of change several measurements were calculated. The fraction of cell counts below a certain threshold, as observed on the graph that fell between the two peaks, was calculated to determine the lower fluorescence peak (referred to from this point as the fraction small peak). This was to establish if there was any shift between the distribution of cells at different fluorescence peaks from the ancestor. This difference was established by comparing the fraction below this threshold for each evolved line to the ancestor. Secondly, the position of each peak was measured to determine if any shift in the location of the peaks occurs (referred to as position of small peak and position of large peak). Only those peaks that equate to more than 20% of the total count are recognised as peaks in the analysis. To analyse these measurements statistically, linear models were used to compare the evolved lines to the ancestors in both DAPI and SYTOX samples. The remainder of this chapter will review these results.

5.7.1.1 *Kodamaea* A1

From the graphs shown in Appendix D.3 it would appear that A1 ancestor demonstrates only a single peak in the DAPI stain and this is reflected in the replicate and in SYTOX also. When the data is analysed through linear models (Appendix D.3 Table D.3Cseveral points of interest may be noted. Firstly, in terms of the fraction of cells that makes up the lower peak, it is evident that there is no observed statistical change in DAPI signal for any of the lines compared to the ancestor. For SYTOX however, lines A1.3-6 show a statistically significant decrease in the fraction that is the lower peak compared to the ancestor. As demonstrated by Figure 5.4, where DAPI stain was used, lines A1.1-5 all appear to have a relative increase but not statistically significantly, while the SYTOX stain shows exactly the opposite trend with decreases in all but only statistically significantly in the lines A1.3-6.

In terms of peak locations, the DAPI stained samples showed no change in the position of the lower peak across all lines, while only line A1.6 in the SYTOX stained samples showed a statistically significant increase in position, i.e. it moved closer to the higher peak. For the higher peak location, there were no results for either DAPI or SYTOX due to there not being a higher peak present in the measurements. From this it would appear there is no consistent, notable change in the measured DNA content of yeast population A1.

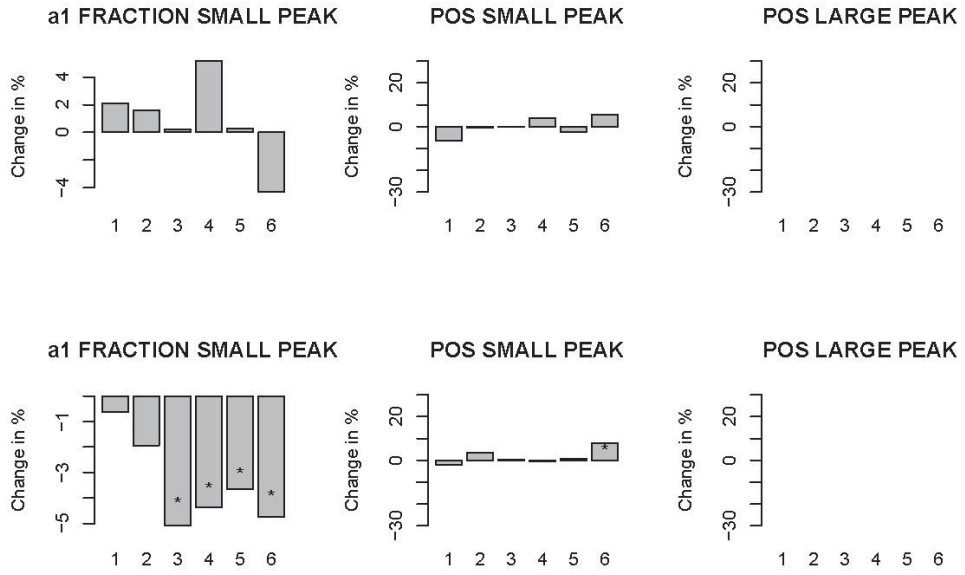


Figure 5.4: A1 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.** The large peak position is missing due to there being no higher peak present.

5.7.1.2 *Kodamaea* A8

From the graphs shown in Appendix D.4 it would appear that A1 ancestor demonstrates only a single peak in the DAPI stain and this is reflected in the replicate and in SYTOX also. When the data is analysed (Appendix D.4 Table D.4C) it is evident that, firstly, in terms of the fraction that makes up the lower peak, in DAPI there is no observed statistical change in any of the lines compared to the ancestor. For SYTOX however only line A8.5 showed a statistically significant decrease in the fraction within the lower peak compared to the ancestor. As demonstrated by Figure 5.5, where DAPI stain was used, lines A8.3, A8.4 and A8.6 all appear to have a relative

increase but not statistically significantly, while the SYTOX stain shows exactly the opposite trend with decreases in all but only statistically significantly in line A8.5.

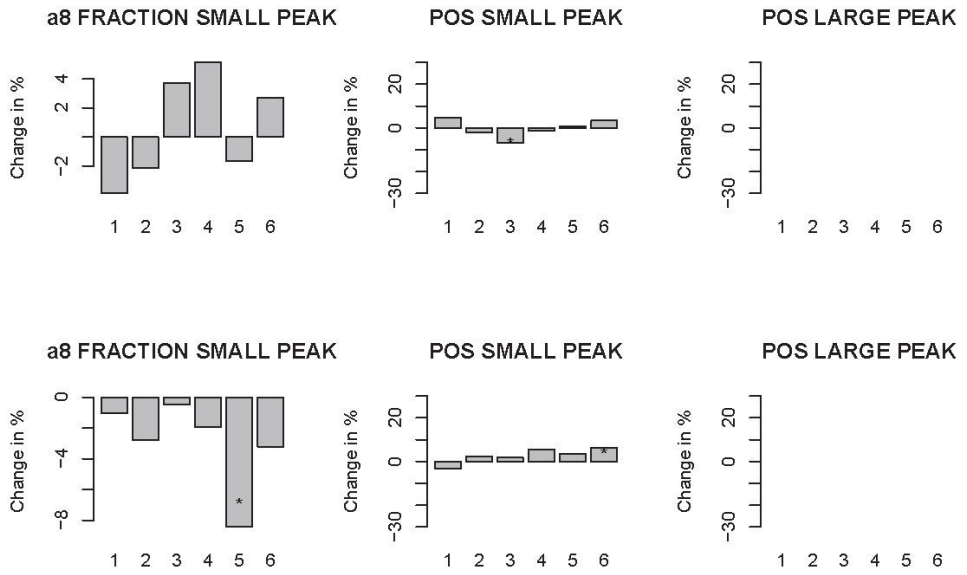


Figure 5.5: A8 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.** The large peak position is missing due to there being no higher peak present.

In terms of peak locations, the DAPI stained samples only showed statistically significant change in the position of the lower peak in line A8.3, which decreased, while only line A8.6 in the SYTOX stained samples showed a statistically significant increase in position. For the higher peak location, there were no results for either DAPI or SYTOX due to there not being a higher peak present in the measurements. From this it would appear there is no consistent, notable change in the measured DNA content of yeast population A8.

5.7.1.3 *Issatchenkia* A11

From the graphs shown in Appendix D.5 it would appear that A11 ancestor demonstrates two peaks in the DAPI stain and this is reflected in the replicate and in SYTOX to a smaller extent also. When the data is analysed (Appendix D.5 Table D.5C) it can be observed that, in terms of the fraction that makes up the lower peak, in DAPI there is there is only one statistically significant change in the lines compared to the ancestor. This is a decrease shown in line A11.2. For SYTOX

there are five lines that showed statistically significant decreases in the fraction that is the lower peak compared to the ancestor. These were lines A11.1-3, A11.5 and A11.6. As demonstrated by Figure 5.6, where DAPI stain was used, these lines appear to all have a relative decrease but only statistically significantly in line A11.2, while the SYTOX stain supports this with overall decreases, but only statistically significantly in lines A11.1-3, A11.5 and A11.6.

When it comes to the peak locations, the DAPI stained samples showed statistically significant changes in the position of the lower peak in lines A11.1, A11.4 and A11.5 which increased, while lines A11.2, A11.3 and A11.5 in the SYTOX stained samples showed a statistically significant increase in position. For the higher peak location, lines A11.2, A11.5 and A11.6 demonstrated statistically significant increase, while SYTOX showed no statistically significant results. These results suggest that there appears to be a trend across A11 of decrease in the fraction making up the lower peak which is especially prominent in SYTOX, and Figure 5.6 suggests a shift in position of the lower peak towards the higher peak and is measured in both DAPI and SYTOX samples but across different lines and only consistently in line A11.5.

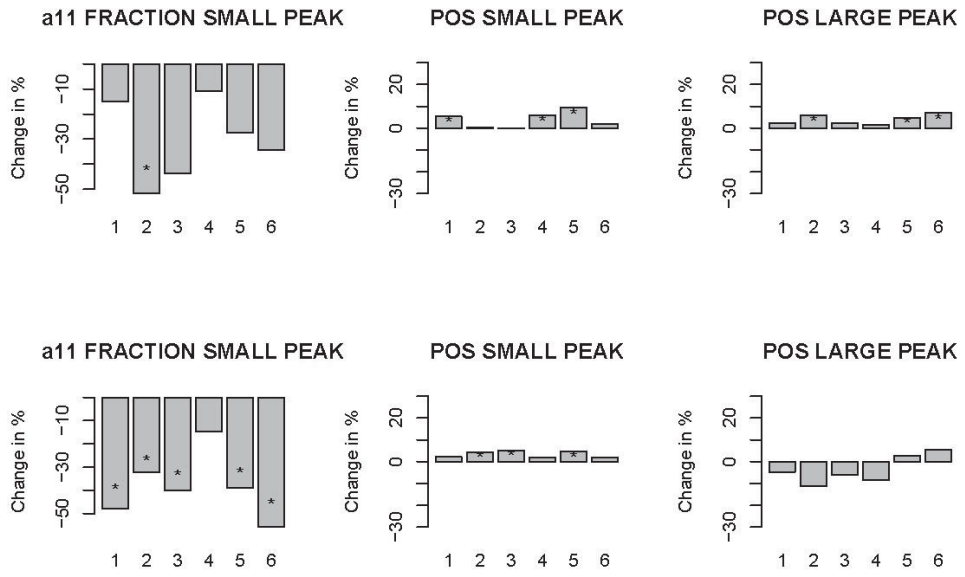


Figure 5.6: A11 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.**

5.7.1.4 *Candida railenensis* B3

The graphs shown in Appendix D.6 shows that B3 ancestor exhibits two peaks with a large proportion at the higher peak in the DAPI stain and this is reflected in the replicate. While SYTOX shares the two peaks format the distribution does not specifically mirror that of the DAPI. Upon analysis of the data as shown in Appendix D.6 Table D.6C, it is evident that of the fraction that makes up the lower peak, in DAPI, only line B3.5 changes statistically significantly and increases. For SYTOX this trend is statistically significant too, while one more line decreases statistically significantly also, namely line B3.3.

In terms of peak location, the DAPI stained samples showed no statistically significant changes in the position of the lower peak, while lines B3.2, B3.4 and B3.6 in the SYTOX stained samples showed a statistically significant decrease in position. For the higher peak location, only line B3.6 demonstrated statistically significant increase, while SYTOX showed statistically significant increase in lines B3.1, B3.2 and B3.3. Overall these results do not seem to reflect anything consistent or reliable across the two stains other than possibly a likelihood for change in the position of the higher peak as can be seen in Figure 5.7.

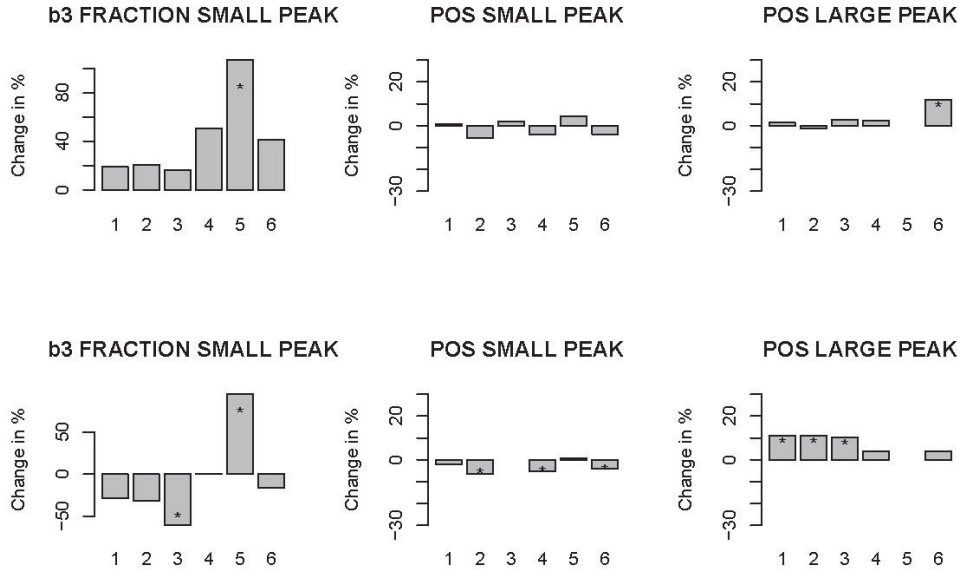


Figure 5.7: B3 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.**

5.7.1.5 *Pichia kluveri* B5

The graphs shown in Appendix D.7 shows that B3 ancestor exhibits two peaks with a large proportion at the higher peak in the DAPI stain and this is reflected in the replicate. Upon analysis of the data as shown in Appendix D.7 Table D.7C, the fraction that makes up the lower peak, in DAPI, shows only line B5.5 changes statistically significantly and increases. For SYTOX lines B5.1, B5.2 and B5.3 all show a statistically significant increase.

In terms of peak location, the DAPI stained samples showed no statistically significant changes in the position of the lower peak, which is the case for the SYTOX stained samples too. For the higher peak location, only line B5.4 demonstrated statistically significant increase, while SYTOX showed statistically significant increase in lines B5.4 and B5.5. Overall these results do not seem to reflect anything consistent across the two stains other than possibly a change in the position of the higher peak as can be seen in Figure 5.8.

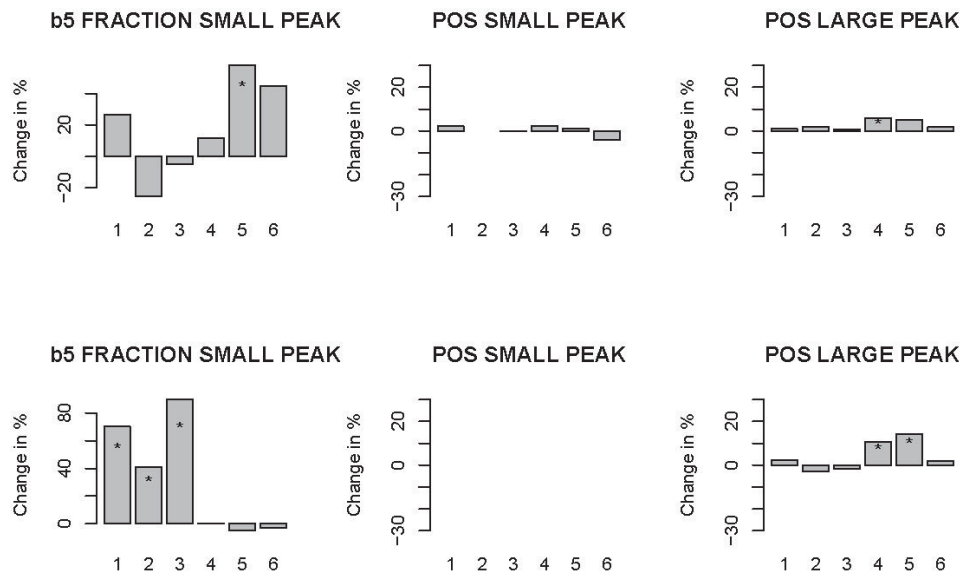


Figure 5.8: B5 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.** The small peak position is missing due to there being no higher peak present in the SYTOX measurements.

5.7.1.6 *Issatchenkia orientalis Kudravtsev* C10

In the graph presented in the Appendix D.8 it would appear that C10 ancestor demonstrates two peaks in DAPI, this is supported by the replicate but to a lesser degree in SYTOX with a very small higher peak. Some of the evolved lines in DAPI appear to lose the lower peak which is not reflected in the SYTOX samples. When looking at the statistical results in Appendix D.8 Table D.8C, four evolved lines, C10.1, C10.2, C10.4 and C10.6, evolved towards a decrease in the fraction that was the lower peak in DAPI, save for line 4 which evolved towards an increase. In the SYTOX samples there were three lines where the lower peak fraction decreased statistically significantly, lines C10.1, C10.2 and C10.4. From Figure 5.9 it would appear that there is a common trend towards decreased lower peak fraction, each with one increasing line, however these do not occur in the same line and SYTOX does not show this increase to a statistically significant level.

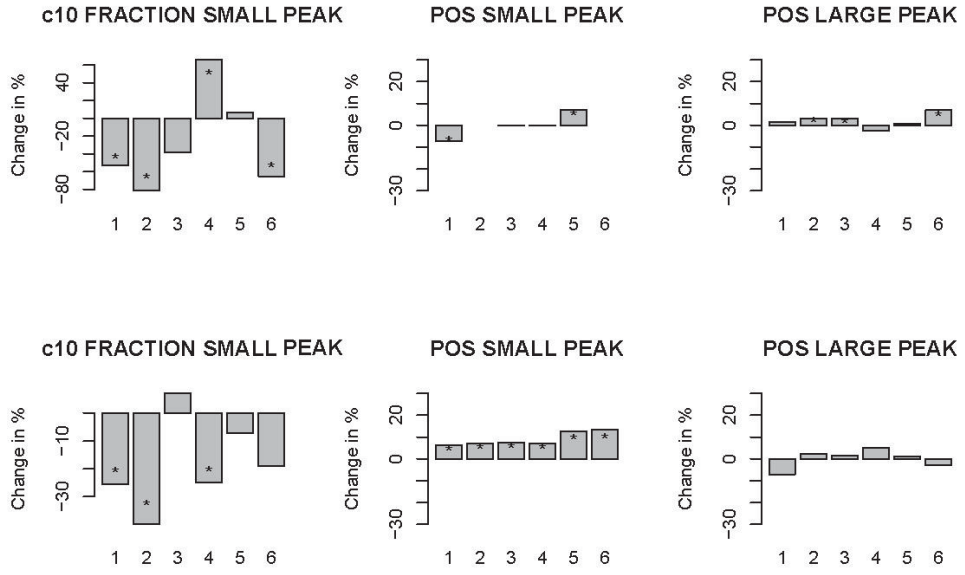


Figure 5.9: C10 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.**

From the analysis of the position of the peaks, the DAPI samples show two lines with statistically significant changes line C10.1 with a decrease and line C10.5 with an increase in position of the lower peak. SYTOX samples however show an all-round statistically significant increase in the lines for the lower peak. In the higher peak, lines C10.2, C10.3 and C10.6 all show statistically significant

increases in the DAPI samples, while the SYTOX samples show no statistically significant changes. No consistent trend is apparent in these results from the evolved lines.

5.7.1.7 *Kluyveromyces nonfermentans* F8

The graph in Appendix D.9 shows the F8 ancestor as having what could be described as two peaks in the DAPI stained samples, however there is very little clear separation between the two peaks. In SYTOX samples while there is one clearly defined high peak in the ancestor, there also appears to be some similarity to the DAPI samples with a low level of signal between where a lower peak would be observed and the higher peak although not to a level that the statistical analysis registers it. When looking at the fraction that makes up the lower peak it is shown that for DAPI stained samples all evolved lines show a statistically significant increase. Looking at Figure 5.10, for SYTOX stained samples all lines show an increase in lower peak fraction, although this is only statistically significant at p=0.008 for line F8.5.

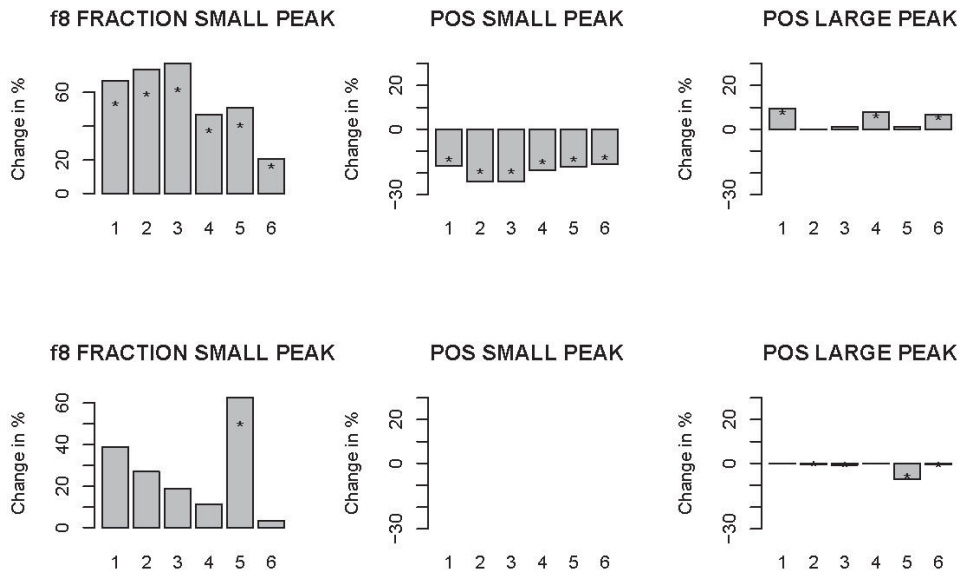


Figure 5.10: F8 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.** The small peak position is missing due to there being no higher peak present in the SYTOX measurements.

Appendix D.9 Table D.9C demonstrates that in terms of peak position, the lower peak in the DAPI samples all lines have statistically significant decrease in position compared to the ancestor. This

is not supported by the SYTOX samples due to the lack of lower peak in the ancestor to compare to. The position of the higher peak in the DAPI samples shows statistically significant increase in three evolved lines, F8.1, F8.4 and F8.6, while the SYTOX samples show statistically significant decrease in lines F8.2, F8.3, F8.5 and F8.6. From this data, there does not appear to be consistency in the changes in position of the peaks, but there does seem to be support for the changes in fraction that makes up the lower peak.

5.7.1.8 *Saccharomyces cerevisiae* SCE2

The graphs shown in Appendix D.10 show that the ancestor starts with two peaks for DAPI, but the consistency of the proportions of each peak between replicates is questionable. When it comes to SYTOX the higher peak appears to be very low in proportions and would not appear to count as a peak in the statistical analysis hence being assigned NA in Appendix D.10 Table D.10C. In terms of measuring the fraction of which makes up the lower peak, DAPI samples show an across the board statistically significant change towards increased fraction as the lower peak. However, SYTOX samples exhibit the exact opposite unanimous change in the evolved lines, statistically significantly decreasing in lower peak fraction.

If the peak position is considered, a number of notable trends can be observed and are demonstrated in Appendix D.10 Table D.10C and Figure 5.11. Firstly, the position of the lower peak in the evolved lines appear to increase in both DAPI and SYTOX stained samples, although this change Is only statistically significant in lines SCE2.5 and 6 for DAPI and SCE2.1, SCE2.3, SCE2.4 and SCE2.6 in SYTOX. For the higher peak, however, DAPI samples do not demonstrate the presence of a higher peak, while in SYTOX the ancestor does not have a double peak while the evolved lines do. This does mean that the statistical results cannot be used as there is no ancestor peak to compare to but the presence of these peaks in SYTOX evolved lines in itself suggests a change from the ancestor. From these results, we can conclude that there are no consistent changes across the two stains.

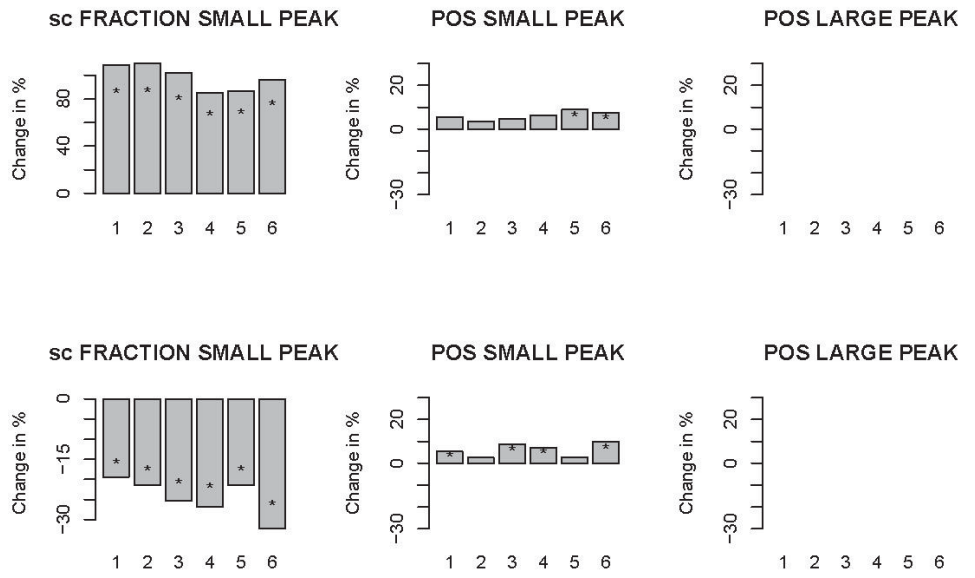


Figure 5.11: SCE2 Relative Change. Fraction of lower peak and position of both the lower and higher peaks in both DAPI and SYTOX as compared to the ancestor**.** The large peak position is missing due to there being no higher peak present.

5.7.1.9 Summary

Here the DAPI and SYTOX DNA stain data was presented and analysed using linear models. From this analysis, observed number of observations may be made. Firstly, the populations presented different patterns, either demonstrating two observable single peaks or only one. The peak with the most counts also differed depending on yeast. Each yeast appeared to have a somewhat unique peak pattern. In most populations, the replicates would be similar and support the same pattern. However, there were often differences between the two staining methods, which, rather than adding robustness to the results, were frequently contradictory and even statistically significant ones, such as the change in the fraction that made up the lower peak in SCE2.

Binomial tests were performed on the results from the two DNA stains to establish if there were any statistically significant difference from random chance in the results. As binomial tests require a minimum of six values, these results were not limited down to only statistically significant results like in previous chapters. From Table 5.12 it would appear that A1, A8 and C10 in DAPI staining do not have any statistically significant trends while in SYTOX this is the case for B3 and

B5. For the DAPI stain all evolved lines of B3, F8 and SCE2 show a movement of cell count fraction towards the lower peak, while A11 shows systematic movement of evolved lines towards the higher peak. When considering the position of the peaks, F8 evolved lines consistently show a decrease in the position of the lower peak, while SCE2 shows a consistent increase in the position of the lower peak in all evolved lines. In terms of the higher peak, both A11 and B5 show consistent increase in the higher peak location in all evolved lines.

	YEAST	Fraction	P-value	Pos. L Peak	P-value	Pos. H Peak	P-value
DAPI	**A1**	5/6	0.2188	2/6	0.6875	NA	NA
	A8	3/6	1.3125	3/6	1.3125	NA	NA
	A11	**0/6**	**0.0313**	5/6	0.2188	**6/6**	**0.0313**
	B3	**6/6**	**0.0313**	3/6	1.3125	4/6	0.6875
	B5	4/6	0.6875	3/6	1.3125	**6/6**	**0.0313**
	C10	2/6	0.6875	1/6	0.2188	5/6	0.2188
	F8	**6/6**	**0.0313**	**0/6**	**0.0313**	5/6	0.2188
	SCE2	**6/6**	**0.0313**	**6/6**	**0.0313**	NA	NA
	YEAST	**Fraction**	**P-value**	**Pos. L Peak**	**P-value**	**Pos. H Peak**	**P-value**
SYTOX	**A1**	**0/6**	**0.0313**	4/6	0.6875	NA	NA
	A8	**0/6**	**0.0313**	5/6	0.2188	NA	NA
	A11	**0/6**	**0.0313**	**6/6**	**0.0313**	2/6	0.6875
	B3	2/6	0.6875	1/6	0.2188	5/6	0.2188
	B5	3/6	1.3125	4/6	0.6875	4/6	0.6875
	C10	1/6	0.2188	**6/6**	**0.0313**	4/6	0.6875
	F8	**6/6**	**0.0313**	1/6	0.2188	1/6	0.2188
	SCE2	**0/6**	**0.0313**	**6/6**	**0.0313**	2/6	0.6875

Table 5.12: Binomial Statistical Outcomes for all Populations. Testing the statistical significance of increases in fraction of data in the lower peak and increase in the position of each peak compared to the ancestor. Statistically significant results are highlighted in bold.

SYTOX on the other hand shows some different patterns overall than the DAPI stain. The fraction of cells for A1, A8, A11 and SCE2 all appear to move towards an increase in the higher peak, while F8 shows a consistent move of the fraction of cells to the lower peak in all evolved lines. The position of the lower peak in A11, C10 and SCE2 increase consistently over the evolved lines, while no consistent changes are observed in the position of the higher peak.

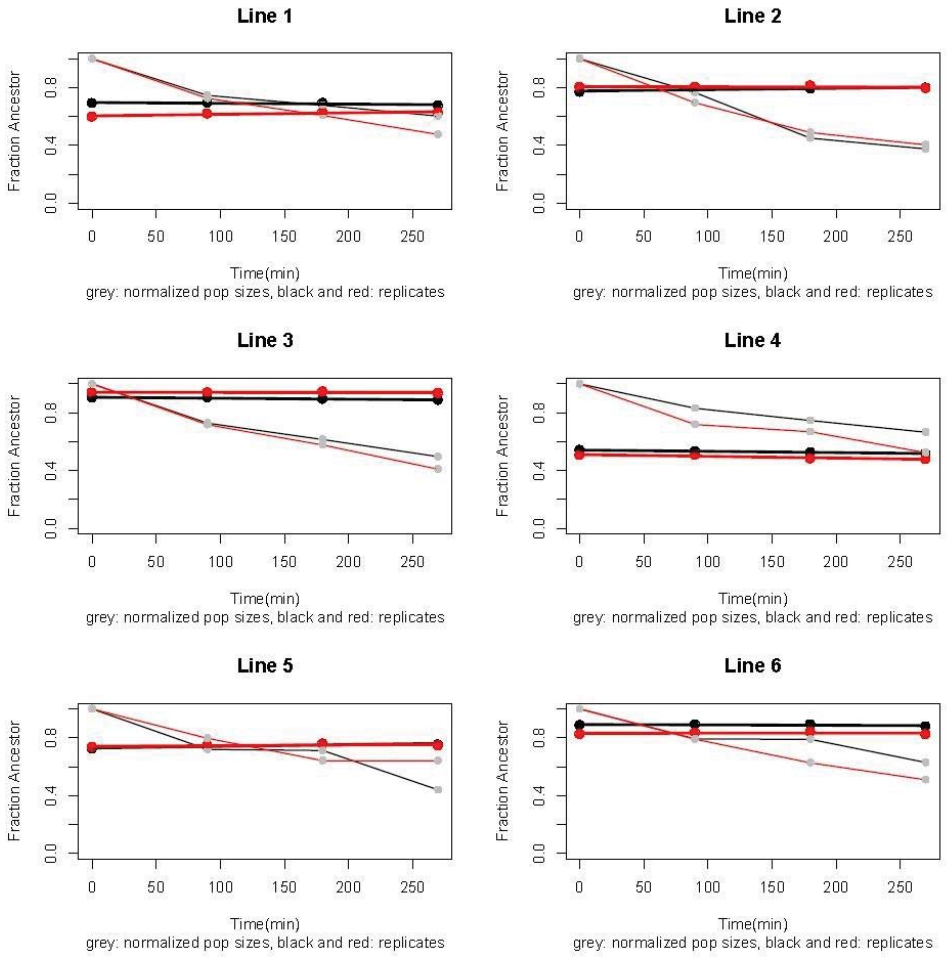
While there is little consistency between DAPI and SYTOX there is consistency observed between replicates of each dye (see Appendix D for the graphs) and between evolved lines. Thus, there are clearly some consistent changes occurring, but the interpretation of these changes remains elusive.

5.7.2 Cell Tracker and Fitness

The use of the Cell Tracker™ was a method utilised in an attempt to examine the fitness changes in the evolved lines versus the ancestor. The resulting measures of each stain signal over a period of exponential growth can be seen in Appendix D.11. To statistically analyse the results, a linear model was used to determine ancestor frequency in each experimental line over time to establish whether the ancestor increases or decreases statistically significantly in the mixed culture. If the evolved lines outcompete the ancestor it would be expected that the ancestor would decrease in frequency as the population increased. The overall frequency of the ancestor over the course of the experiment is presented below.

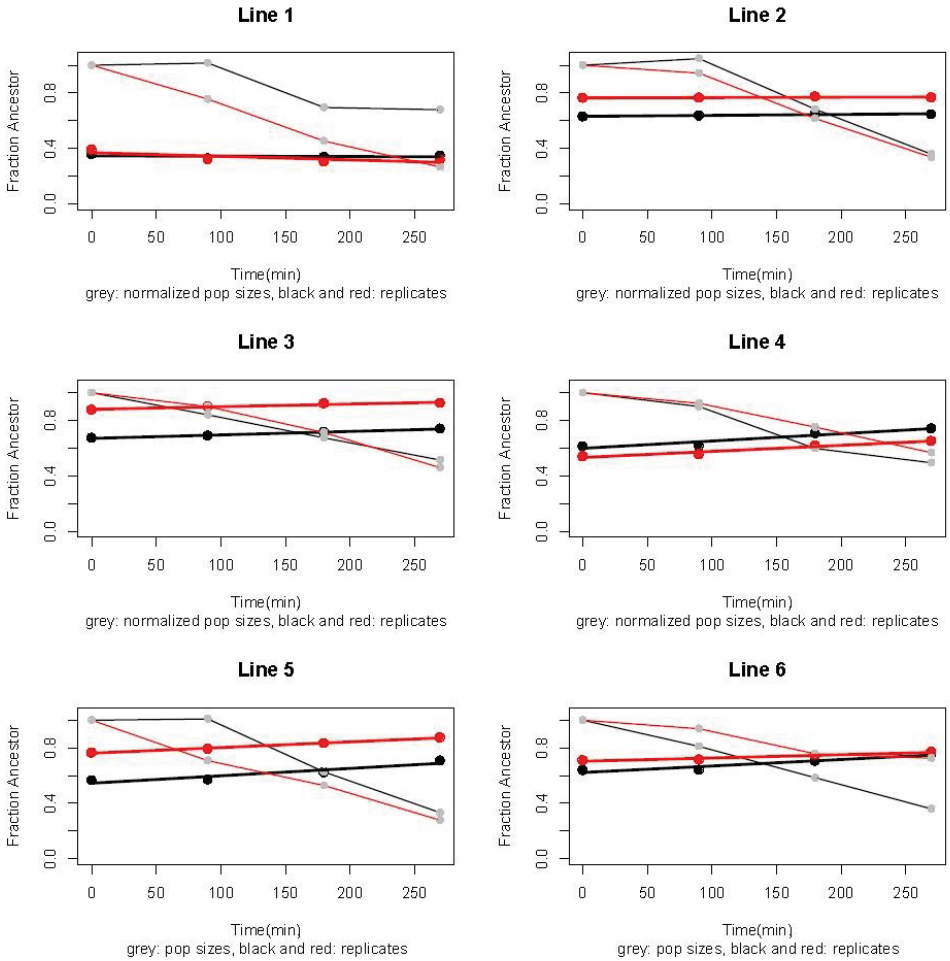
In the A8 experimental data, the population size appears to decrease over the experiment's duration of four and a half hours. This is unexpected as with exponential growth, the population size is anticipated to increase. In Figure 5.13 it would appear that in all the lines the overall frequency of the ancestor does not change statistically significantly or consistently across replicates.

For B5, the population size once again decreases over time for all of the experimental mixes. However, unlike A8, some of the lines show statistically significant results in the frequency change in the ancestor. In Figure 5.14 the table shows statistically significant changes in frequency of the ancestor in lines B5.3, B5.4 and B5.6. These results are consistent across repeats in the three lines. All these changes are increases in the frequency of the ancestor. This might suggest a decrease in fitness in the evolved lines compared to the ancestor, especially as the population size decreases over the course of the experiment.



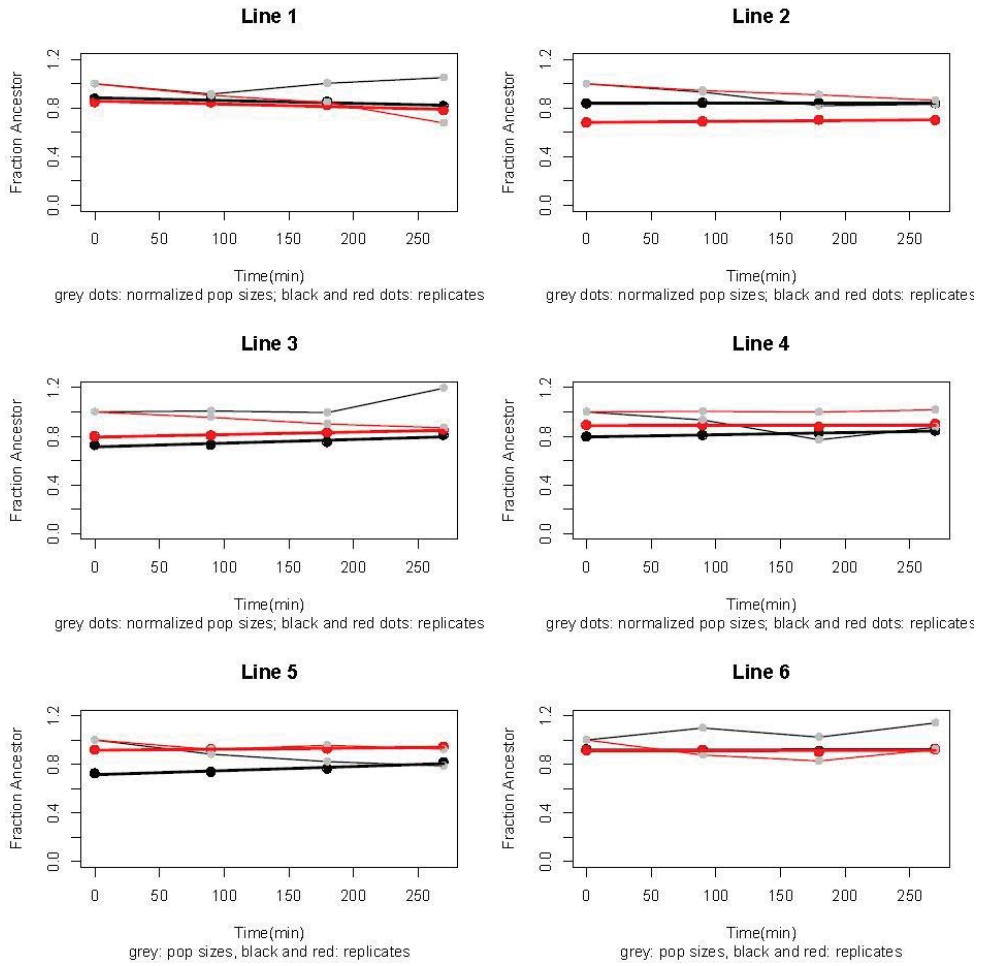
Line	Replicate	Frequency change	P-value
1	**1**	-0.005	0.252
	2	0.009	0.139
2	**1**	0.009	0.202
	2	-0.001	0.740
3	**1**	-0.006	0.071
	2	0.000	0.909
4	**1**	**-0.008**	**0.003**
	2	-0.011	0.104
5	**1**	0.010	0.155
	2	0.004	0.409
6	**1**	-0.002	0.518
	2	0.000	0.992

Figure 5.13: A8 Cell Tracker Experimental Data. Replicates in the graphs are represented by black and red. Grey dots represent the population size, while the red and black dots represent the fraction of the ancestor over time. Linear model results for the frequency of the ancestor are also presented for each line and replicate. Statistically significant results are highlighted in bold.



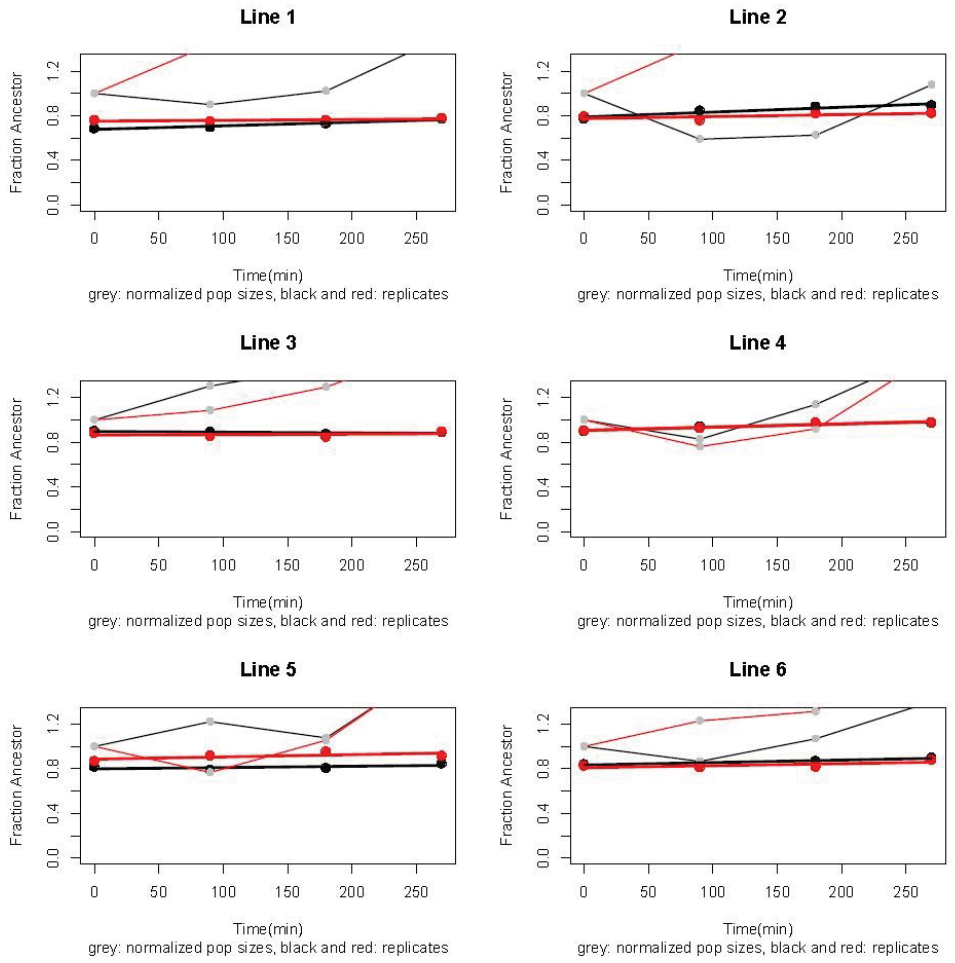
Line	Replicate	Frequency change	P-value
1	**1**	-0.002	0.782
	2	-0.023	0.221
2	**1**	0.006	0.146
	2	0.002	0.443
3	**1**	**0.023**	**0.006**
	2	**0.017**	**0.038**
4	**1**	**0.047**	**0.049**
	2	**0.039**	**0.024**
5	**1**	0.048	0.060
	2	**0.037**	**0.003**
6	**1**	**0.042**	**0.047**
	2	**0.021**	**0.030**

Figure 5.14: B5 Cell Tracker Experimental Data. Replicates in the graphs are represented by black and red. Grey dots represent the population size, while the red and black dots represent the fraction of the ancestor over time. Linear model results for the frequency of the ancestor are also presented for each line and replicate. Statistically significant results are highlighted in bold.



Line	Replicate	Frequency change	P-value
1	**1**	-0.021	0.056
1	**2**	-0.023	0.077
2	**1**	0.000	0.858
2	**2**	0.007	0.094
3	**1**	0.028	0.093
3	**2**	**0.019**	**0.027**
4	**1**	**0.016**	**0.007**
4	**2**	0.001	0.880
5	**1**	**0.030**	**0.044**
5	**2**	0.008	0.062
6	**1**	0.000	0.908
6	**2**	0.000	0.938

Figure 5.15: C10 Cell Tracker Experimental Data. Replicates in the graphs are represented by black and red. Grey dots represent the population size, while the red and black dots represent the fraction of the ancestor over time. Linear model results for the frequency of the ancestor are also presented for each line and replicate. Statistically significant results are highlighted in bold.



Line	Replicate	Frequency change	P-value
1	**1**	**0.029**	**0.027**
1	**2**	0.007	0.311
2	**1**	0.039	0.059
2	**2**	0.016	0.348
3	**1**	-0.006	0.352
3	**2**	0.004	0.787
4	**1**	**0.026**	**0.049**
4	**2**	**0.027**	**0.042**
5	**1**	0.010	0.431
5	**2**	0.018	0.330
6	**1**	**0.020**	**0.035**
6	**2**	0.016	0.324

Figure 5.16: SCE2 Cell Tracker Experimental Data. Replicates in the graphs are represented by black and red. Grey dots represent the population size, while the red and black dots represent the fraction of the ancestor over time. Linear model results for the frequency of the ancestor are also presented for each line and replicate. Statistically significant results are highlighted in bold.

In yeast population C10 the population size appears to remain relatively constant in all the lines rather than decreasing. In terms of frequency changes, while there are several statistically significant changes, as can be seen in Figure 5.15, none of these statistically significant changes are consistent over the repeats. Therefore, it is unlikely that these observed changes are anything other than noise produced from sample variance.

Finally, in SCE2, the population size appears to increase over most of the lines. This would be the expected behaviour during exponential growth. In terms of observed fitness change we would therefore expect decreases in the ancestor with the population increase, if the evolved lines had increased in fitness. As can be seen in Figure 5.16, there is only one observed statistically significant change that is consistent across repeats. Line 4 displays a statistically significant increase in frequency of the ancestor.

5.7.3 Summary

The experimental data from the cell tracker experiment has not revealed much in terms of whether fitness of the evolved lines has changed in comparison to the ancestor. Looking at the frequency change of the ancestor, it would be expected that if the evolved lines increase in fitness the ancestor frequency would decrease. However, the population size in all save SCE2 decreases and therefore any results must be considered carefully. Out of the four yeasts measured, only B5 produced replicated statistically significant changes in frequency in the ancestor, an increase in a decreasing population. This might suggest that the evolved line has a decrease in fitness or that the stain may be affecting the evolved lines growth.

The experimental method suggests the potential for producing a greater insight into the fitness of the populations, the issue of when the samples are being measured being the biggest hurdle to overcome. If the samples are not in exponential growth, the fitness of the samples cannot be accurately measured. Another hurdle that may have to be investigated is whether the stain is having a toxic effect on the lines.

5.8 Conclusion

The experiments discussed above aimed to address two questions. Is the increase in cell size due to an increase in DNA content in the cells, and have the evolutionary changes observed in the populations increased the fitness of the evolved lines compared to their ancestors. It was predicted that with increasing cell size, ploidy would likely also increase. With this increase in ploidy it would be expected that in the long run fitness would increase, but for a period of time after the change in ploidy fitness would decrease. If on the other hand the data from previous chapters are used to predict the expectation of fitness change, it would be likely fitness overall would have increased in the lines with the increase in rate at the cost of yield observed in Chapter Two.

The data presented in this chapter are exploratory, to glean some understanding of the observed changes in previous chapters. The data here on DNA stain is difficult to interpret in terms of ploidy. This is due to, firstly, it being unclear what each peak represents in the data, and secondly, whether the peaks truly represent DNA quantity when the two stains often contradict each other or do not statistically significantly correlate. This raises the question as to whether the stains signal is proportional to DNA content in these results. If it was representing ploidy the anticipated results would demonstrate one peak, haploid ancestors changing to two peaks, diploid evolved lines or similar for diploid lines changing towards tetraploid. This would be proportional between the peaks, i.e. the high peak would be two times the lower peak. This is not the case and therefore brings in to question what the results are quantifying if not the ploidy of the DNA. The fact that the ancestors often show the same peak structure as the evolved lines suggests that if this does show ploidy that no ploidy change has occurred in these lines (such as in A1 and A8).

If, therefore, observed peaks are not due to ploidy an alternative explanation for such results must be sought. It is possible that the results are representing budding in the yeasts although this seems unlikely due to sampling in stationary phase and not exponential growth. Otherwise these results may be issues with experimental procedure. With the use of natural yeasts there is a lack

of experimental testing and optimisation of commonly used components on these particular species. It is possible that the stains have not successfully dyed the DNA content of the yeasts or that the fixing procedure using formaldehyde has not effectively permeabilised the yeast and the dye has therefore been unable to permeate the cells properly. It is apparent therefore that further investigation should be carried out on determining the DNA quantity in these samples. The most effective and reliable way to do this would be to sequence the strains, and indeed samples of A8 have been submitted, however the data will not be presented in this thesis.

Looking at the data presented in the Cell Tracker™ experiment a similar story is observed as with the DNA stain. This experiment was again an exploratory test to see if competitive fitness could be established between ancestor and evolved. This would allow for the conclusion that while ethanol production did not appear to increase in the populations, the rate yield trade-off that was often observed and the change in cell size, did in fact, result in a fitness advantage. As there was no evidence for ploidy changes in the DNA stain experiment, fitness would be expected to show signs of increase if the lines had evolved an advantage compared to the ancestor. The most reliable measure to analyse was the frequency of the ancestor to establish if the evolved lines outcompeted the ancestor resulting in a decrease in ancestor frequency. However, no consistent changes were observed save in B5 where the ancestor appeared to increase in three of the mixes. Surprisingly population size seemed to decrease overall in the populations and only increased in SCE2. This is strange as exponential growth would see the population size increase What can be established is that the individual lines and ancestor can be clearly tracked through the experiment up to four and a half hours, as can be seen by the graphs in Appendix D.11. There are two clear populations in most of the experiments. However, as time goes on the populations move inwards towards each other and begin to merge, thus making detection over time increasingly more difficult. In pilot studies at 24 hours it was almost impossible to detect the Cell Tracker™ stains separately from each other. This provides a small window of sampling opportunity.

The lack of fitness measurements could be the result of sampling time, lines may still be in lag phase growth and not in exponential growth, resulting in no population increase within the time

points measured. Measuring for longer would perhaps solve this but the dyes become harder to detect, hindering this. It is also plausible that inadequate cell permeation occurred and the dye was not as effective at intended (See Chapter 6 limitations for further discussion). Another possibility, although the dyes used were specifically not supposed to be, is that the dyes may be slightly toxic to the yeasts. Once again with the use of natural yeasts the components have not been optimised for use with these specific yeasts and could therefore be having unknown effects on the growth of the populations. From this data, there can be no conclusion drawn on the fitness of the populations and further investigation should be carried out to establish this. It is possible that fitness might be measured through the use of a genetic marker such as using a URA3 vector, a technique often used in yeasts (Goffrini et al. 2002; Ishchuk et al. 2016; Brown & Lindquist 2009).

This chapter presented exploratory data that has unfortunately not clarified the questions raised from the main experimental data of this thesis. It is clear further investigative work can be carried out to investigate these questions, but this is beyond the scope that this thesis can address. The Discussion chapter will now present the summary analysis of the data presented in these results chapters and will deliberate the conclusions that can be drawn overall from the experimental work that has been presented.

CHAPTER SIX

Discussion and Synthesis

''Someone else always has to carry on the story.''

-Bilbo Baggins, The Lord of the Rings.

6.1 Introduction

The predominant aim of this thesis was to investigate whether the evolution of the Crabtree effect could be a strategy to respond to resource competition by increasing the rate of ATP production at the cost of growth yield. This has importance in a number of general fields such as metabolic biochemistry, social evolution theory of microorganisms and the commercial use of yeasts. It also has important implications for the study of tumour cell metabolism.

6.2 Summary of Findings

In Chapter Two the main experimental focus was presented for this thesis. An experimental evolution study was conducted in high sugar medium for 1500 generations on seven yeast populations and a *S. cerevisiae*. For each of those starting isolates, six replicate lines were evolved in parallel. Chapter Two reports the growth rate and yield of the lines over time. In the evolved lines of all yeasts, of the statistically significant changes, 30/32 lines increase in growth rate compared to the ancestor. When measuring growth yield, 23/25 statistically significant changes developed a decrease in yield compared to the ancestor (See Chapter Two Table 2.19 for binomial results). This is as anticipated in relation to the expectation of the RYT theory. While the yeast population A11 data was not included in the rate and yield analysis in Chapter Two due to growth curve noise, the population was included in all further investigations.

In Chapter Three spectrophotometric assays were used to analyse the metabolic capabilities of the populations and how they may have changed. Primarily the focus of this chapter was the analysis of ethanol production. When investigating the ethanol concentration over a 24-hour period, the only notable changes that were witnessed were an increase in ethanol production in B5, a decrease in A8 and a shift in when ethanol was produced in SCE2.

In Chapter Four, the phenotypic qualities were quantified. CFUs, FACS count and cell size were measured for each evolved line and ancestor. From this there was strong support from the CFUs and FACS counts for the growth yield decrease measured in Chapter Two, with 45/47 statistically

significant CFUs decreasing and 37/37 statistically significant FACS counts decreasing (see Chapter Four Table 4.17 for binomial results). Of the statistically significant measurements, 25/35 evolved lines of all the yeasts increased in cell size (see Chapter Four Table 4.17 for binomial results) which was an unexpected observation.

Finally, Chapter Five was an exploratory chapter to investigate both DNA quantity and fitness of the evolved lines compared to the ancestor using stains that could be detected by flow cytometry. This chapter did not provide any statistically significant conclusions and highlighted the need for other techniques to answer these questions.

6.3 Correlation of Results

The aim of this thesis set out to demonstrate that a metabolic trade-off between rate and yield was an evolutionary advantage in high sugar environments, a probable explanation for the evolution of the Crabtree effect. It would therefore be predicted that Crabtree-negative yeasts would evolve towards having a faster growth rate at the cost of growth yield. As discussed, the populations used in this study were more likely to increase in growth rate than decrease and were more likely to decrease in growth yield than increase. However, it would be of interest to establish if this was directly correlated i.e. a trade-off occurred for this to happen. It is also of interest to establish if, for example, any correlation between growth rate and yield is found, and if any of the other observed traits through the chapters are correlated to the changes in growth rate and growth yield, e.g. if cell size was a trait that developed along with the changes in growth yield or a consequence of the change in growth rate. In this section, the following questions are asked:

- Are there any correlations between the changes of rate, yield, CFUs, FACS count, size and DNA stains over all the lines?

Correlations were calculated for all the results to find any relation between the factors presented in the previous chapters. Since this involves a large number of tests, false positives are likely. This is combatted through the use of Bonferroni correction, adjusting the p-value to 0.05 divided by

the number of tests carried out. These tests were carried out across all traits tested in the results chapters and then also on a species level.

Out of these tests, the only significant correlations observed (after the Bonferroni correction) were between size and the fraction of the lower peak in DAPI, rate and the location of the lower peak and finally significant correlation was observed between size and FACS count in C10.

In terms of correlations with the DNA stains two observed significant tests are seen, as demonstrated in Figure 6.1. This is important as no other correlations are seen with rate or size after the correction for false positives. The main driving force behind this seems to be in SCE2 and F8. Both show the largest gains in size and also the most change in the fraction that is the lower peak in DAPI leading to a positive correlation. The location of the peak, correlating negatively with the rate, is once again driven by the yeasts SCE2 and F8, but this time with drastically opposing changes, SCE2 having a shift of the lower peak towards the higher peak and no change in rate while F8 shows a shift away from the higher peak and a significant increase in rate. The reason behind these correlations is hard to interpret without further understanding of what the peaks actually represent. The Figure 6.1 shows that these correlations are largely because of variation between the species.

Otherwise no other significant correlations were observed when adjusting for false positives with the Bonferroni correction. If the correction is removed, a positive correlation could be observed between CFUs and FACS count and a negative correlation between FACS count and size.

What is clarified from these correlations is that, while growth yield decreases when there is a selection for growth rate in the experimental evolution study, the two are not directly correlated.

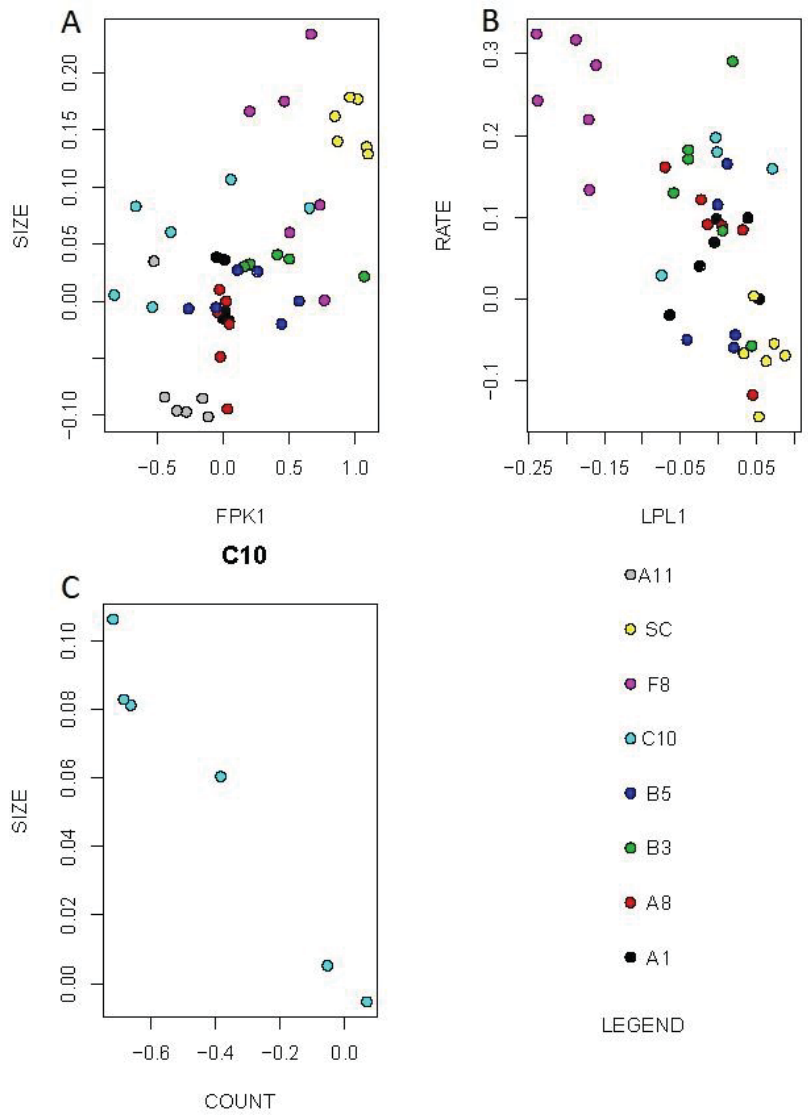


Figure 6.1 Observed Correlations. This figure presents the three correlations that had significance across all tested traits and species-specific tests. Graph A shows a correlation between the size of the cells when measured with flow cytometry and the fraction of the lower peak (FPK) in the DAPI stain. Graph B shows a correlation between rate and the location of the lower peak (LPL) in the DAPI stain. Graph C is the only species-specific correlation, between size and count.

The statistical output is as thus: FPK1 versus size r-value = 0.59, p-value= <0.001, LPL1 versus rate r-value = -0.67, p-value = <0.001. C10 size versus count r-value = -0.984, p-value= <0.001.

- Are there any correlations between the changes in rate, yield, CFUs, FACS count and size for each species?

As is shown in Figure 6.1 the only statistically significant correlation observed on a species level was the negative correlation of cell size with the FACS count in C10.

- Are there any correlations between the 6 changing variables from the DAPI & SYTOX experiment?

There were no observable significant correlations between the variables measured in the DNA stains experiment presented in Chapter Five.

- Is there a relation between ancestral value and change for any of rate, yield, CFUs, FACS count and size?

When investigating the relative change of the evolved lines from the ancestor for each trait, it is apparent, from Figure 6.2, that both rate and yield relative changes are dependent on the ancestral values. Species that started out relatively fast, changed little while the slower species had more significant gains in growth rate. With growth yield, the higher starting yield species lost more significantly than the already low yield species. This could suggest that having a fast rate makes it more difficult to get faster, while for yield, the higher the starting yield the more yield that can be lost.

There is no obvious dependence in CFUs or FACS counts on the starting number in the ancestor as to what change is observed. There is however a driving force acting on the species to decrease growth yield, but it is not relative to what yield the lines started on.

When looking at the relative change in size, it would appear that starting both as the smallest and largest species leads to the largest increase in size. There is obviously selection occurring here but it does not appear that the selection is towards an optimum size.

It is of interest to note that, when correlating the rate and yield estimates as obtained by the linear model carried out to analyse the data in Chapter 2 (see tables 2.3 - 2.16) for the ancestors

of each yeast population, there is no trade-off observed. As shown in Figure 6.3, the correlation is positive (0.58), though not significant (p=0.17). The reference *S. cerevisiae* population began as a low yield strain, however a number of the other yeast populations started at the same rate. This could indicate that, relatively speaking, it may not be a high rate strain, or that these other populations were high rate Crabtree-negative strains.

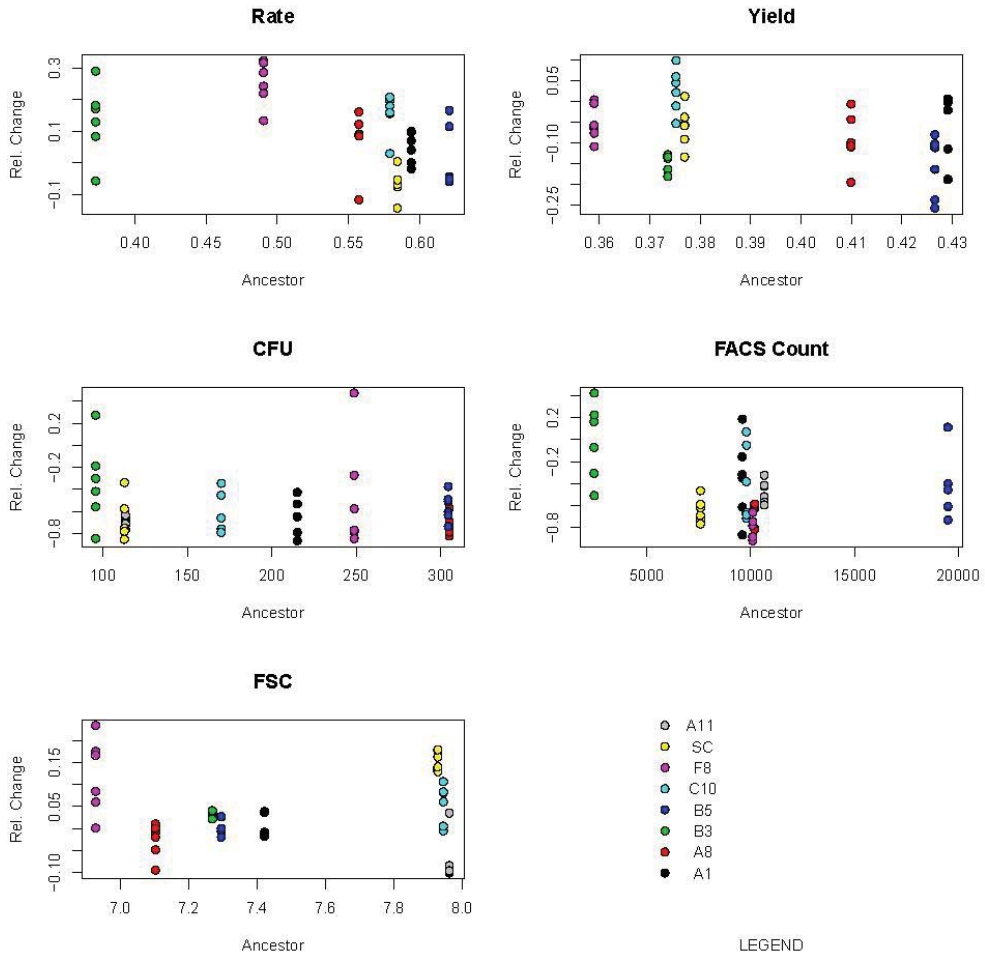


Figure 6.2 Summary of Relative Change of Evolved Lines of each Yeast from the Ancestor. Here the relative change of each line of each yeast population is plotted against the starting value of the ancestor. Each significant trait that was presented in the previous chapters is summarised in a graph which includes: rate, yield, CFU, FACS counts and size. Statistically it is established that of the traits investigated rate and yield show a dependence on the ancestor starting value.

Linear model statistical output: Rate: estimate = -0.6062, t-value= -2.734 and p-value = 0.00927 while for yield: estimate= -0.9984, t-value= -2.185 and p-value= 0.0348.

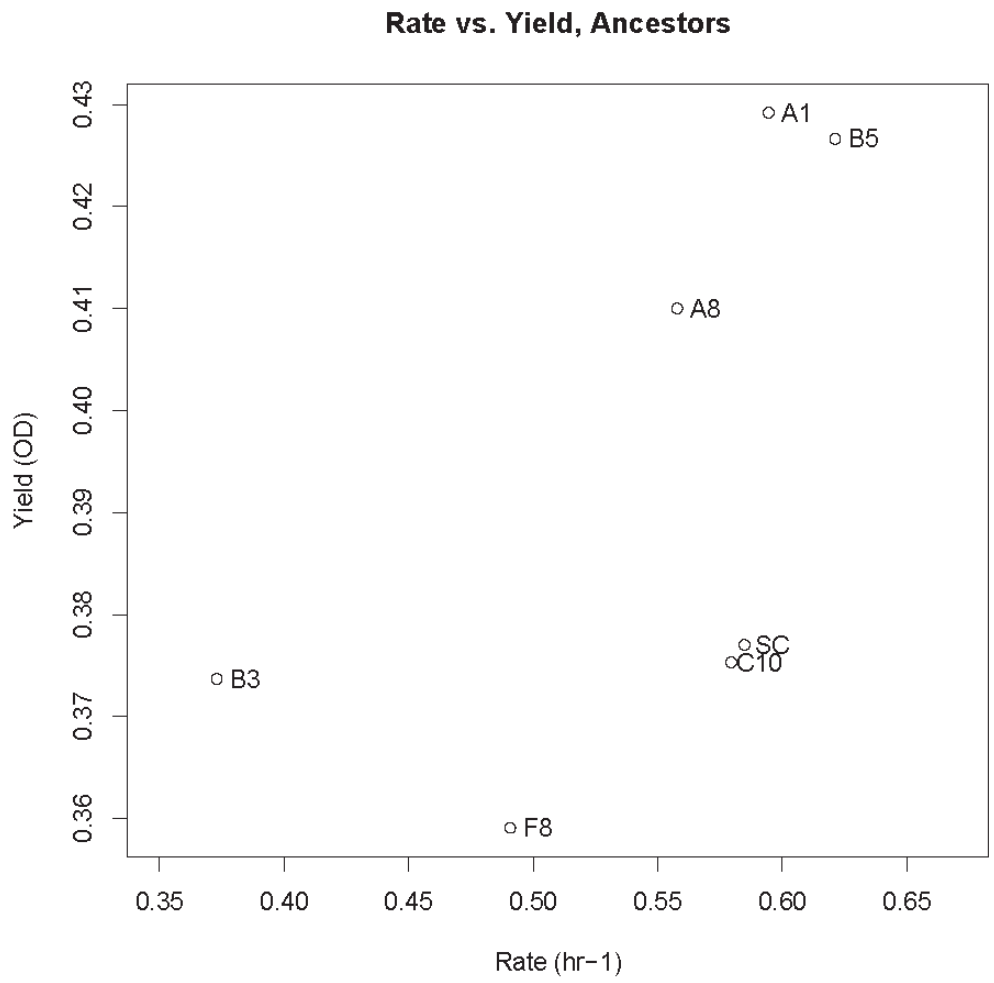


Figure 6.3 Rate vs Yield Correlation of all Ancestor Populations. Derived from the data obtained from the linear model in Chapter 2. The correlation between rate and yield across the yeast populations is positive although not significant (p-value =0.17).

6.4 Review of the Results

6.4.1 Relevance to the Field

When looking at these results as a whole, there is clearly strong selective pressure acting upon the populations over the experimental evolution study. Specifically, the traits that appear to be favoured are not what would be associated with cooperative, efficient behaviour. While there is no evidence for the direct correlation between rate and yield as predicted by the trade-off theory, the behaviours of most of the populations mirrors the expected changes. Rate increases while

yield decreases. This change in rate would be seen as a selfish trait not beneficial towards the overall population (Hardin 1968; Maclean 2008). Although the investigation in to ethanol production didn't produce much in the way of conclusive data, one observation of note is the distinct lack of decrease in the ethanol production of a natural *S. cerevisiae* population. With the MAC hypothesis as presented in the Introduction, it would be expected that with the lack of bacterial competition, ethanol production would be a costly trait to maintain when not necessary. All public goods are generally assumed to be costly to produce for the cooperative individual but overall beneficial to the group (Maclean & Gudelj 2006; Rainey & Rainey 2003; Goel et al. 2012). If there is no bacterial competitor deterring benefit to the group and cheaters can exploit the free secondary carbon resource, then the trait is an unnecessary cost to the cooperative individual. It is likely therefore that some other selective advantage is responsible for the metabolic trait.

Similar results in rate and yield have been observed in several other studies (Maclean & Gudelj 2006; Jasmin et al. 2012; Novak et al. 2006). One particular study demonstrated trade-offs in growth rate and growth yield within populations of *E. coli* (Novak et al. 2006). While the number of generations studied was larger, achieved with the faster generation time of the microorganism, the methods employed were similar.

The most surprising changes occurred in terms of cell size change. Other studies have been conducted on specifically measuring this trait (Jasmin & Zeyl 2012). What has been observed is that often cell size is associated to ploidy changes (Wu et al. 2010). It has been previously speculated that selection may favour larger cell size although it is also possible that cell size is a correlated response to some other trait selection and the result of genetic drift (Lenski & Travisano 1994). Cell enlargement has been observed quite frequently in experimental evolution studies but the reason for its emergence and its effects are yet to be investigated thoroughly (Dhar et al. 2011; Lenski & Travisano 1994; Philippe et al. 2009). It has been observed that with increasing growth rate, both cell size and ribosomal quantity increase, with a shift towards more inefficient metabolism (Molenaar et al. 2009). In another study observing cell size in a selection of *S. cerevisiae* strains, large cell size was associated with high specific glucose consumption rates

and selfish resource allocation i.e. favouring their own cell size over efficient resource utilisation allowing for overall increases in population size (Spor et al. 2009b). Therefore, the literature would suggest that the development of such a trait, while the benefit is unclear, is possibly a sign of selfish resource allocation and an indication that growth rate is shifting towards faster inefficient metabolic routes.

6.4.2 Alternative Explanations

With the expectation that ethanol production would increase with a rate/yield trade-off, and the apparent lack of change observed in Chapter Three and no statistical correlation between rate and yield as discussed in section 6.3, it brings into question what driving force was acting upon the yeasts in the experiment. It is apparent that cell size has an effect but, with the lack of evidence in ploidy changes, again it is unclear in what way. As an alternative explanation, it is plausible that another selective advantage may be occurring that has not been monitored that results in the increased cell size and increased rate or decreases the yield. One explanation could be the production of another metabolite, such as glycerol which was not quantified in this experiment but has been shown to be produced by yeasts (Merico et al. 2009; Petit et al. 2000; Lin et al. 2010; Pérez-Nevado et al. 2006). It is also possible that while both rate and yield reflect what was expected if a trade-off occurred, this may be completely unrelated changes that are the result of some other change such as membrane or binding protein changes in the metabolic pathway (Bruggeman & Teusink 2015). It is plausible that no metabolic changes are occurring and that there is a selective advantage in increased cell size completely unrelated to the metabolic capabilities of the yeasts as cell size has been shown to influence many functional properties of an organism (Lenski & Travisano 1994).

6.5 Limitations

The experimental studies conducted as part of this thesis have several limitations that could be further accommodated for if research on this topic were to continue. Firstly, one of the biggest limitations of this study was the number of replicates that could be maintained per yeast

population or yeast population line, and the sample volume of these replicates. Due to the wish to maintain a statistically viable number of lines per yeast population, there was a compromise in sample size, only 200µl per sample. This was also the best way to gain continuous measurements over 24 hours as it allowed the use of a spectrophotometer plate reader. However, this proved a detriment in the quantification of such things as protein and ethanol. If this experiment was to be rerun it would be of great benefit to carry out the study with larger sample sizes which would also allow for larger sampling over the experiment and more samples of the same culture over a 24-hour period giving more accuracy and less sample to sample variability. Measuring optical density over a 24-hour period would have to be altered as a flask culture of say 100ml, would not be measurable in a spectrophotometer and a method that allowed the monitoring of multiple cultures at once would have to be found. This was the great benefit of the method chosen for this study, the growth of a large number of samples could be measured at once with relative ease and accuracy. With the number of samples, due to the large quantity of lines that were maintained, running repeats on that many populations and lines became an unachievable task due to time and other constraints of this study. Therefore, in some tests, statistical robustness was lost due to the obtaining of only two repeats for some experiments. If there was more time to carry out further testing, more repetitions of experiments would be a top priority. This thesis has been a high throughput, low resolution set up that has highlighted where more labour intensive but higher resolution studies should focus in the future.

More repetitions could potentially counteract some of the measurement variability encountered in the experiment. Measurement variability could also be counteracted with further optimisation of the experiments, for example, there was a lot of plate-to-plate variance observed with the growth curves. While methods were implemented to avoid condensation, there could have been an issue as simple as the quality of 96-well plate being used having an effect on the readings or potentially the clumping of cells playing a role. With further pilot studies testing consumable quality, more tailored media recipes etc. this variability might be removed.

Some of the limitations of the study, such as the inconsistencies experienced in the DNA staining experiment, could be the result of using the natural yeast populations. As these yeasts have not been through the same long "domestication" as the commonly used laboratory yeast strains, certain dyes and reagents may not interact with these natural yeasts in the same way. For example, though not presented in this thesis, when conducting DNA extractions on *Kodamaea sp.* A8, the standard methods implemented in specialist yeast DNA extraction kits (Gentra Puregene Yeast/Bact. Kit and the YeaStar™ Genomic DNA Kit) to break down the cell wall, did not work. This meant a far harsher technique was eventually used. A similar issue is suspected to explain the results from experiments that required the cells to uptake dyes. If the uptake of the dyes was not occurring as expected this would result in misleading and probably replicate variable results. Therefore, further optimisation for these experiments would be extremely beneficial if any further testing were to be carried out.

While fitness advantages were not observed due to what would appear to be optimisation issues in the exploratory experiments, future tests could be carried out with some prerequisite experiments in mind. For better optimisation of the Cell Tracker data a pilot could be conducted using an impaired growth mutant against one of the populations to see if fitness could be monitored between the two. This would allow for a reference test of known fitness difference to be compared to the Cell Tracker results between ancestor and evolved. It is also plausible that an alternative marker could be used, such as URA3, if the Cell Tracker continued to prove inconclusive.

In principle the optical density measurements could have been replaced by or benefited from the support of further techniques of monitoring growth such as CFUs. However due to the labour intensiveness of the experiments already undertaken such methods have fallen out of use in favour of the high throughput automated option that the optical density reading spectrophotometers provide (Hall et al. 2014).

Many of the issues experienced in this study might potentially be solved by the use of more expensive and/or complicated techniques. Ethanol quantification would more than likely have improved accuracy and reliability with the use of HPLC. Finally, for a fitness study, inserting genetic markers would easily allow for more reliable tracking of samples without the worry of sample signal merging after a few hours.

6.6 Concluding Remarks and Future Research

For future research, it would be of interest to continue the experimental evolution to investigate what further phenotypic, and possibly genetic changes the natural yeast populations exhibit. If there was more time for this thesis it would be of personal interest to investigate further into the genetic mutations that may have occurred throughout the evolution experiment through sequence analysis. This would also allow for more detailed investigation into DNA quantification, an analysis that unfortunately yielded no definitive results in the DNA stain experiments carried out in Chapter Five. If an experiment could be designed that allowed for the selection of yield, such as the use of emulsion-based propagation regimes in *Saccharomyces cerevisiae,* this would allow for an alternative approach to investigate the dynamics of the origin of the Crabtree effect (Bachmann et al. 2016). Lastly, due to the lack of directly correlated trade-off found, and the selection observed in the change of cell size with no apparent trend towards an optimum, it would be interesting to investigate what other differences may have been occurring in the populations. Investigating whether the yeasts produce glycerol for example, whether there has been increased membrane proteins, carrying out a larger scale investigation into the cell size changes in different environments or in fact manipulating cell size in some way, all may help in establishing if another trade-off is occurring that could explain these results and the observed increase in growth rate.

In conclusion, this thesis has highlighted that in an experimental glucose rich setting a selection of natural yeasts demonstrate most often an increased rate of growth and decreased growth yield in about 1500 generations. There is also evidence of a selective advantage in the evolution towards a change in cell size, though the advantage of this is unclear. Though it has proven more

difficult than expected to address the evolutionary advantage of the Crabtree effect, this thesis has illustrated the importance of studying the competitive behaviours of microorganisms in isolation, where selfish traits appear to thrive.

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APPENDIX A

A.1 Metabolic Capabilities of Yeast Populations

Test	ox/assim	K.sp	P.kl	I.o k	C.r	K.n	S.c
water	ox	(-)	(-)	(-)	(-)	(-)	(-)
acetic acid	ox	w	n	n	n	n	(-)
formic acid	ox	(-)	n	n	n	n	(-)
propionic acid	ox	w	n	n	n	n	(-)
succinic acid	ox	(-)	n	n	n	n	(-)
succinic acid mono-methyl ester	ox	w	n	n	n	n	(-)
l-aspartic acid	ox	w	n	n	n	n	(-)
l-glutamic acid	ox	w	n	n	n	n	(-)
l-proline	ox	(+)	n	n	n	n	(-)
d-gluconic acid	ox	(-)	n	n	n	n	(-)
dextrin	ox	(-)	n	n	n	n	(-)
inulin	ox	(-)	n	n	n	n	w
d-cellobiose	ox	(+)	n	n	n	n	(-)
gentiobiose	ox	(+)	n	n	n	n	(-)
maltose	ox	(+)	(-)	(-)	(-)	n	(+)
maltriose	ox	(+)	n	n	n	n	(-)
d-melezitose	ox	(-)	n	n	n	n	(-)
d-meleibiose	ox	(-)	n	n	n	n	(-)
palatinose	ox	(+)	n	n	n	n	(-)
d-raffinose	ox	(+)	(-)	(-)	(-)	n	(+)
stachyose	ox	(+)	n	n	n	n	(+)
sucrose	ox	(+)	(-)	(-)	(-)	n	(+)
d-trehalose	ox	w	(-)	(-)	(+)	n	(+)
turanose	ox	(+)	n	n	n	n	(+)
n-acetyl-d-glucosamine	ox	(+)	n	n	n	n	(-)
α-d-glucose	ox	(+)	v	(+)	(+)	(-)	(+)
d-galactose	ox	(+)	(-)	(-)	(+)	n	(+)
d-psicose	ox	w	n	n	n	n	w
l-sorbose	ox	w	n	n	n	n	(-)
salicin	ox	w	n	n	n	n	(-)

d-mannitol	ox	(+)	n	n	n	n	(-)
d-sorbitol	ox	(+)	n	n	n	n	(-)
d-arabitol	ox	w	n	n	n	n	(-)
xylitol	ox	(-)	n	n	n	n	(-)
glycerol	ox	(+)	n	n	n	n	(-)
tween 80	ox	(-)	n	n	n	n	(+)
water	assim	(-)	(-)	(-)	(-)	(-)	(-)
fumaric acid	assim	v	n	n	n	n	(-)
l-malic acid	assim	(-)	n	n	n	n	(-)
succinic acid mono-methyl ester	assim	w	n	n	n	n	(-)
bromosuccinic acid	assim	(-)	n	n	n	n	(-)
l-glutamic acid	assim	w	n	n	n	n	(-)
ϒ-aminobutyric acid	assim	w	n	n	n	n	(-)
α-ketoglutaric acid	assim	(-)	n	n	n	(-)	(-)
2-keto-d-gluconic acid	assim	v	(-)	(-)	(+)	(-)	(-)
d-gluconic acid	assim	W	v	(-)	(+)	(-)	(-)
dextrin	assim	(-)	n	n	n	n	(-)
inulin	assim	(-)	(-)	(-)	(-)	(-)	(+)
d-cellobiose	assim	(+)	(+)	(-)	(+)	v	(-)
gentiobiose	assim	(+)	n	n	n	n	(-)
maltose	assim	(+)	(+)	(-)	(-)	(-)	(+)
maltotriose	assim	(+)	n	n	n	n	(-)
d-melezitose	assim	(-)	(+)	(-)	(+)	(-)	(-)
d-melibiose	assim	(-)	(-)	(-)	(-)	(-)	(-)
palatinose	assim	(+)	n	n	n	n	(-)
d-raffinose	assim	(+)	(-)	(-)	(-)	(-)	(+)
stachyose	assim	(+)	n	n	n	n	(+)
sucrose	assim	(+)	(-)	(-)	(+)	n	(+)
d-trehalose	assim	(+)	(-)	(-)	(+)	(-)	(+)
turanose	assim	(+)	n	n	n	n	(+)
n-acetyl-d-glucosamine	assim	(+)	(-)	(+)	(+)	n	(-)
d-glucosamine	assim	(+)	(-)	(+)	(+)	n	(-)

α-d-glucose	assim	(+)	(+)	(+)	(+)	(+)	(+)
d-galactose	assim	(+)	(-)	(-)	(+)	(+)	(+)
d-psicose	assim	w	n	n	n	n	w
l-rhamnose	assim	(-)	(+)	(-)	(-)	(-)	(-)
l-sorbose	assim	w	(-)	(-)	(+)	n	(-)
α-methyl-d-glucoside	assim	(+)	(+)	(-)	(+)	(-)	(-)
β-methyl-d-gluoside	assim	(+)	n	n	n	(-)	(-)
amygdalin	assim	(-)	n	n	n	n	(+)
arbutin	assim	(+)	n	n	(+)	n	w
salicin	assim	(+)	(+)	(-)	(+)	v	(-)
maltitol	assim	(+)	n	n	n	n	(-)
d-mannitol	assim	(+)	(+)	(-)	(+)	(+)	(-)
d-sorbitol (d-glucitol)	assim	(+)	(+)	(-)	(+)	(-)	(-)
adonitol	assim	(+)	n	n	n	n	(-)
d-arabitol	assim	(+)	n	n	n	n	(-)
xylitol	assim	(-)	n	n	(+)	n	(-)
i-erythritol	assim	(-)	(-)	(-)	(-)	(-)	(-)
glycerol	assim	(+)	(+)	(+)	(+)	(-)	w
tween 80	assim	(-)	n	n	n	n	(+)
l-arabinose	assim	(-)	(-)	(-)	(-)	(-)	(-)
d-arabinose	assim	(-)	(-)	(-)	(+)	(-)	(-)
d-ribose	assim	(-)	(-)	(-)	(-)	n	(-)
d-xylose	assim	(-)	(+)	(-)	(+)	(-)	(-)
succinic acid mono-methyl ester plus d-xylose	assim	w	n	n	n	(-)	(-)
n-acetyl-l-glutamic acid plus d-xylose	assim	w	n	n	n	n	(-)
quinic acid plus d-xylose	assim	w	n	n	n	n	w
d-glucuronic acid plus d-xylose	assim	w	n	n	n	(-)	(+)
dextrin plus d-xylose	assim	w	n	n	n	n	(-)
α-d-lactose plus d-xylose	assim	w	n	n	n	(-)	w
d-melibiose plus d-xylose	assim	w	n	n	n	n	(-)
d-galactose plus d-xylose	assim	(+)	n	n	n	n	(+)
m-inositol plus d-xylose	assim	w	n	n	n	(-)	(-)

1,2-propanediol plus d-xylose	assim	w	n	n	n	n	(-)
acetoin plus d-xylose	assim	w	n	n	n	n	(-)

Table A.1A: Oxidation and assimilation tests for all yeast populations. Results were compiled from the Biolog results and the following references (Kurtzman et al. 2011; Ramirez & Gonzalez 1984; Nagahama et al. 1999; Ganter et al. 2000; Yamada et al. 1995). Ox= oxidation, assim= assimilation. (+) positive, (-) negative, w= weak, v= variable and n= no data.

A.2 Experimental Evolution Plate Plan

PLATE ONE

H	G	F	E	D	C	B	A	
Blank	A1.1	Blank	Blank	Blank	B5.1	Blank	Blank	1
Blank	Blank	A1.2	Blank	Blank	Blank	B5.2	Blank	2
Blank	Blank	Blank	A1.3	Blank	Blank	Blank	B5.3	3
Blank	A8.1	Blank	Blank	Blank	C10.1	Blank	Blank	4
Blank	Blank	A8.2	Blank	Blank	Blank	C10.2	Blank	5
Blank	Blank	Blank	A8.3	Blank	Blank	Blank	C10.3	6
Blank	A11.1	Blank	Blank	Blank	F8.1	Blank	Blank	7
Blank	Blank	A11.2	Blank	Blank	Blank	F8.2	Blank	8
Blank	Blank	Blank	A11.3	Blank	Blank	Blank	F8.3	9
Blank	B3.1	Blank	Blank	Blank	Sce2.1	Blank	Blank	10
Blank	Blank	B3. 2	Blank	Blank	Blank	Sce2.2	Blank	11
Blank	Blank	Blank	B3.3	Blank	Blank	Blank	Sce2.3	12

PLATE TWO

H	G	F	E	D	C	B	A	
Blank	A1.4	Blank	Blank	Blank	B5.4	Blank	Blank	1
Blank	Blank	A1.5	Blank	Blank	Blank	B5.5	Blank	2
Blank	Blank	Blank	A1.6	Blank	Blank	Blank	B5.6	3
Blank	A8.4	Blank	Blank	Blank	C10.4	Blank	Blank	4
Blank	Blank	A8.5	Blank	Blank	Blank	C10.5	Blank	5
Blank	Blank	Blank	A8.6	Blank	Blank	Blank	C10.6	6
Blank	A11.4	Blank	Blank	Blank	F8.4	Blank	Blank	7
Blank	Blank	A11.5	Blank	Blank	Blank	F8.5	Blank	8
Blank	Blank	Blank	A11.6	Blank	Blank	Blank	F8. 6	9
Blank	B3.4	Blank	Blank	Blank	Sce2.4	Blank	Blank	10
Blank	Blank	B3.5	Blank	Blank	Blank	Sce2.5	Blank	11
Blank	Blank	Blank	B3.6	Blank	Blank	Blank	Sce2.6	12

Table A.2A: Plate plans for experimental evolution experiment. Layout of the 96-well plates for the long-term experimental evolution. Plate one contains lines 1-3 for all populations and the *S. cerevisiae*, separated by blanks to reduce contamination and evaporation. Plate two contains lines 4-6 in the exact same locations. There are six lines for each yeast isolate.

A.3 Events Database Visualisation

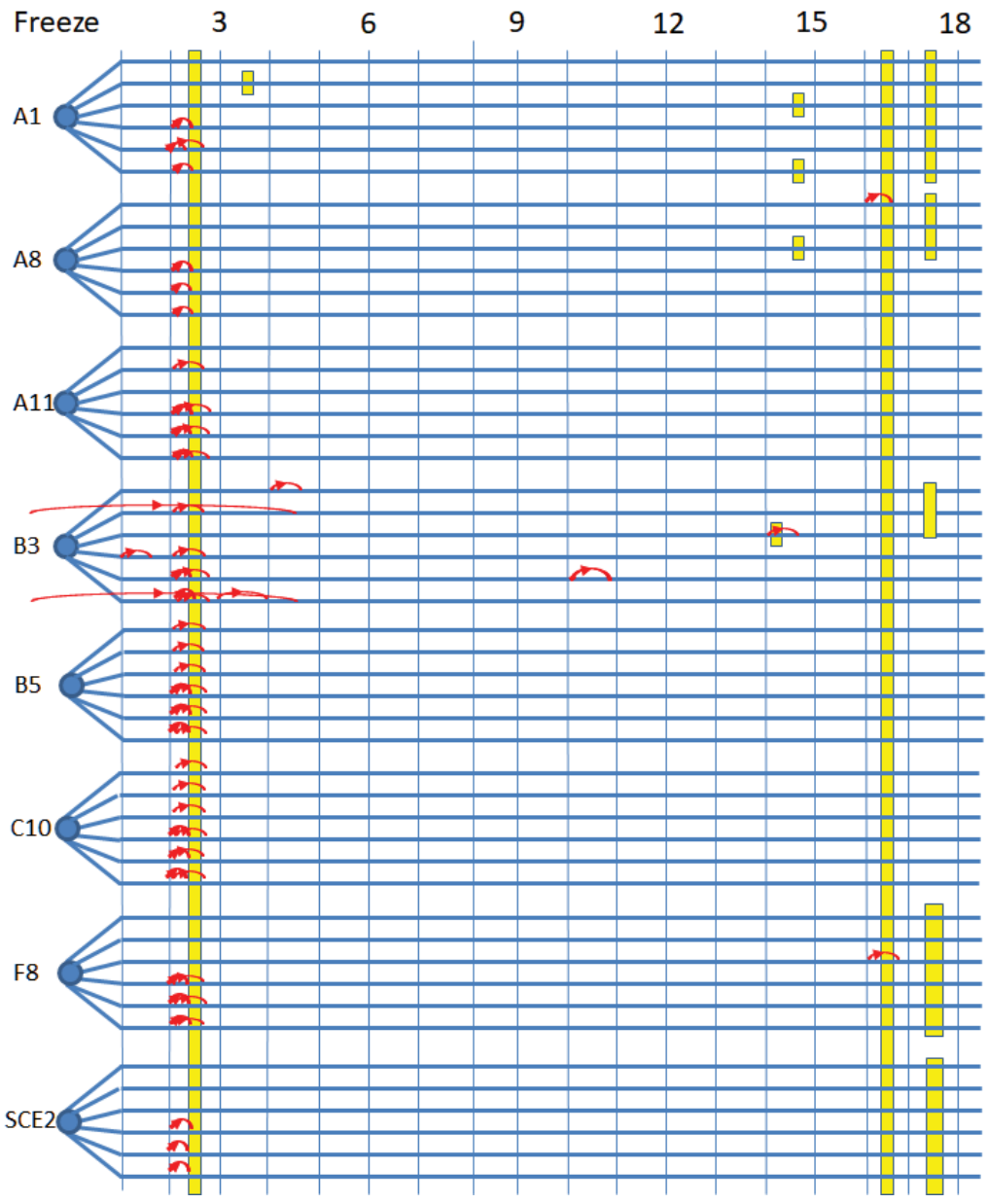

Figure A.3A: Event database visualisation. Each yeast population is labelled at the side. One thick blue line symbolises a line 1-6. Each thin blue line represents a freeze event 1-18 (when the lines were sampled and stored). Red arrows symbolise when a line was contaminated/died and replaced with a previous freeze. Yellow blocks symbolise growth events (mass low growth).

APPENDIX B

B.1 Biolog Results for Ancestors and Reference Species.

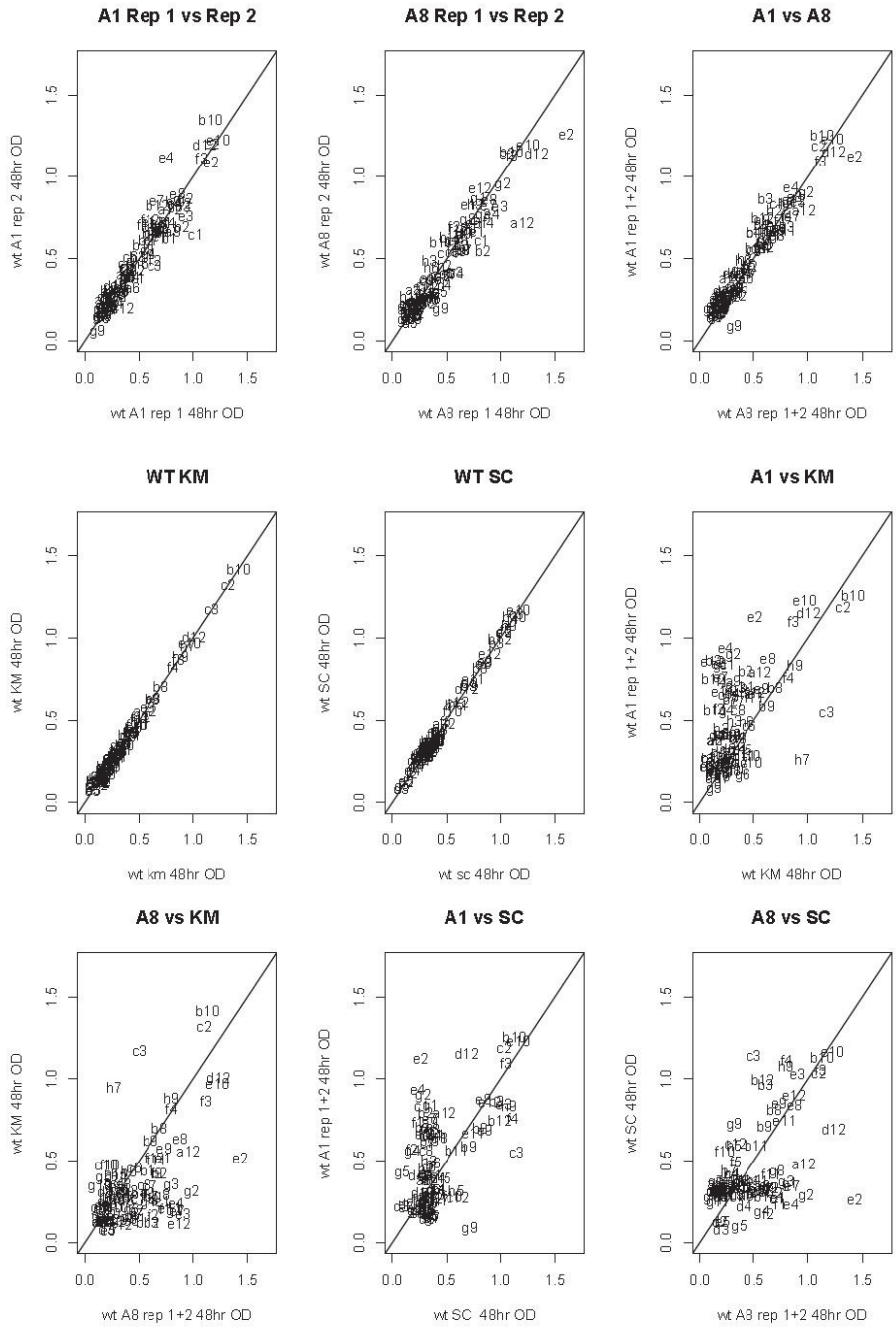

Figure B.1A: Comparisons for Ancestors of A1 and A8 repeats and Reference Species. Repeats of the ancestors were compared for inconsistencies between repeats. The ancestors of A1 and A8 were compared. The reference species repeats were compared for consistency between repeats. The ancestors were then compared with the reference species *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*.

B.2 Biolog Results for A1.

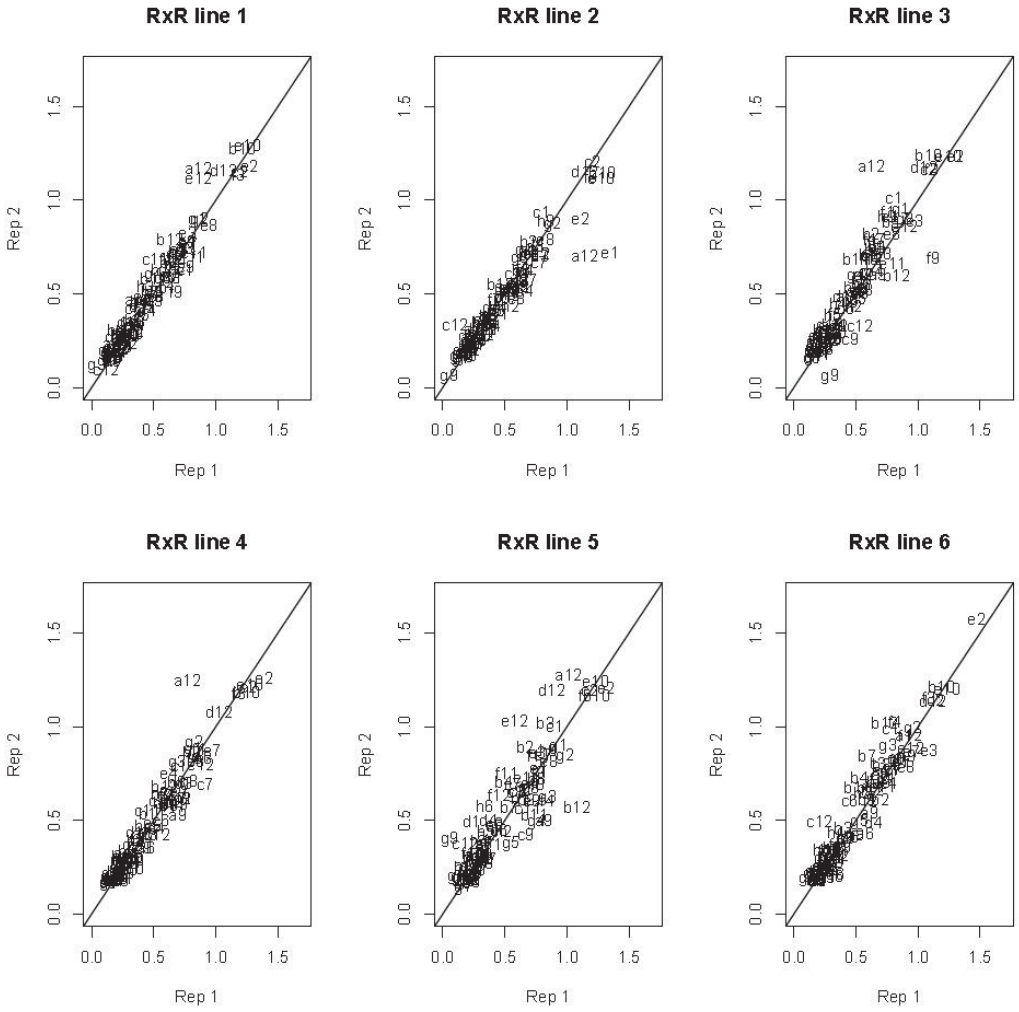

Figure B.2A: Repeat comparison of Ancestor and evolved lines. Looking at A1 evolved, lines A1.1, A1.2, A1.3, A1.4 and A1.5 test A12 is not consistent across replicates suggesting any further analysis of this may be questionable. This is also the case for E2 and E1 (d-cellobiose assimilation) in line A1.2, F9 (β-methyl-d-gluoside assimilation) in A1.3, E12 (turanose assimilation) in lines A1.1 and A1.5, and D12 (inulin assimilation) and B12 (turanose oxidation) in line A1.5.

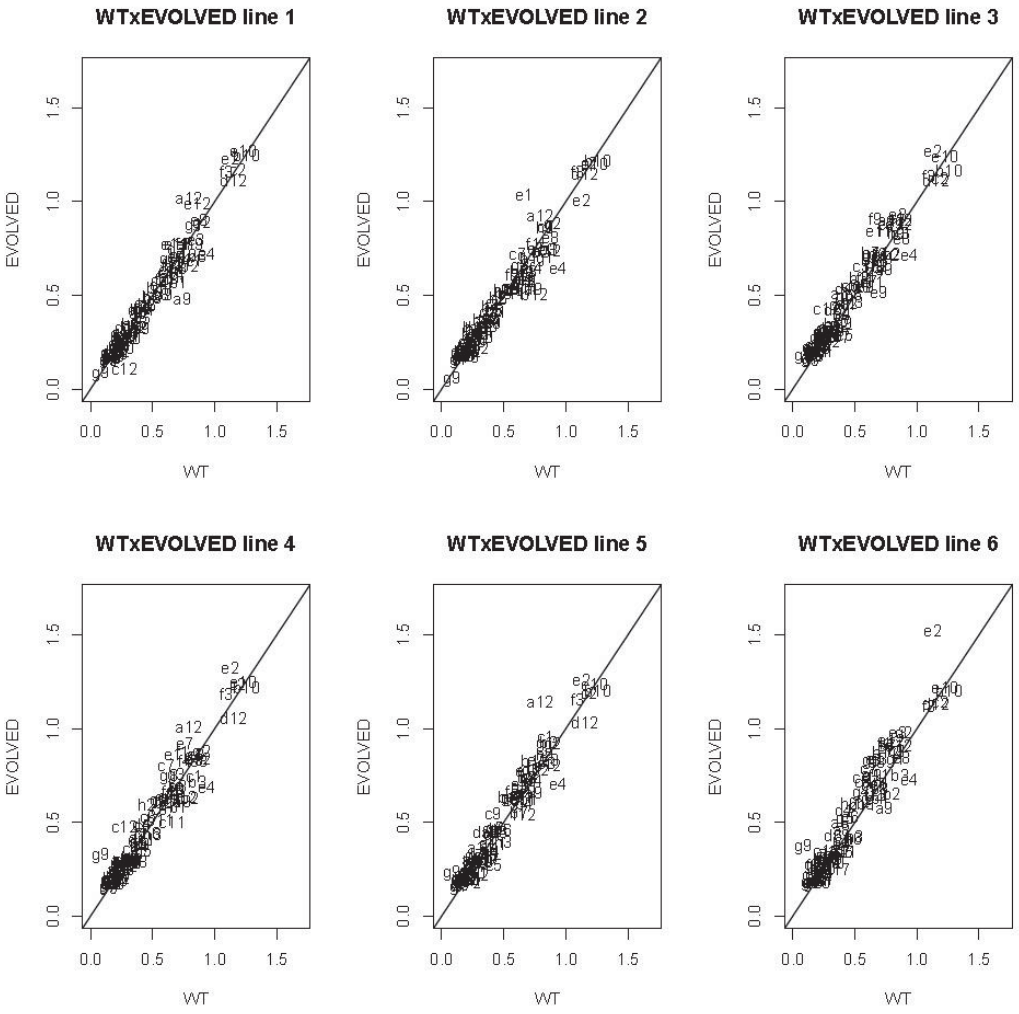

Figure B.2B: Ancestor compared to Evolved lines for A1. Upon comparison of A1 ancestor with A1 evolved lines several tests stand out: line A1.2 E1, line A1.5 A12 and line A1.6 E2. When looking at replicate consistency presented in the previous graph, line A1.5 A12 would not be a reliable difference. Nor would line A1.2 E1. E2 however was both consistent in the A1 ancestor replicates and A1.6 replicates suggesting this may be a notable change, and therefore a possible marker for line A1.6.

B.3 Biolog Results for A8.

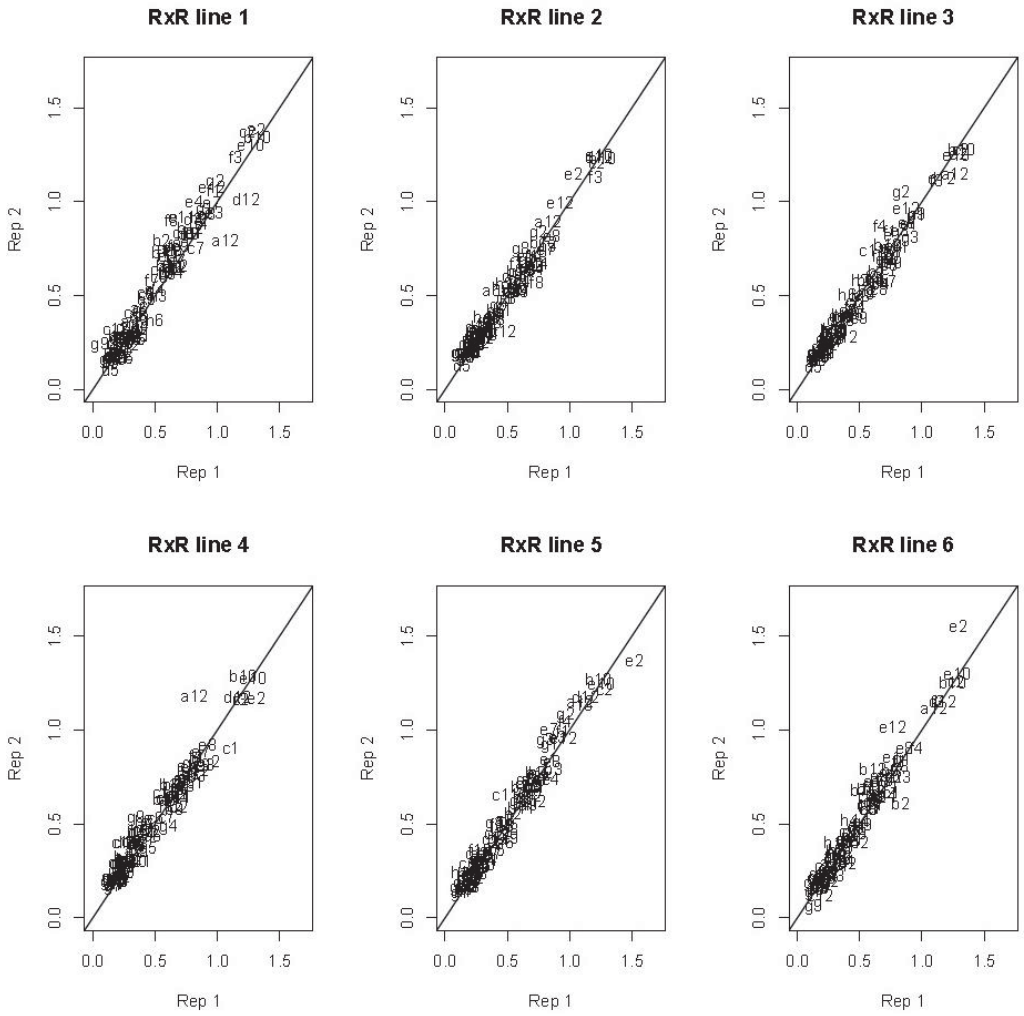

Figure B.3A: Repeat comparison for evolved lines in A8. When comparing replicates of the A8 evolved lines the tests that are brought to attention are: D12 and A12 in line A8.1, G2 (d-mannitol assimilation) in line A8.3, A12 in line A8.4, E2 in line A1.5 (Figure 3.5 E) and lastly E2 and E12 in line A8.6.

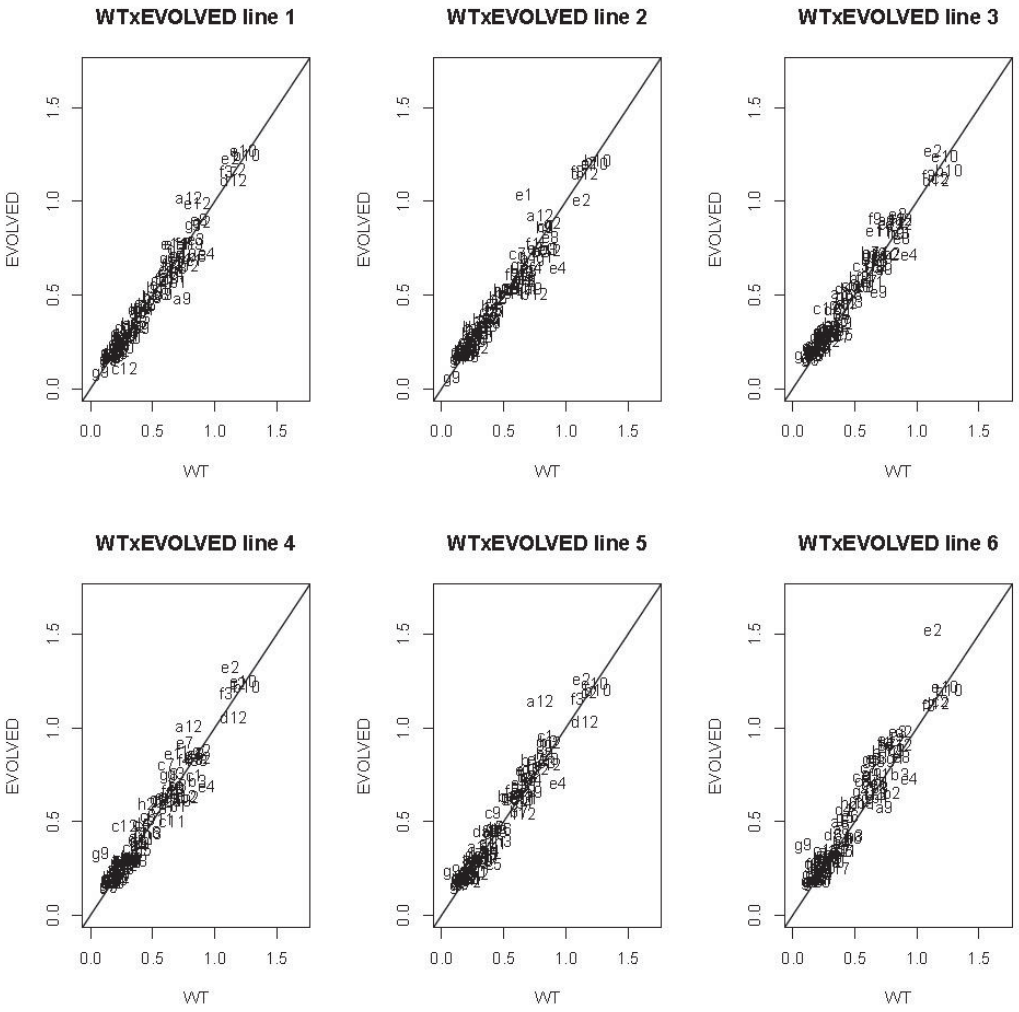

Figure B.3B: Comparison of ancestor with evolved lines of A8. When comparing A8 evolved lines with the ancestor there seem to be only a few notable tests: line A8.2 E1, line A8.5 A12 and A8.4 E2. However, A12 and E2 were tests that showed inconsistencies between the replicates of the ancestor and therefore cannot reliably be classed as a change. E1 however could be a potential marker.

APPENDIX C

C.1 Yeast Population FSC Against Count.

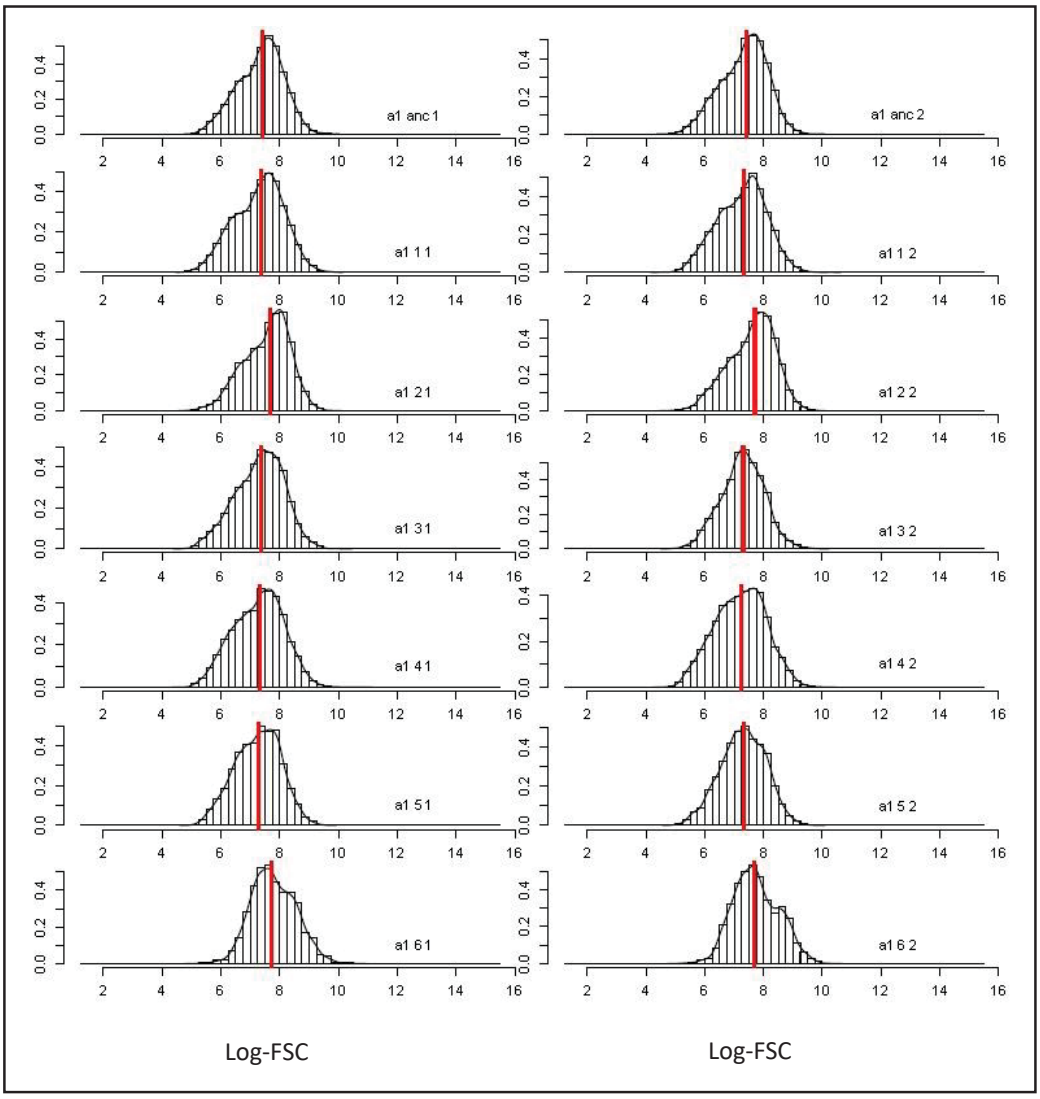

Figure C.1A: A1 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

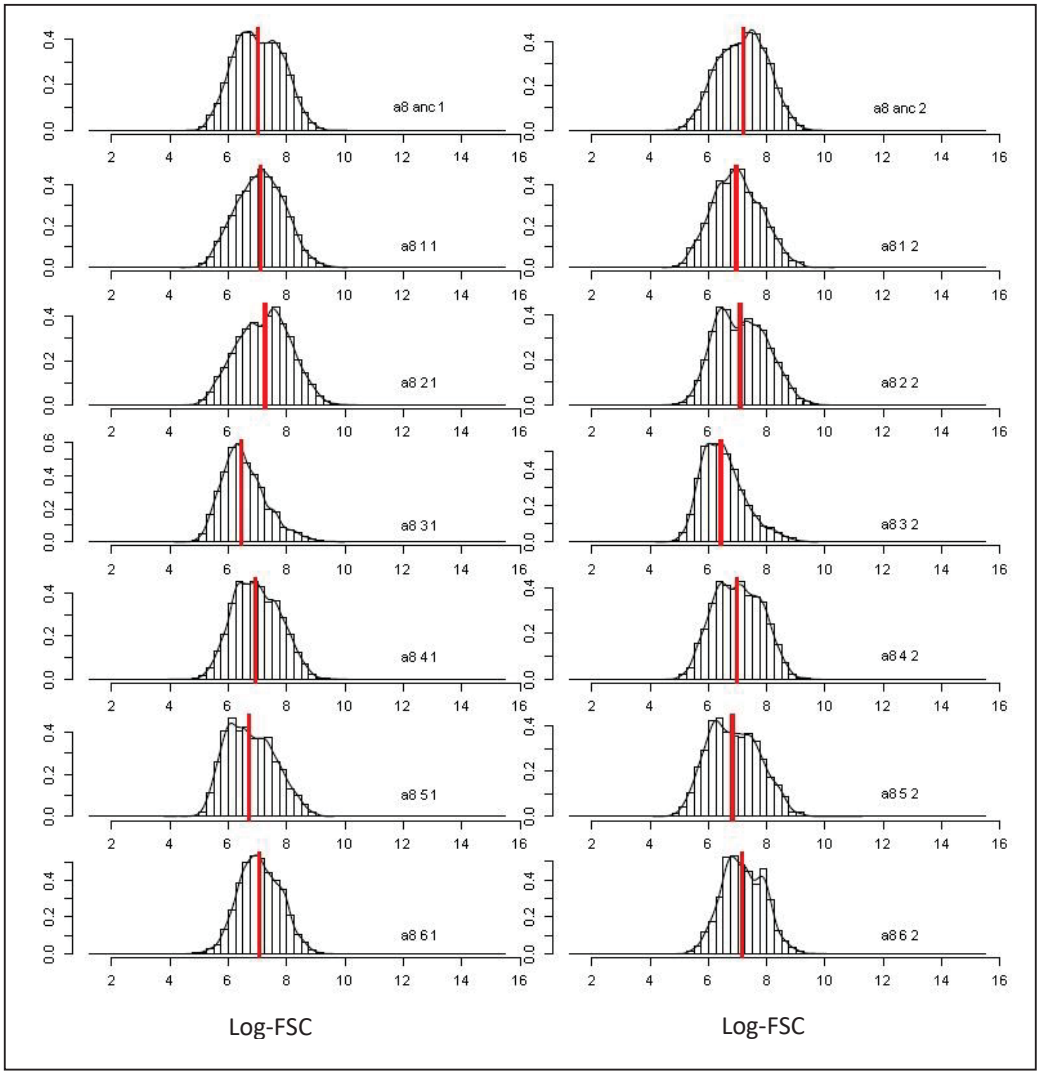

Figure C.1B: A8 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

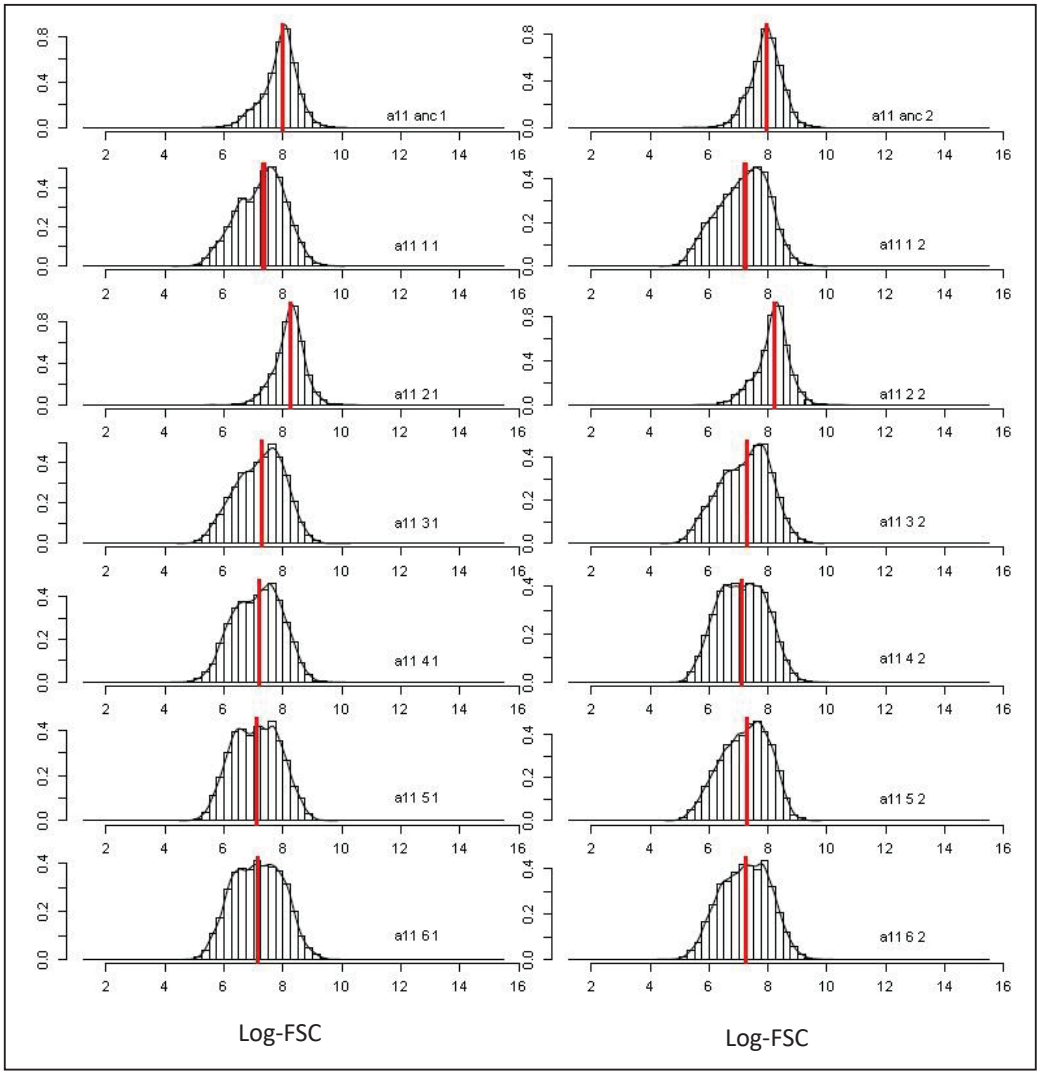

Figure C.1C: A11 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

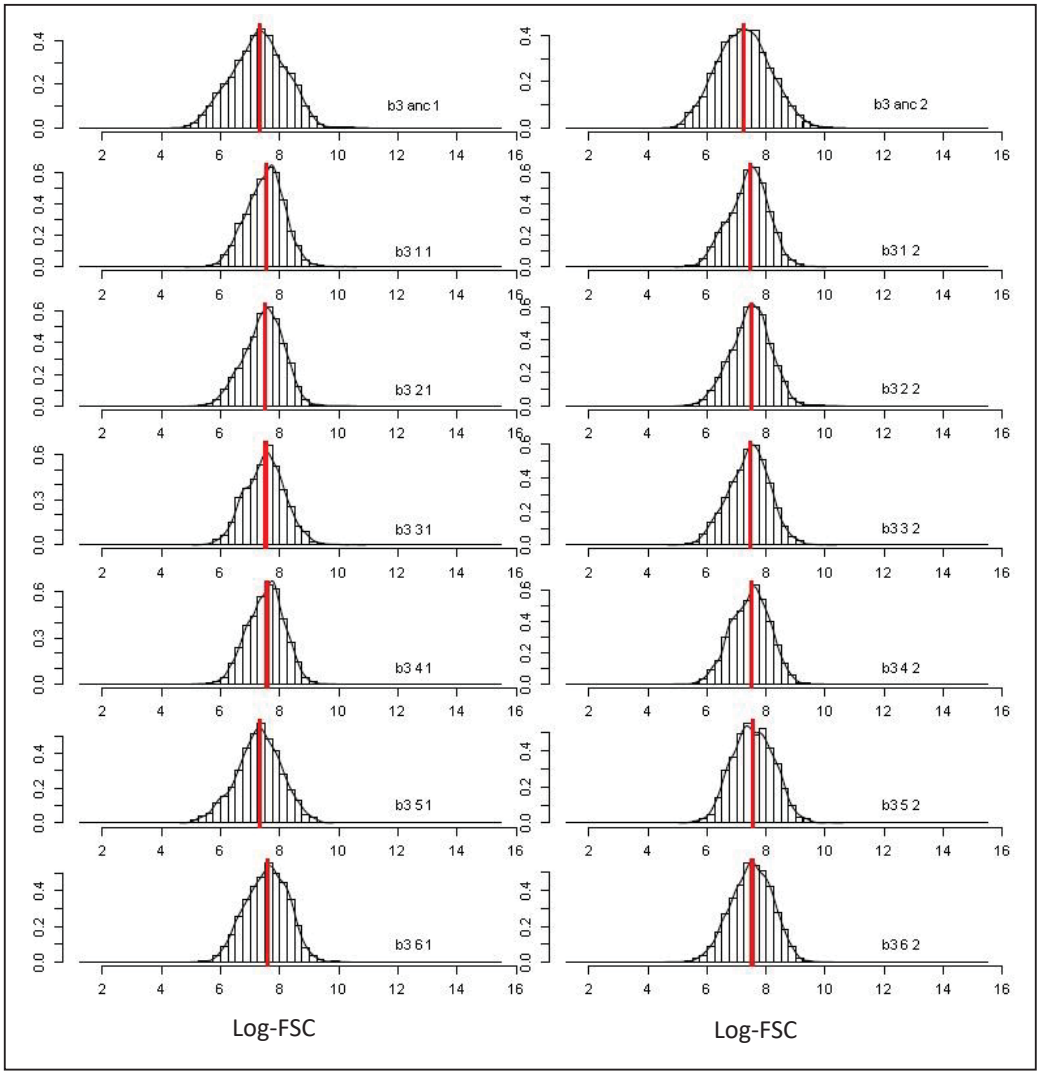

Figure C.1D: B3 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

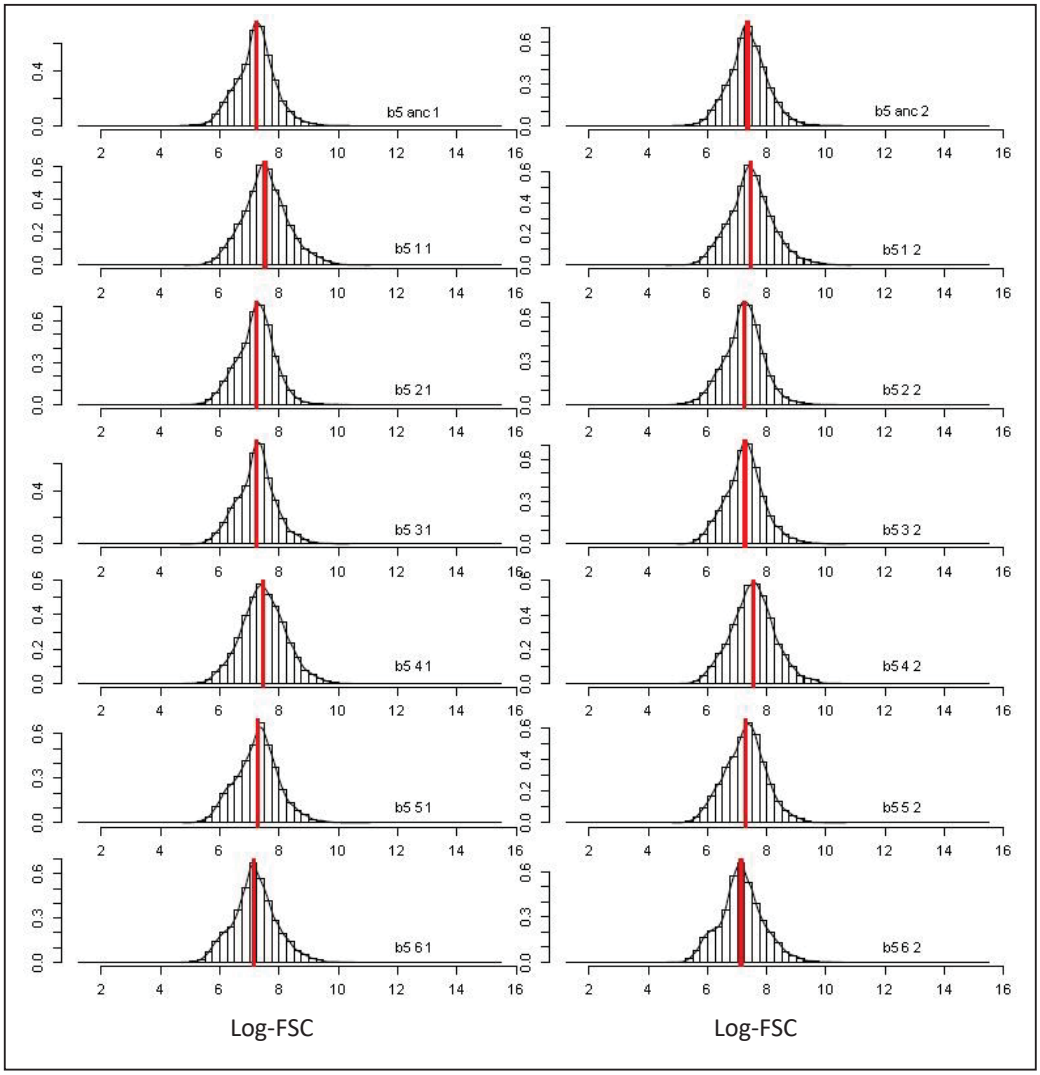

Figure C.1E: B5 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

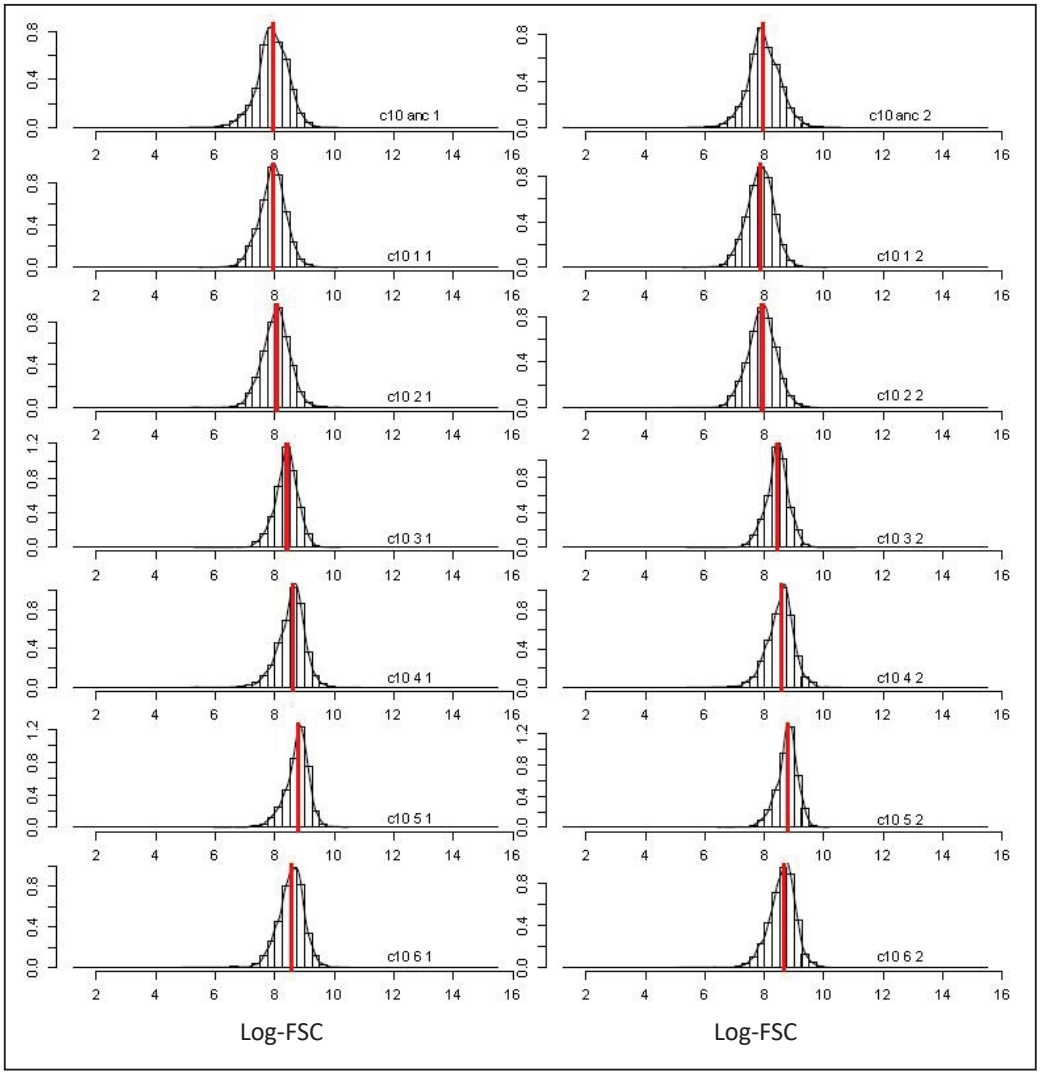

Figure C.1F: C10 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

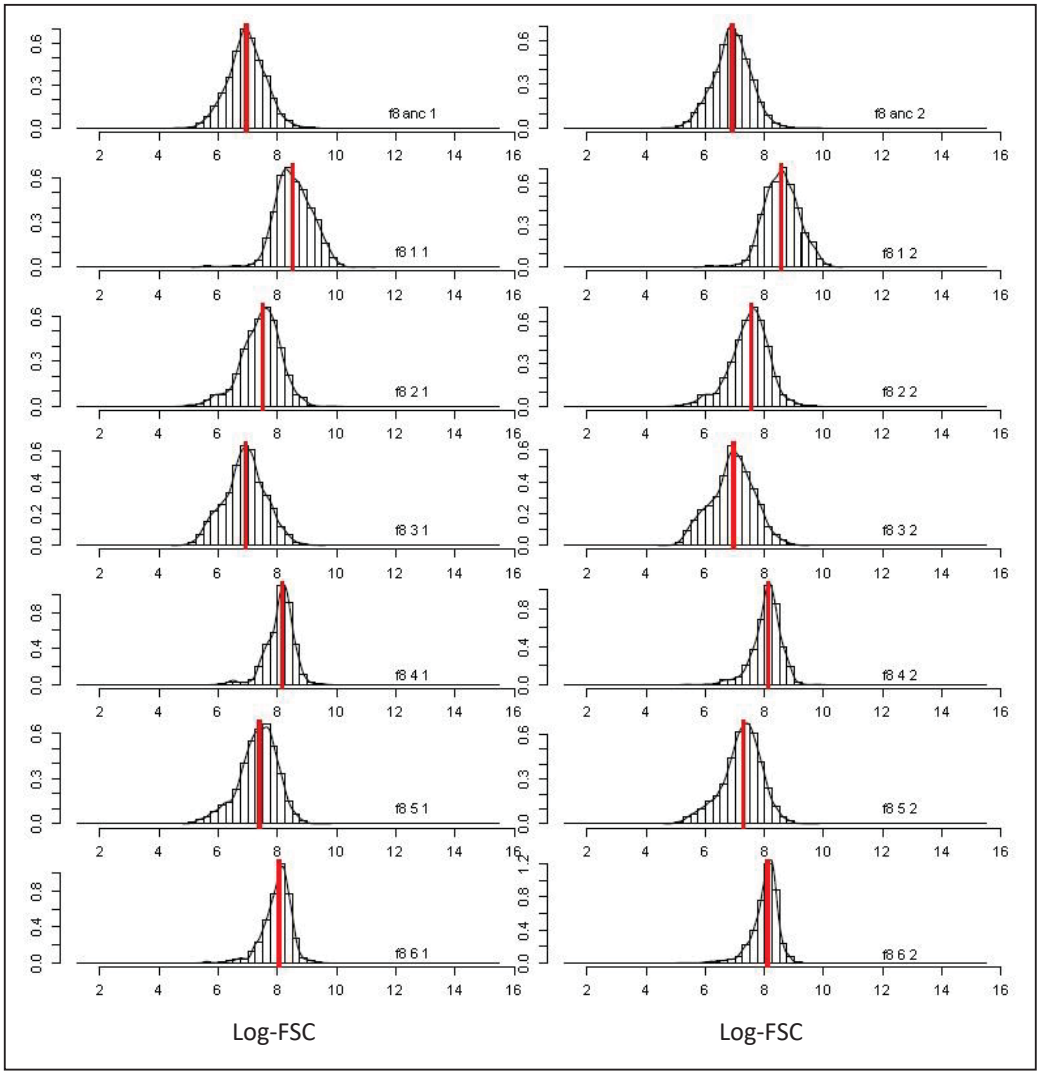

Figure C.1G: F8 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

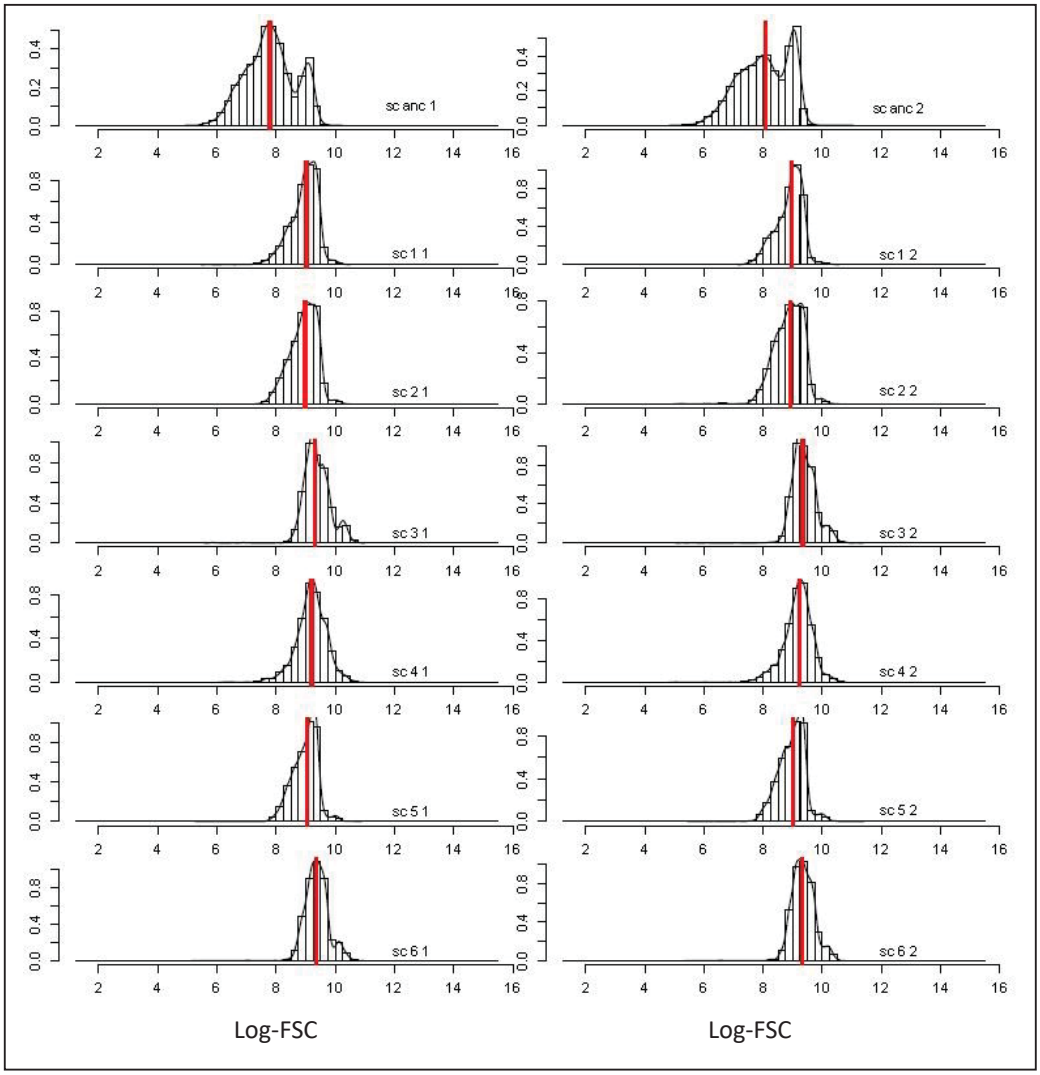

Figure C.1H: SCE2 FSC Output versus Count. Ancestor and evolved lines, repeats 1 and 2.

C.2 FSC to Size Conversion and CFU per 200µl Calculation

Line ID	A1.0	A1.1	A1.2	A1.3	A1.4	A1.5	A1.6
repeat 1	4.322	4.264	4.684	4.269	4.219	4.172	4.773
repeat 2	4.315	4.218	4.729	4.180	4.120	4.204	4.701
Line ID	A8.0	A8.1	A8.2	A8.3	A8.4	A8.5	A8.6
repeat 1	3.903	3.997	4.136	3.581	3.851	3.701	3.942
repeat 2	4.081	3.869	3.974	3.570	3.894	3.777	4.034
Line ID	A11.0	A11.1	A11.2	A11.3	A11.4	A11.5	A11.6
repeat 1	5.298	4.227	6.101	4.167	4.068	3.989	4.033
repeat 2	5.211	4.098	5.962	4.175	4.002	4.153	4.117
Line ID	B3.0	B3.1	B3.2	B3.3	B3.4	B3.5	B3.6
repeat 1	4.190	4.485	4.432	4.450	4.520	4.194	4.559
repeat 2	4.103	4.354	4.420	4.363	4.424	4.472	4.461
Line ID	B5.0	B5.1	B5.2	B5.3	B5.4	B5.5	B5.6
repeat 1	4.111	4.448	4.126	4.121	4.358	4.169	4.040
repeat 2	4.239	4.353	4.118	4.139	4.468	4.177	4.016
Line ID	C10.0	C10.1	C10.2	C10.3	C10.4	C10.5	C10.6
repeat 1	5.179	5.201	5.475	6.643	7.583	8.731	7.403
repeat 2	5.248	5.039	5.160	6.804	7.430	8.689	7.755
Line ID	F8.0	F8.1	F8.2	F8.3	F8.4	F8.5	F8.6
repeat 1	3.867	7.151	4.402	3.838	5.753	4.279	5.491
repeat 2	3.832	7.415	4.470	3.868	5.678	4.174	5.598
Line ID	SCE2.0	SCE2.1	SCE2.2	SCE2.3	SCE2.4	SCE2.5	SCE2.6
repeat 1	4.887	10.601	10.223	14.267	12.647	10.921	14.826
repeat 2	5.519	10.178	9.695	14.482	12.805	10.573	14.304

Table C.2A: FSC Converted to Size in µm. The FSC was converted to size in R using the following calculation: diameter = (exp(peak_loc)+10000)x2/5618x2.

To calculate the CFUs per 200µl of culture one must use the following calculations:

Number of colonies on petri dish x 100000 x2 = 2˜6 x 10e6 ˜ 7

Because working back, the mixture was diluted four times 1/10, and then 1/10 volume was inoculated onto the petri dish giving a x 100000, on top of this only half of the original 200µl culture was taken therefore we must times by 2 to calculate back to the original quantity. This calculation should determine the CFU count for the entire saturated culture.

APPENDIX D

D.1 DAPI Signal Measurement Output

yeast	No. cells	FSC Median	Fraction < 8	L_PEAK	H_PEAK	FSC_MED H	FSC_MED L
b3.0	1820	7.141	0.428	5.582	9.122	7.365	6.792
b3.0	1586	7.029	0.405	5.720	8.820	7.304	6.609
b3.1	1287	7.054	0.672	5.655	9.277	7.671	6.857
b3.1	728	6.904	0.319	5.722	8.904	7.047	6.528
b3.2	1560	6.901	0.661	5.041	8.654	7.230	6.758
b3.2	1144	7.086	0.344	5.604	9.063	7.274	6.730
b3.3	1040	7.357	0.490	5.834	9.254	7.632	6.898
b3.3	793	7.199	0.479	5.689	9.155	7.424	6.945
b3.4	806	7.194	0.818	5.538	NA	NA	7.074
b3.4	715	7.333	0.435	5.324	9.169	7.602	6.976
b3.5	507	7.471	0.830	5.889	NA	NA	7.345
b3.5	585	7.372	0.894	5.917	NA	NA	7.314
b3.6	1274	6.999	0.615	5.283	9.972	7.329	6.750
b3.6	1183	7.040	0.563	5.579	10.122	7.287	6.786
a1.0	832	7.545	0.899	5.825	NA	NA	7.506
a1.0	936	7.569	0.920	5.845	NA	NA	7.514
a1.1	910	7.570	0.901	5.616	NA	NA	7.510
a1.1	1183	7.452	0.956	5.314	NA	NA	7.421
a1.2	962	7.820	0.894	5.817	NA	NA	7.779
a1.2	702	7.757	0.954	5.802	NA	NA	7.743
a1.3	767	7.504	0.890	5.876	NA	NA	7.429
a1.3	845	7.524	0.933	5.772	NA	NA	7.459
a1.4	1157	7.453	0.948	6.202	NA	NA	7.426
a1.4	1157	7.422	0.965	5.932	NA	NA	7.380
a1.5	1404	7.469	0.895	5.799	NA	NA	7.388
a1.5	442	7.306	0.930	5.594	NA	NA	7.264
a1.6	299	7.765	0.906	5.929	NA	NA	7.701
a1.6	247	7.916	0.834	6.383	NA	NA	7.759
f8.0	5279	7.038	0.320	4.243	9.771	7.113	6.879
f8.0	9674	7.111	0.390	4.029	9.583	7.272	6.912
f8.1	1769	7.732	0.336	7.245	10.394	7.914	7.081
f8.1	1820	7.599	0.454	4.941	10.301	8.011	6.789
f8.2	2836	7.032	0.336	7.427	9.271	7.181	6.790
f8.2	8282	6.924	0.456	4.138	9.207	7.105	6.738
f8.3	3121	6.935	0.335	4.864	9.557	7.050	6.672
f8.3	1990	7.138	0.460	7.109	9.713	7.421	6.774
f8.4	1524	7.233	0.489	5.429	10.414	7.773	6.641
f8.4	2034	7.757	0.400	4.649	10.457	8.074	6.734
f8.5	1937	7.173	0.506	5.004	9.606	7.478	6.764
f8.5	5161	6.980	0.372	6.497	9.443	7.157	6.776

f8.6	1599	7.490	0.464	5.412	10.522	7.819	6.894
f8.6	2405	7.446	0.333	5.218	10.085	7.618	6.899
a8.0	793	7.469	0.903	5.992	NA	NA	7.400
a8.0	754	7.468	0.942	5.838	NA	NA	7.420
a8.1	585	7.304	0.901	6.158	NA	NA	7.235
a8.1	650	7.258	0.872	6.219	NA	NA	7.189
a8.2	455	7.436	0.912	5.752	NA	NA	7.407
a8.2	468	7.384	0.893	5.818	NA	NA	7.306
a8.3	273	7.173	0.945	5.343	NA	NA	7.109
a8.3	156	6.959	0.968	5.666	NA	NA	6.993
a8.4	741	7.505	0.964	5.764	NA	NA	7.491
a8.4	286	7.513	0.976	5.906	NA	NA	7.467
a8.5	403	7.304	0.911	6.173	NA	NA	7.174
a8.5	260	7.045	0.904	5.724	NA	NA	6.935
a8.6	208	7.597	0.918	6.016	NA	NA	7.452
a8.6	169	7.678	0.976	6.206	NA	NA	7.650
a11.0	7709	7.855	0.675	5.263	9.519	7.955	7.802
a11.0	4238	7.870	0.421	5.398	9.673	7.918	7.818
a11.1	3367	7.956	0.560	5.467	9.726	8.102	7.802
a11.1	3276	8.062	0.371	5.764	9.903	8.163	7.876
a11.2	1573	8.015	0.302	5.447	9.978	8.096	7.802
a11.2	1508	8.120	0.227	5.261	10.331	8.135	8.065
a11.3	2158	7.947	0.255	5.317	9.864	8.004	7.803
a11.3	2847	7.943	0.362	5.340	9.805	8.005	7.836
a11.4	1495	8.088	0.520	5.602	9.567	8.232	7.934
a11.4	1313	8.057	0.458	5.696	9.895	8.131	7.905
a11.5	1898	8.246	0.454	5.830	9.982	8.360	8.098
a11.5	1469	8.289	0.342	5.852	10.109	8.381	8.107
a11.6	988	8.297	0.290	5.538	10.374	8.365	8.028
a11.6	728	8.230	0.429	5.340	10.150	8.370	7.925
b5.0	2028	7.173	0.262	6.170	9.132	7.241	7.015
b5.0	3354	7.188	0.243	6.186	9.215	7.262	6.984
b5.1	1521	7.278	0.258	6.230	9.446	7.326	7.122
b5.1	2392	7.231	0.381	6.387	9.073	7.275	7.152
b5.2	5252	7.377	0.191	NA	9.525	7.457	NA
b5.2	7501	7.334	0.185	NA	9.194	7.405	NA
b5.3	2873	7.192	0.171	NA	9.124	7.218	NA
b5.3	1885	7.260	0.308	6.178	9.376	7.344	7.130
b5.4	2600	7.281	0.246	6.317	9.806	7.293	7.218
b5.4	1274	7.273	0.317	6.326	9.625	7.315	7.211
b5.5	1131	7.274	0.383	6.280	9.794	7.354	7.148
b5.5	1846	7.308	0.414	6.227	9.481	7.408	7.161
b5.6	1859	7.219	0.342	6.097	9.522	7.330	7.047
b5.6	3016	7.123	0.389	5.761	9.140	7.206	7.021

c10.0	2808	7.910	0.328	6.828	10.579	7.943	7.866
c10.0	1716	7.884	0.371	6.680	10.636	7.927	7.834
c10.1	3211	7.833	0.045	NA	10.982	7.841	NA
c10.1	2548	7.805	0.285	6.255	10.563	7.873	7.652
c10.2	1846	7.946	0.059	NA	10.987	7.961	NA
c10.2	1365	7.895	0.069	NA	10.919	7.910	NA
c10.3	1781	8.326	0.253	6.736	10.885	8.326	8.341
c10.3	1729	8.297	0.174	NA	10.973	8.298	NA
c10.4	1313	8.428	0.552	6.675	10.390	8.564	8.300
c10.4	1092	8.323	0.606	6.825	10.344	8.494	8.163
c10.5	494	8.625	0.385	7.241	10.721	8.725	8.353
c10.5	559	8.639	0.358	7.241	10.633	8.658	8.619
c10.6	1144	8.635	0.132	NA	11.272	8.654	NA
c10.6	663	8.697	0.110	NA	11.409	8.701	NA
sce2.0	1092	8.765	0.679	6.806	9.642	7.824	8.994
sce2.0	676	7.956	0.225	6.842	9.985	7.866	8.824
sce2.1	1794	8.892	0.938	7.338	NA	NA	8.894
sce2.1	2106	8.850	0.949	7.051	NA	NA	8.829
sce2.2	1859	8.884	0.941	7.381	NA	NA	8.867
sce2.2	2067	8.762	0.956	6.735	NA	NA	8.762
sce2.3	832	9.235	0.921	7.141	NA	NA	9.217
sce2.3	1079	9.259	0.905	7.155	NA	NA	9.231
sce2.4	1599	8.984	0.825	7.191	NA	NA	8.979
sce2.4	1170	9.016	0.845	7.327	NA	NA	9.010
sce2.5	1677	8.906	0.919	7.381	NA	NA	8.899
sce2.5	1209	8.868	0.768	7.476	11.128	8.789	8.903
sce2.6	1066	9.231	0.896	7.340	NA	NA	9.202
sce2.6	949	9.161	0.876	7.322	NA	NA	9.149

Table D.1A: Flow Cytometry Output of DAPI Measurements. Presented here are the number of cells measured, the median FSC output, the fraction of measures measured as part of the lower peak, the position of the lower peak, the position of the higher peak, the median FSC of the lower peak and the median FSC of the higher peak.

D.2 SYTOX Signal Measurement Output

yeast	No. cells	FSC Median	Fraction < 8	L_PEAK	H_PEAK	FSC_MED_H	FSC_MED_L
b3.0	2013	7.560	0.516	5.569	8.457	7.757	7.315
b3.0	1991	7.625	0.462	5.637	9.188	7.918	7.287
b3.1	1309	7.611	0.268	5.460	9.877	7.700	7.283
b3.1	1331	7.773	0.428	5.502	9.726	7.942	7.486
b3.2	2805	7.320	0.255	5.198	9.780	7.385	7.074
b3.2	1749	7.589	0.407	5.294	9.802	7.815	7.293
b3.3	4565	7.292	0.185	NA	9.498	7.358	NA
b3.3	2717	7.410	0.195	NA	9.980	7.507	NA
b3.4	5555	7.294	0.438	5.232	9.216	7.390	7.173
b3.4	1441	7.420	0.547	5.379	9.147	7.667	7.187
b3.5	1716	7.448	0.965	5.571	NA	NA	7.428
b3.5	1705	7.478	0.945	5.719	NA	NA	7.450
b3.6	3322	7.096	0.506	5.340	8.993	7.117	7.066
b3.6	3531	6.990	0.315	5.394	9.335	7.021	6.921
a1.0	7282	7.435	0.993	5.695	NA	NA	7.432
a1.0	6039	7.416	0.991	5.664	NA	NA	7.410
a1.1	6083	7.407	0.989	5.571	NA	NA	7.402
a1.1	4631	7.468	0.983	5.550	NA	NA	7.459
a1.2	3377	7.615	0.977	5.771	NA	NA	7.610
a1.2	2508	7.734	0.968	5.993	NA	NA	7.725
a1.3	2673	7.575	0.942	5.675	NA	NA	7.558
a1.3	1364	7.530	0.941	5.740	NA	NA	7.508
a1.4	7469	7.232	0.966	5.754	NA	NA	7.209
a1.4	7084	7.283	0.931	5.545	NA	NA	7.245
a1.5	2277	7.573	0.954	5.731	NA	NA	7.545
a1.5	1815	7.455	0.958	5.707	NA	NA	7.435
a1.6	704	7.942	0.938	6.227	NA	NA	7.906
a1.6	528	7.856	0.953	6.035	NA	NA	7.841
a8.0	7887	7.276	0.982	5.286	NA	NA	7.267
a8.0	7304	7.285	0.982	5.496	NA	NA	7.273
a8.1	2618	7.091	0.977	5.197	NA	NA	7.081
a8.1	2354	7.149	0.966	5.216	NA	NA	7.134
a8.2	2827	7.370	0.940	5.584	NA	NA	7.337
a8.2	1870	7.304	0.970	5.467	NA	NA	7.278
a8.3	693	7.210	0.984	5.302	NA	NA	7.204
a8.3	1078	7.218	0.970	5.667	NA	NA	7.216
a8.4	6325	7.306	0.944	5.662	NA	NA	7.272
a8.4	5731	7.330	0.982	5.712	NA	NA	7.319
a8.5	1771	7.411	0.899	5.710	NA	NA	7.371
a8.5	1155	7.190	0.899	5.431	NA	NA	7.120

a8.6	913	7.607	0.929	5.762	NA	NA	7.546
a8.6	1221	7.478	0.972	5.702	NA	NA	7.471
a11.0	21835	7.987	0.745	5.420	9.402	8.056	7.961
a11.0	20526	7.975	0.752	5.390	10.438	8.007	7.966
a11.1	9427	8.047	0.463	5.503	9.173	8.153	7.913
a11.1	6710	8.074	0.316	5.541	9.732	8.137	7.932
a11.2	5005	8.145	0.438	5.777	9.299	8.240	8.030
a11.2	7051	8.059	0.574	5.513	8.302	8.174	7.967
a11.3	10527	7.972	0.497	5.667	9.422	8.038	7.905
a11.3	8063	7.962	0.399	5.691	9.210	8.020	7.880
a11.4	9757	8.150	0.700	5.535	9.114	8.247	8.108
a11.4	6017	8.106	0.578	5.479	9.025	8.194	8.039
a11.5	7447	8.248	0.428	5.625	9.620	8.364	8.090
a11.5	13024	8.265	0.488	5.673	10.782	8.370	8.146
a11.6	11253	8.027	0.286	5.537	10.453	8.088	7.870
a11.6	9009	8.046	0.374	5.480	10.467	8.116	7.927
b5.0	10186	7.118	0.169	NA	9.784	7.179	NA
b5.0	10769	7.093	0.213	5.138	9.531	7.164	6.900
b5.1	7546	7.243	0.321	5.363	9.829	7.341	7.076
b5.1	6787	7.313	0.330	5.276	9.927	7.397	7.171
b5.2	5324	7.143	0.234	7.156	9.253	7.234	6.895
b5.2	6644	7.214	0.306	5.173	9.504	7.331	6.996
b5.3	5797	7.246	0.404	5.303	9.581	7.354	7.144
b5.3	4598	7.234	0.322	5.461	9.409	7.297	7.125
b5.4	4917	7.248	0.204	5.271	10.562	7.304	7.060
b5.4	4532	7.226	0.178	NA	10.832	7.263	NA
b5.5	4961	7.394	0.188	NA	10.808	7.482	NA
b5.5	7029	7.483	0.174	NA	11.269	7.558	NA
b5.6	3058	7.068	0.188	NA	9.668	7.130	NA
b5.6	2387	7.104	0.182	NA	10.035	7.176	NA
c10.0	5610	7.881	0.770	5.286	11.123	7.859	7.884
c10.0	5522	7.961	0.646	5.467	10.429	7.967	7.956
c10.1	5577	7.880	0.559	5.750	9.669	7.905	7.859
c10.1	3663	7.922	0.495	5.680	10.359	7.949	7.891
c10.2	12639	7.950	0.482	5.759	10.553	7.904	7.995
c10.2	9900	7.947	0.368	5.751	11.515	7.949	7.944
c10.3	1705	8.341	0.735	5.791	10.345	8.107	8.378
c10.3	4081	8.498	0.781	5.770	11.502	8.461	8.515
c10.4	5258	8.120	0.609	5.682	10.962	8.149	8.100
c10.4	3300	8.119	0.454	5.848	11.648	8.118	8.120
c10.5	1837	8.636	0.694	6.036	10.263	8.595	8.651
c10.5	1925	8.682	0.620	6.062	11.499	8.674	8.685
c10.6	1595	8.472	0.586	6.058	10.715	8.418	8.508
c10.6	1628	8.615	0.560	6.128	10.243	8.597	8.628

f8.0	3036	8.281	0.162	NA	12.442	8.326	NA
f8.0	5181	8.365	0.136	NA	12.460	8.406	NA
f8.1	3179	8.594	0.166	NA	12.466	8.693	NA
f8.1	2772	8.491	0.247	6.550	12.450	8.625	7.959
f8.2	2453	8.025	0.202	6.182	12.412	8.176	7.321
f8.2	4576	8.157	0.176	NA	12.393	8.307	NA
f8.3	1067	8.335	0.183	NA	12.364	8.493	NA
f8.3	1254	8.325	0.171	NA	12.367	8.475	NA
f8.4	3300	8.285	0.156	NA	12.459	8.388	NA
f8.4	2255	8.245	0.175	NA	12.443	8.361	NA
f8.5	1353	7.993	0.245	5.881	11.539	8.172	7.346
f8.5	3388	8.093	0.239	6.161	11.544	8.238	7.530
f8.6	3278	8.472	0.140	NA	12.358	8.527	NA
f8.6	2585	8.387	0.167	NA	12.396	8.453	NA
sce2.0	3454	8.457	0.880	6.698	NA	NA	8.514
sce2.0	3487	8.489	0.862	6.570	NA	NA	8.520
sce2.1	2728	8.769	0.728	6.990	12.329	8.652	8.825
sce2.1	2024	8.781	0.676	6.993	11.721	8.745	8.799
sce2.2	2354	8.745	0.688	6.858	12.399	8.587	8.802
sce2.2	1859	8.664	0.680	6.747	11.536	8.571	8.691
sce2.3	946	9.030	0.658	7.154	12.415	8.954	9.052
sce2.3	1672	9.213	0.642	7.285	12.039	9.346	9.163
sce2.4	814	9.045	0.598	7.282	11.980	9.110	9.029
sce2.4	539	8.957	0.677	6.944	11.623	8.787	9.008
sce2.5	1496	8.791	0.799	6.832	12.267	8.617	8.826
sce2.5	1364	8.728	0.569	6.772	12.433	8.583	8.841
sce2.6	1100	9.176	0.559	7.243	12.221	9.433	9.084
sce2.6	1309	9.211	0.620	7.333	7.659	9.389	9.121

Table D.2A: Flow Cytometry Output of SYTOX Measurements. Presented here are the number of cells measured, the median FSC output, the fraction of measures measured as part of the lower peak, the position of the lower peak, the position of the higher peak, the median FSC of the lower peak and the median FSC of the higher peak.

D.3 A1 DAPI and SYTOX Graphical Output and Statistical Output.

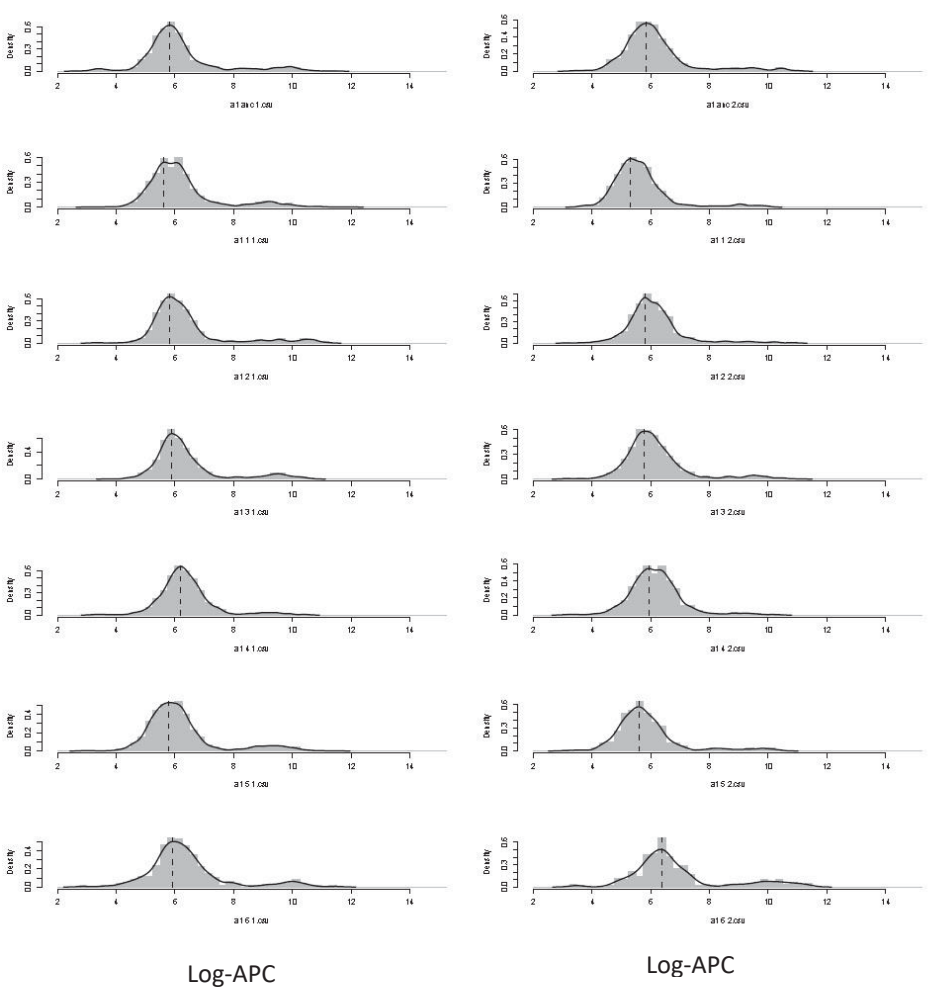

Figure D.3A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of A1.

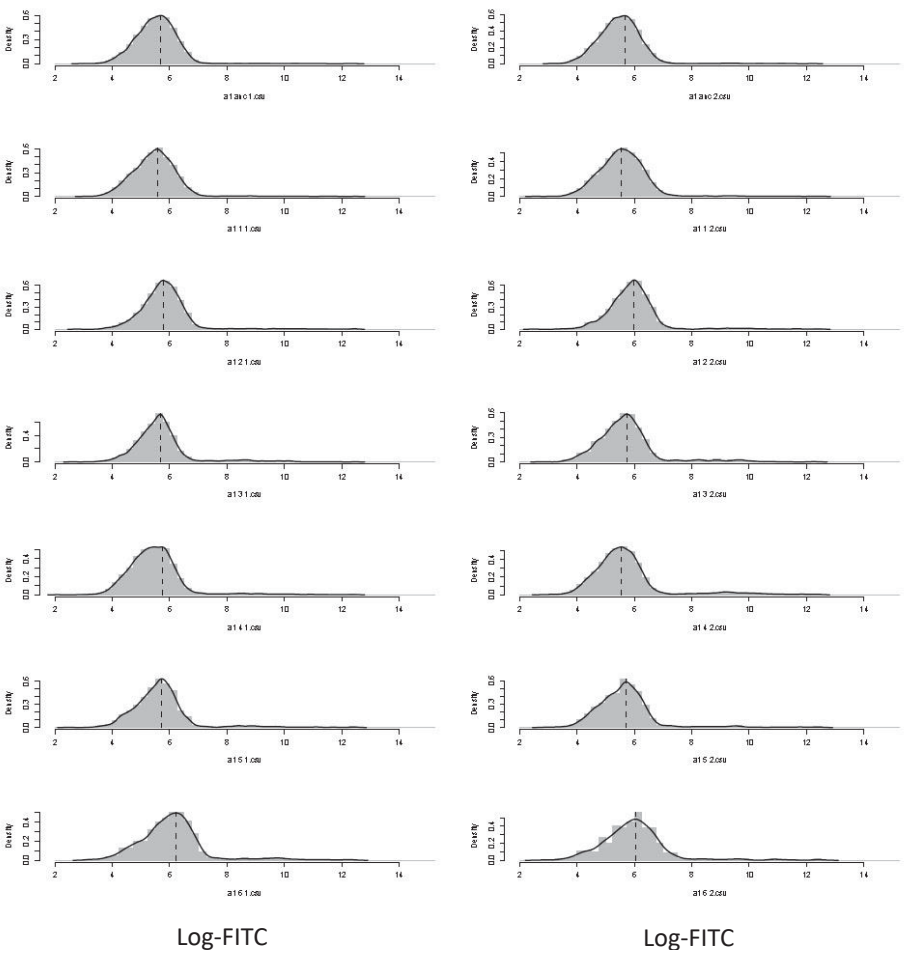

Figure D.3B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of A1.

DAPI	A1	0	1	2	3	4	5	6
Fraction lower peak	Estimate	**0.909**	0.019	0.015	0.002	0.047	0.003	-0.039
	T-value		0.572	0.441	0.062	1.404	0.083	-1.176
	P-value		0.585	0.672	0.953	0.203	0.936	0.278
Position of lower peak	Estimate	**5.835**	-0.370	-0.025	-0.011	0.232	-0.139	0.321
	T-value		-2.129	-0.145	-0.061	1.331	-0.796	1.842
	P-value		0.071	0.889	0.953	0.225	0.452	0.108
Position of higher peak	Estimate	NA	NA	NA	NA	NA	NA	NA
	T-value		NA	NA	NA	NA	NA	NA
	P-value		NA	NA	NA	NA	NA	NA
SYTOX	**A1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Fraction lower peak	Estimate	**0.992**	-0.006	-0.019	**-0.051**	**-0.043**	**-0.036**	**-0.047**
	T-value		-0.587	-1.799	**-4.728**	**-4.048**	**-3.375**	**-4.392**
	P-value		0.575	0.115	**0.002**	**0.005**	**0.012**	**0.003**
Position of lower peak	Estimate	**5.679**	-0.119	0.203	0.028	-0.030	0.040	**0.451**
	T-value		-1.208	2.058	0.284	-0.302	0.402	**4.583**
	P-value		0.266	0.079	0.785	0.772	0.699	**0.003**
Position of higher peak	Estimate	NA	NA	NA	NA	NA	NA	NA
	T-value		NA	NA	NA	NA	NA	NA
	P-value		NA	NA	NA	NA	NA	NA

Table D.3C: A1 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.4 A8 DAPI and SYTOX Graphical Output and Statistical Output.

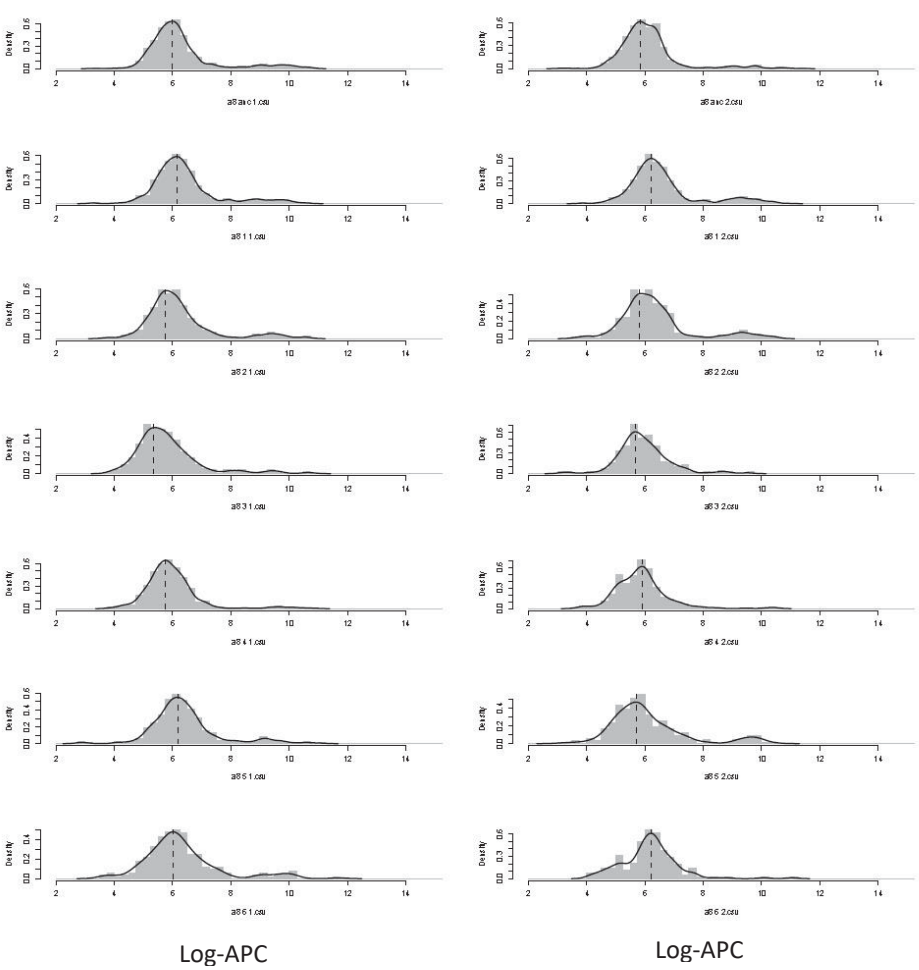

Figure D.4A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of A8.

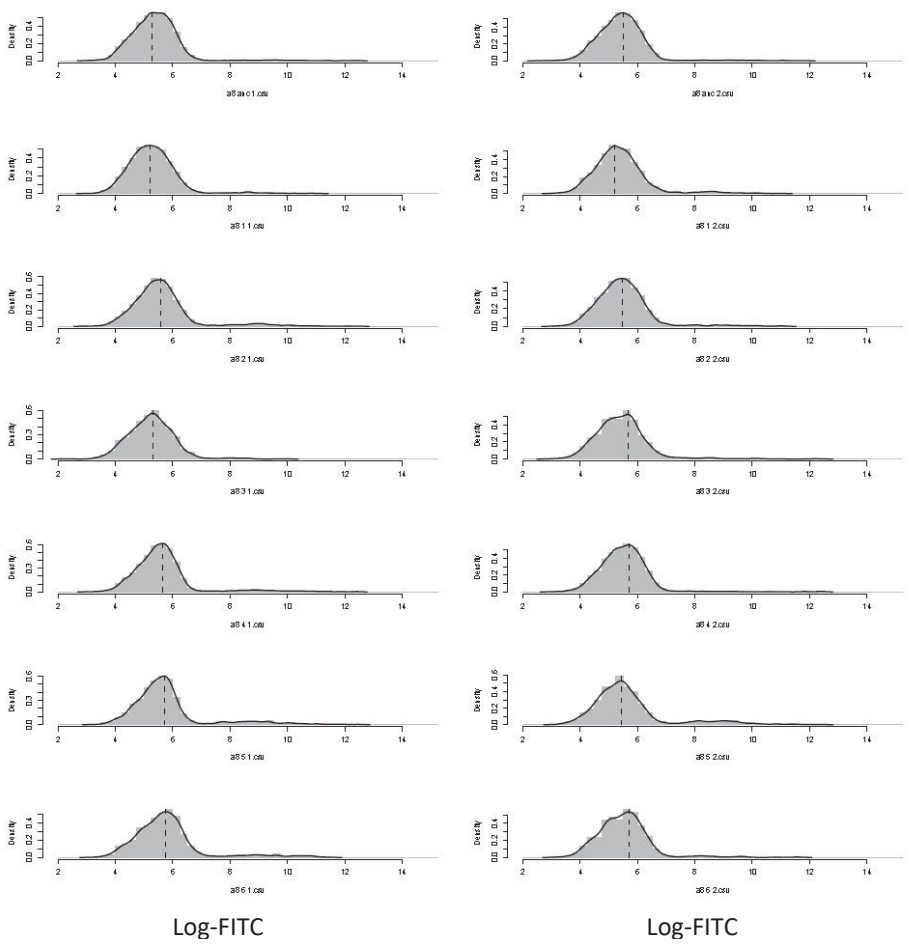

Figure D.4B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of A8.

DAPI	A8	0	1	2	3	4	5	6
Fraction lower peak	Estimate	**0.922**	-0.036	-0.020	0.034	0.047	-0.015	0.025
	T-value		-1.624	-0.894	1.558	2.151	-0.683	1.139
	P-value		0.148	0.401	0.163	0.068	0.516	0.292
Position of lower peak	Estimate	**5.915**	0.274	-0.130	**-0.411**	-0.081	0.033	0.196
	T-value		1.633	-0.777	**-2.450**	-0.481	0.198	1.169
	P-value		0.146	0.463	**0.044**	0.645	0.849	0.281
Position of higher peak	Estimate	NA	NA	NA	NA	NA	NA	NA
	T-value		NA	NA	NA	NA	NA	NA
	P-value		NA	NA	NA	NA	NA	NA
SYTOX	A8	0	1	2	3	4	5	6
Fraction lower peak	Estimate	**0.982**	-0.010	-0.027	-0.005	-0.019	**-0.083**	-0.031
	T-value		-0.568	-1.518	-0.264	-1.059	**-4.613**	-1.753
	P-value		0.588	0.173	0.799	0.325	**0.002**	0.123
Position of lower peak	Estimate	**5.391**	-0.184	0.135	0.094	0.296	0.180	**0.341**
	T-value		-1.314	0.960	0.668	2.108	1.281	**2.429**
	P-value		0.230	0.369	0.525	0.073	0.241	**0.046**
Position of higher peak	Estimate	NA	NA	NA	NA	NA	NA	NA
	T-value		NA	NA	NA	NA	NA	NA
	P-value		NA	NA	NA	NA	NA	NA

Table D.4C: A8 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.5 A11 DAPI and SYTOX Graphical Output and Statistical Output.

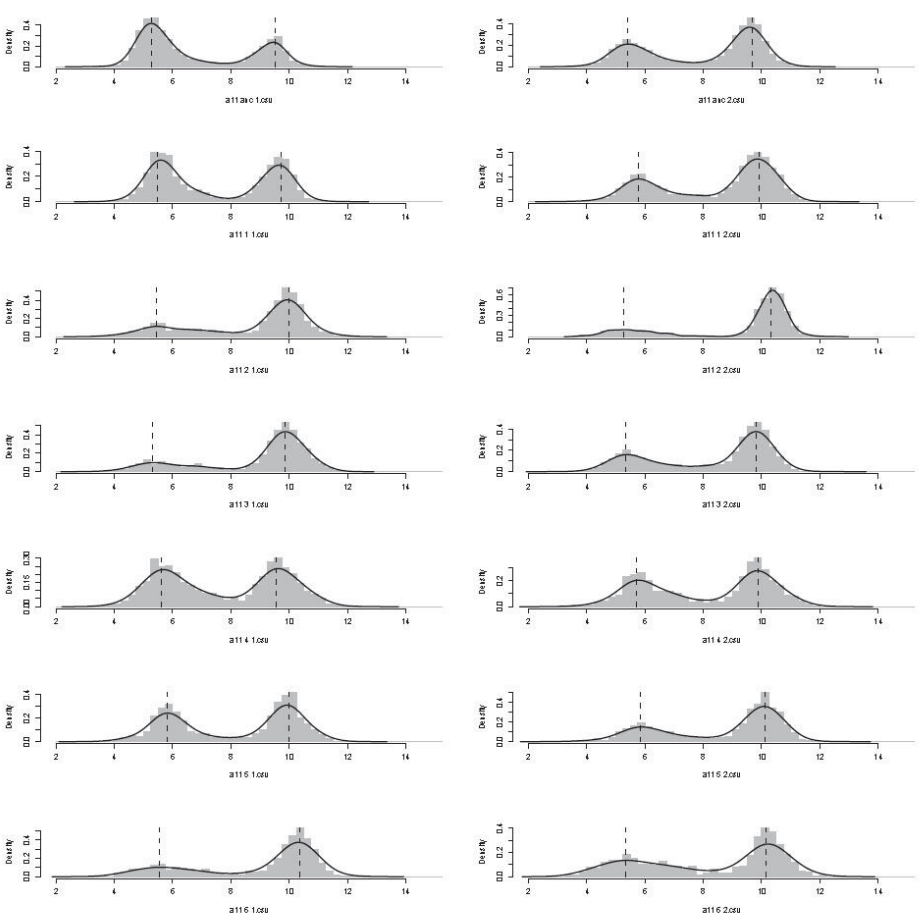

Figure D.5A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of A11.

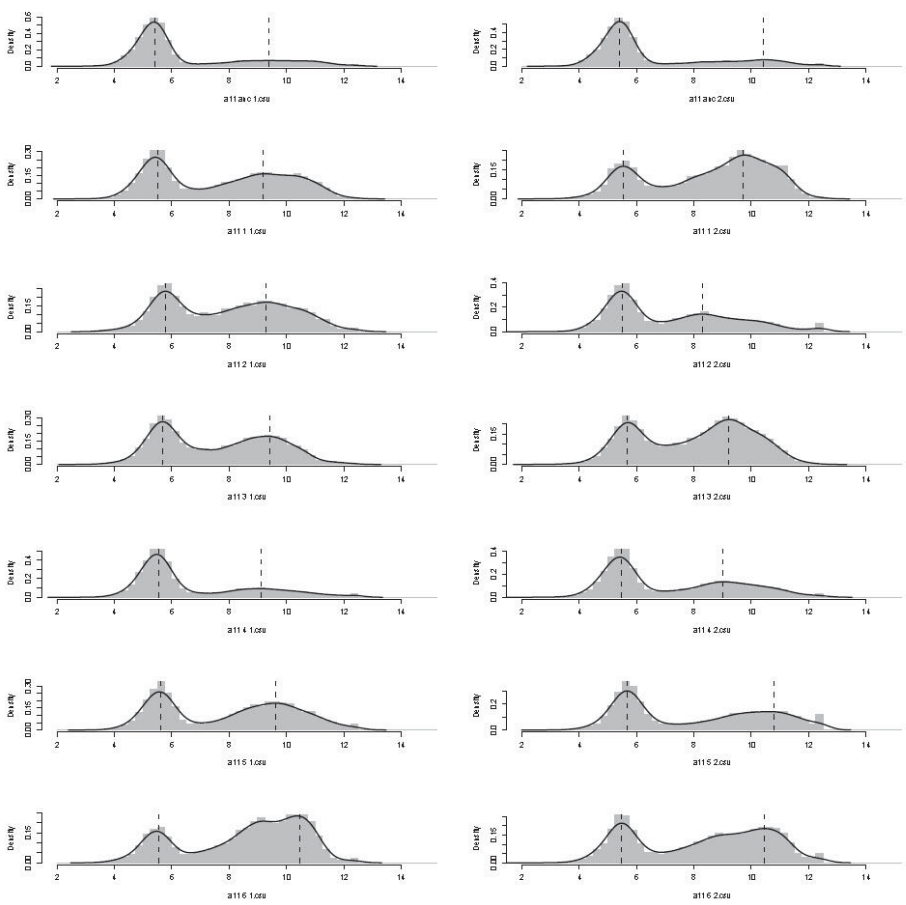

Figure D.5B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of A11.

DAPI	A11	0	1	2	3	4	5	6
Fraction lower peak	Estimate	**0.548**	-0.082	**-0.284**	-0.239	-0.058	-0.150	-0.188
	T-value		-0.791	**-2.720**	-2.295	-0.561	-1.438	-1.807
	P-value		0.455	**0.030**	0.055	0.592	0.194	0.114
Position of lower peak	Estimate	**5.330**	**0.285**	0.024	-0.002	**0.319**	**0.511**	0.109
	T-value		**2.450**	0.202	-0.017	**2.734**	**4.386**	0.934
	P-value		**0.044**	0.846	0.987	**0.029**	**0.003**	0.382
Position of higher peak	Estimate	**9.596**	0.218	**0.558**	0.239	0.135	**0.449**	**0.666**
	T-value		1.368	**3.495**	1.494	0.847	**2.814**	**4.172**
	P-value		0.214	**0.010**	0.179	0.425	**0.026**	**0.004**
SYTOX	**A11**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Fraction lower peak	Estimate	**0.748**	**-0.359**	**-0.242**	**-0.300**	-0.109	**-0.290**	**-0.418**
	T-value		**-4.858**	**-3.283**	**-4.067**	-1.480	**-3.933**	**-5.663**
	P-value		**0.002**	**0.013**	**0.005**	0.182	**0.006**	**0.001**
Position of lower peak	Estimate	**5.405**	0.117	**0.240**	**0.274**	0.101	**0.244**	0.103
	T-value		1.538	**3.154**	**3.604**	1.334	**3.205**	1.355
	P-value		0.168	**0.016**	**0.009**	0.224	**0.015**	0.218
Position of higher peak	Estimate	**9.920**	-0.468	-1.119	-0.604	-0.851	0.281	0.540
	T-value		-0.900	-2.154	-1.163	-1.637	0.540	1.039
	P-value		0.398	0.068	0.283	0.146	0.606	0.333

Table D.5C: A11 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.6 B3 DAPI and SYTOX Graphical Output and Statistical Output.

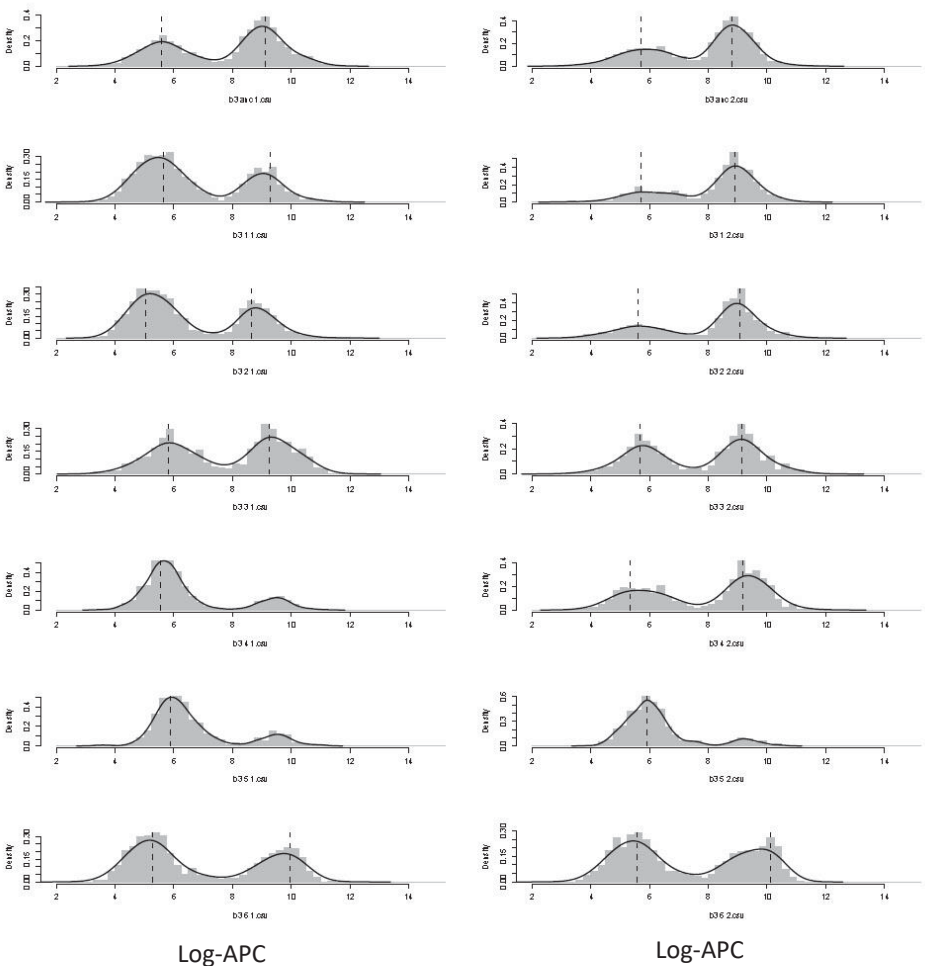

Figure D.6A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of B3.

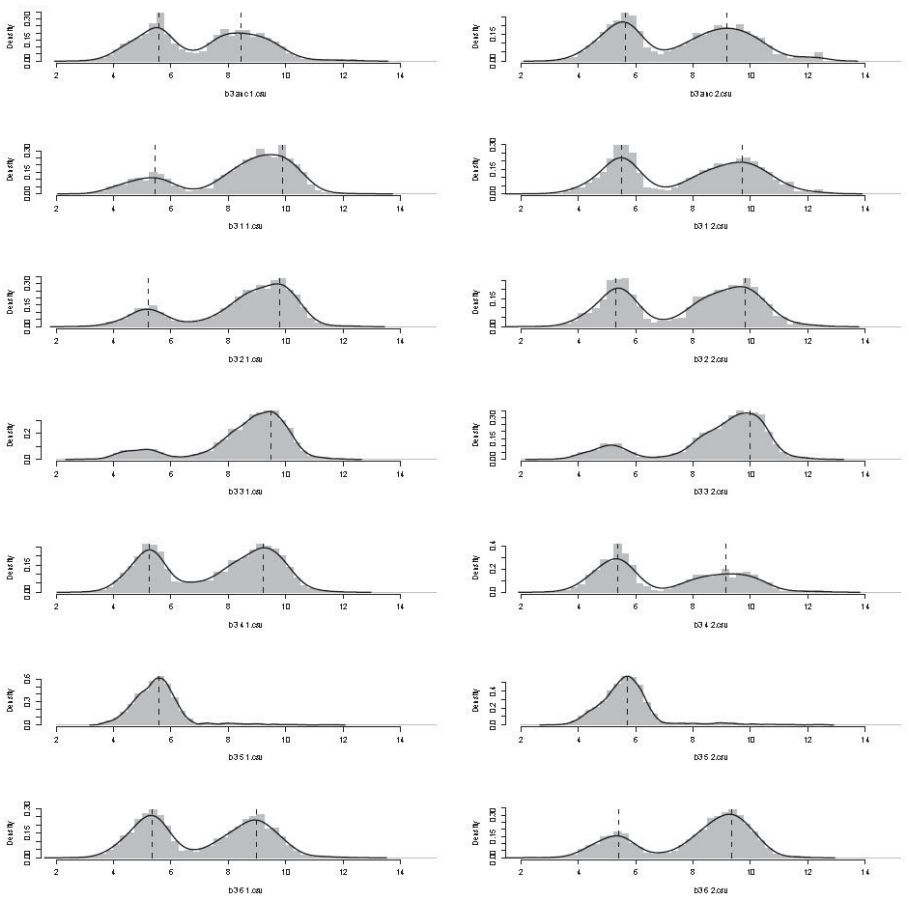

Figure D.6B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of B3.

DAPI	B3	0	1	2	3	4	5	6
Fraction lower peak	Estimate	0.416	0.079	0.086	0.068	0.210	**0.446**	0.173
	T-value		0.480	0.521	0.415	1.275	**2.708**	1.049
	P-value		0.646	0.618	0.690	0.243	**0.030**	0.329
Position of lower peak	Estimate	5.651	0.037	-0.328	0.111	-0.220	0.252	-0.220
	T-value		0.197	-1.744	0.589	-1.167	1.338	-1.170
	P-value		0.849	0.125	0.574	0.281	0.223	0.280
Position of higher peak	Estimate	8.971	0.119	-0.113	0.234	0.198	NA	**1.076**
	T-value		0.574	-0.544	1.125	0.779	NA	**5.184**
	P-value		0.591	0.610	0.312	0.471	NA	**0.004**
SYTOX	**B3**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Fraction lower peak	Estimate	0.489	-0.141	-0.158	**-0.299**	0.004	**0.466**	-0.078
	T-value		-1.659	-1.860	**-3.526**	0.042	**5.497**	-0.923
	P-value		0.141	0.105	**0.010**	0.968	**0.001**	0.387
Position of lower peak	Estimate	5.603	-0.122	**-0.357**	NA	**-0.298**	0.042	**-0.236**
	T-value		-1.693	**-4.950**	NA	**-4.131**	0.581	**-3.269**
	P-value		0.141	**0.003**	NA	**0.006**	0.583	**0.017**
Position of higher peak	Estimate	8.822	**0.979**	**0.969**	**0.917**	0.359	NA	0.342
	T-value		**3.553**	**3.515**	**3.325**	1.301	NA	1.241
	P-value		**0.012**	**0.013**	**0.016**	0.241	NA	0.261

Table D.6C: B3 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.7 B5 DAPI and SYTOX Graphical Output and Statistical Output.

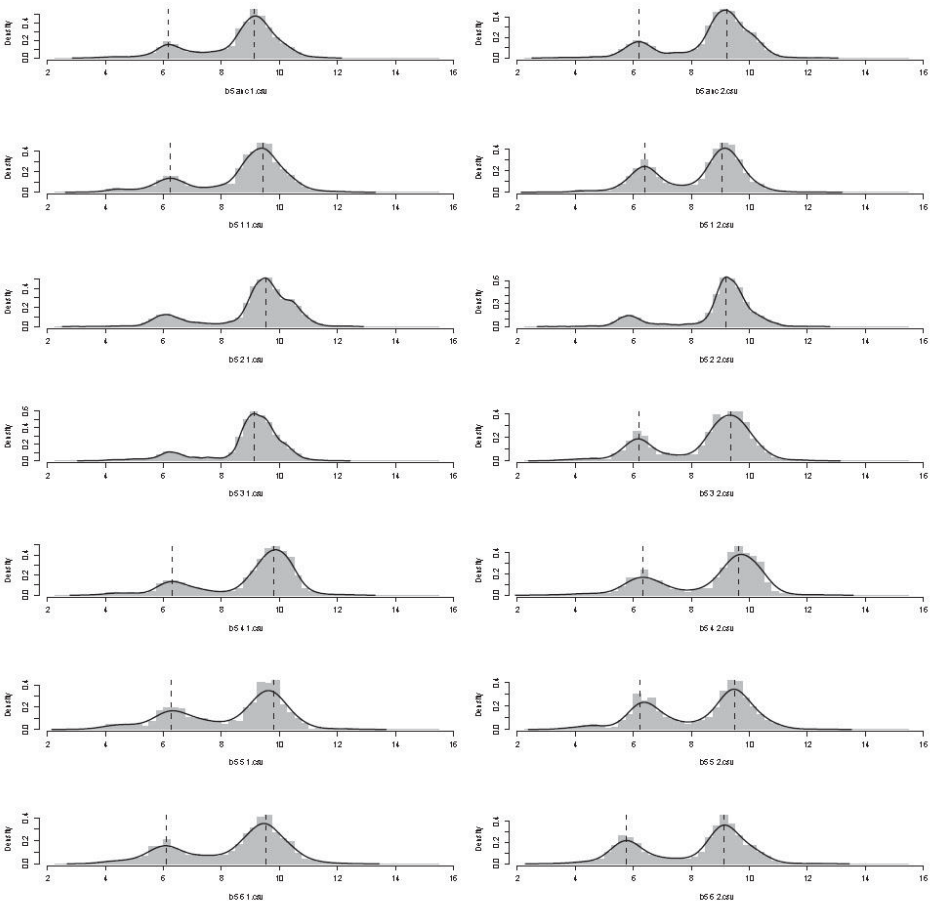

Figure D.7A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of B5.

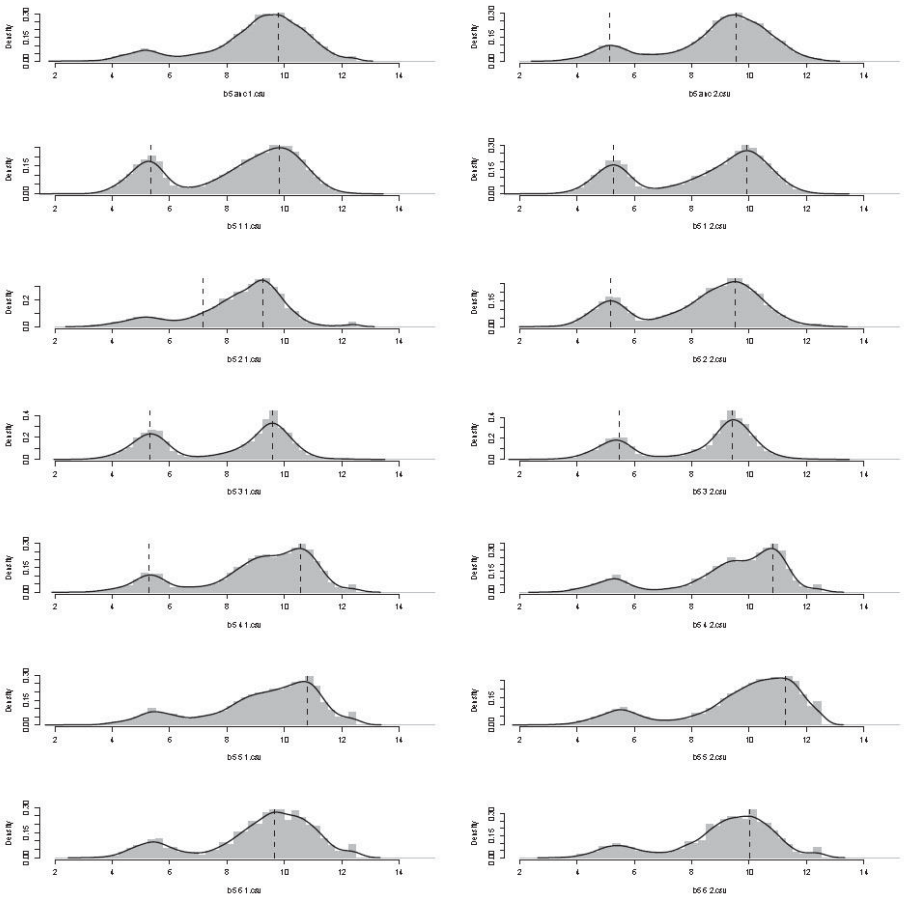

Figure D.7B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of B5.

DAPI	B5	0	1	2	3	4	5	6
Fraction lower peak	Estimate	0.252	0.067	-0.065	-0.013	0.029	**0.146**	0.113
	T-value		1.211	-1.168	-0.230	0.526	**2.648**	2.047
	P-value		0.265	0.281	0.825	0.615	**0.033**	0.080
Position of lower peak	Estimate	6.178	0.130	NA	0.000	0.143	0.075	-0.249
	T-value		1.098	NA	-0.003	1.205	0.634	-2.101
	P-value		0.322	NA	0.998	0.282	0.554	0.090
Position of higher peak	Estimate	9.173	0.086	0.186	0.076	**0.542**	0.464	0.158
	T-value		0.418	0.903	0.370	**2.629**	2.249	0.764
	P-value		0.689	0.397	0.722	**0.034**	0.059	0.470
SYTOX	**B5**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Fraction lower peak	Estimate	0.191	**0.134**	**0.079**	**0.172**	0.000	-0.010	-0.006
	T-value		**4.138**	**2.420**	**5.286**	-0.009	-0.303	-0.186
	P-value		**0.004**	**0.046**	**0.001**	0.993	0.771	0.858
Position of lower peak	Estimate	5.138	0.182	1.027	0.244	0.133	NA	NA
	T-value		0.182	1.031	0.245	0.116	NA	NA
	P-value		0.867	0.378	0.822	0.915	NA	NA
Position of higher peak	Estimate	9.657	0.221	-0.279	-0.162	**1.039**	**1.381**	0.194
	T-value		1.078	-1.364	-0.794	**5.079**	**6.749**	0.950
	P-value		0.317	0.215	0.453	**0.001**	**<0.001**	0.374

Table D.7C: B5 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.8 C10 DAPI and SYTOX Graphical Output and Statistical Output.

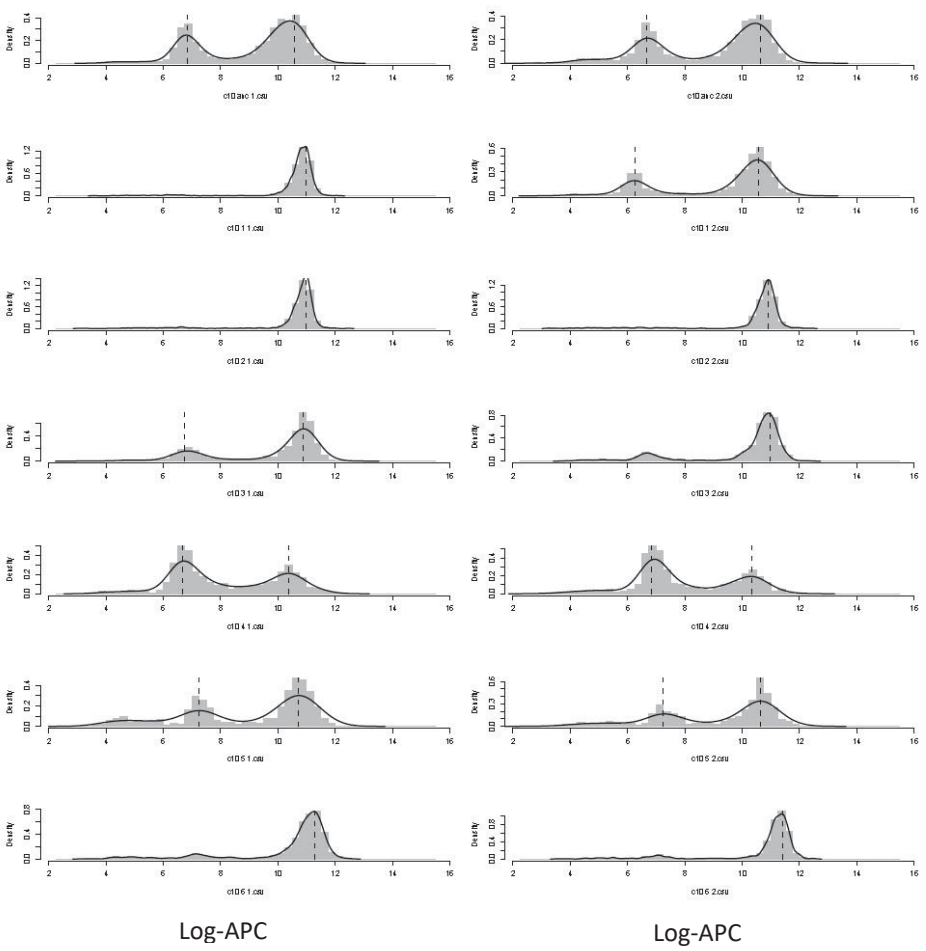

Figure D.8A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of C10.

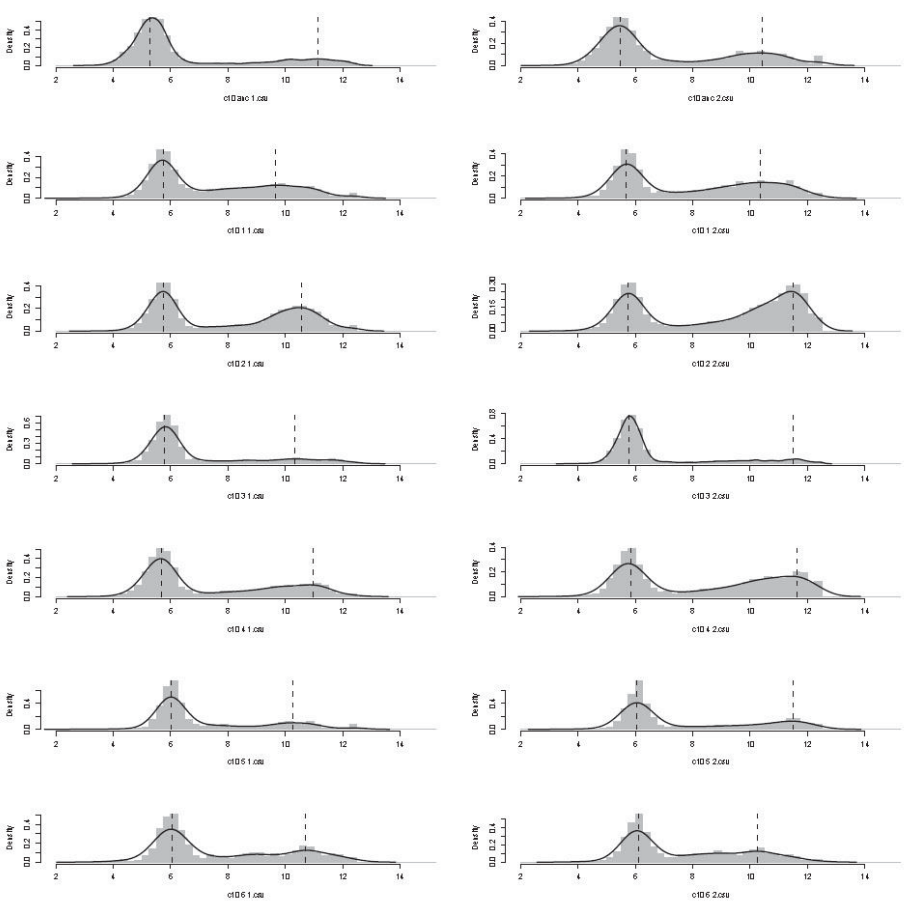

Figure D.8B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of C10.

DAPI	C10	0	1	2	3	4	5	6
Fraction lower peak	Estimate	0.349	**-0.185**	**-0.286**	-0.136	**0.230**	0.022	**-0.228**
	T-value		**-2.620**	**-4.052**	-1.930	**3.257**	0.308	**-3.239**
	P-value		**0.034**	**0.005**	0.095	**0.014**	0.767	**0.014**
Position of lower peak	Estimate	6.754	**-0.499**	NA	-0.017	-0.004	**0.487**	NA
	T-value		**-4.739**	NA	-0.165	-0.047	**5.670**	NA
	P-value		**0.018**	NA	0.879	0.966	**0.011**	NA
Position of higher peak	Estimate	10.607	0.165	**0.346**	**0.322**	-0.240	0.070	**0.733**
	T-value		1.319	**2.763**	**2.572**	-1.916	0.557	**5.859**
	P-value		0.229	**0.028**	**0.037**	0.097	0.595	**0.001**
SYTOX	**C10**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Fraction lower peak	Estimate	0.708	**-0.181**	**-0.283**	0.050	**-0.177**	-0.051	-0.136
	T-value		**-2.655**	**-4.159**	0.734	**-2.594**	-0.748	-1.991
	P-value		**0.033**	**0.004**	0.487	**0.036**	0.479	0.087
Position of lower peak	Estimate	5.376	**0.338**	**0.378**	**0.404**	**0.389**	**0.672**	**0.717**
	T-value		**4.740**	**5.299**	**5.662**	**5.446**	**9.418**	**10.040**
	P-value		**0.002**	**0.001**	**0.001**	**0.001**	**<0.001**	**<0.001**
Position of higher peak	Estimate	10.776	-0.762	0.258	0.147	0.529	0.105	-0.297
	T-value		-1.222	0.414	0.236	0.849	0.169	-0.476
	P-value		0.261	0.692	0.820	0.424	0.871	0.648

Table D.8C: C10 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.9 F8 DAPI and SYTOX Graphical Output and Statistical Output.

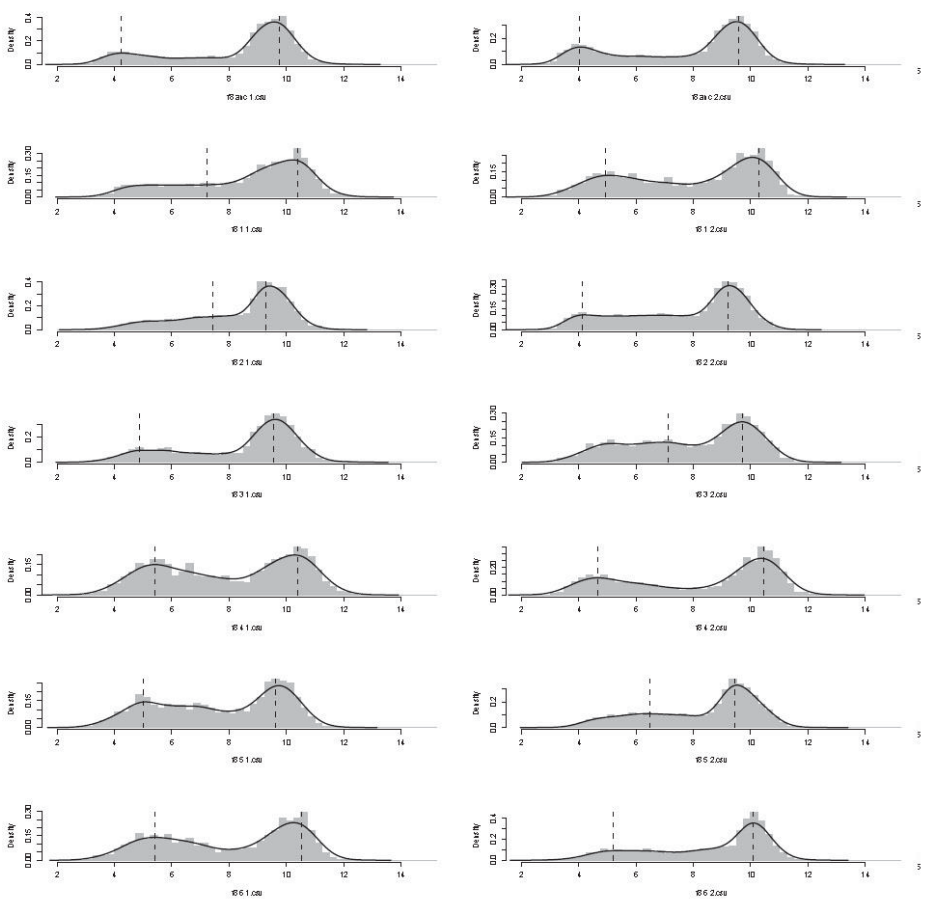

Figure D.9A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of F8.

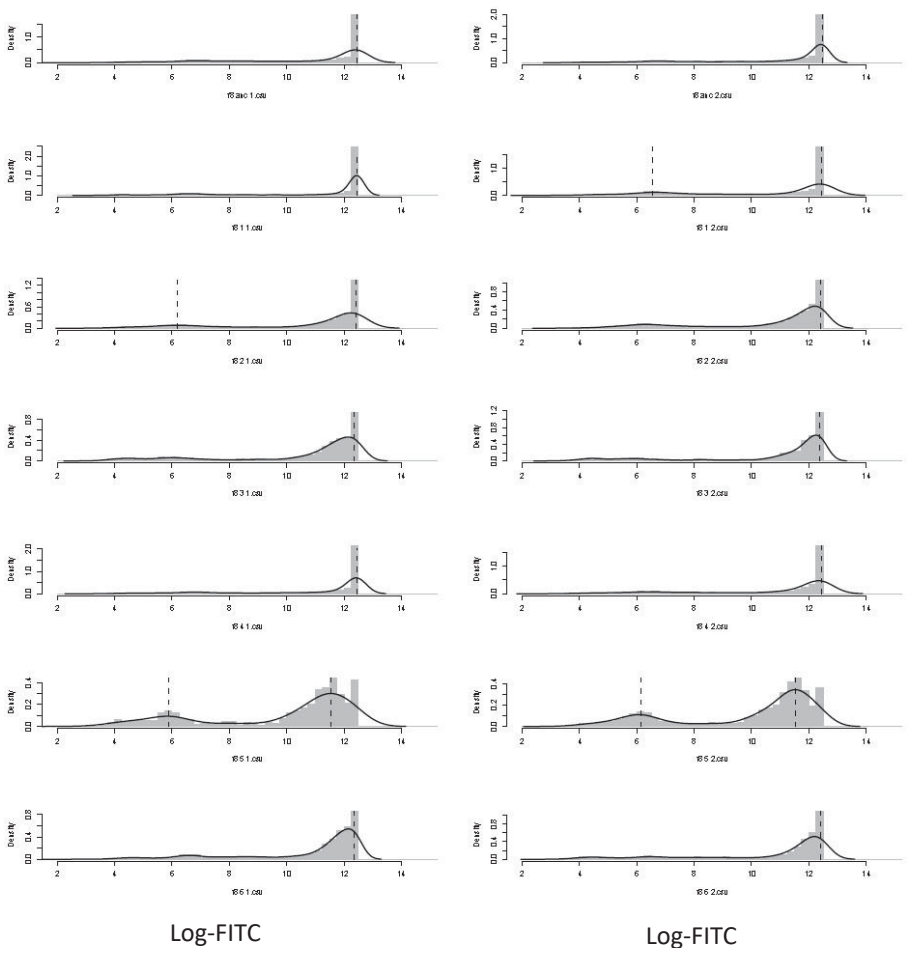

Figure D.9B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of F8.

DAPI	F8	0	1	2	3	4	5	6
Fraction lower peak	Estimate	0.397	**0.266**	**0.292**	**0.306**	**0.185**	**0.202**	**0.082**
	T-value		**9.611**	**10.574**	**11.054**	**6.702**	**7.293**	**2.963**
	P-value		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.021**
Position of lower peak	Estimate	6.078	**-1.028**	**-1.445**	**-1.452**	**-1.136**	**-1.033**	**-0.975**
	T-value		**-3.076**	**-4.324**	**-4.346**	**-3.402**	**-3.093**	**-2.919**
	P-value		**0.018**	**0.003**	**0.003**	**0.011**	**0.018**	**0.022**
Position of higher peak	Estimate	9.737	**0.924**	-0.015	0.093	**0.765**	0.118	**0.651**
	T-value		**6.730**	-0.110	0.678	**5.570**	0.856	**4.741**
	P-value		**<0.001**	0.915	0.520	**0.001**	0.420	**0.002**
SYTOX	**F8**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Fraction lower peak	Estimate	0.149	0.058	0.040	0.028	0.017	**0.093**	0.005
	T-value		2.271	1.569	1.090	0.657	**3.641**	0.186
	P-value		0.057	0.161	0.312	0.532	**0.008**	0.858
Position of lower peak	Estimate	NA	NA	NA	NA	NA	NA	NA
	T-value		NA	NA	NA	NA	NA	NA
	P-value		NA	NA	NA	NA	NA	NA
Position of higher peak	Estimate	12.451	0.007	**-0.049**	**-0.085**	0.000	**-0.910**	**-0.074**
	T-value		0.514	**-3.520**	**-6.172**	-0.001	**-65.744**	**-5.343**
	P-value		0.623	**0.010**	**<0.001**	0.999	**<0.001**	**0.001**

Table D.9C: F8 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.10 SCE2 DAPI and SYTOX Graphical Output and Statistical Output.

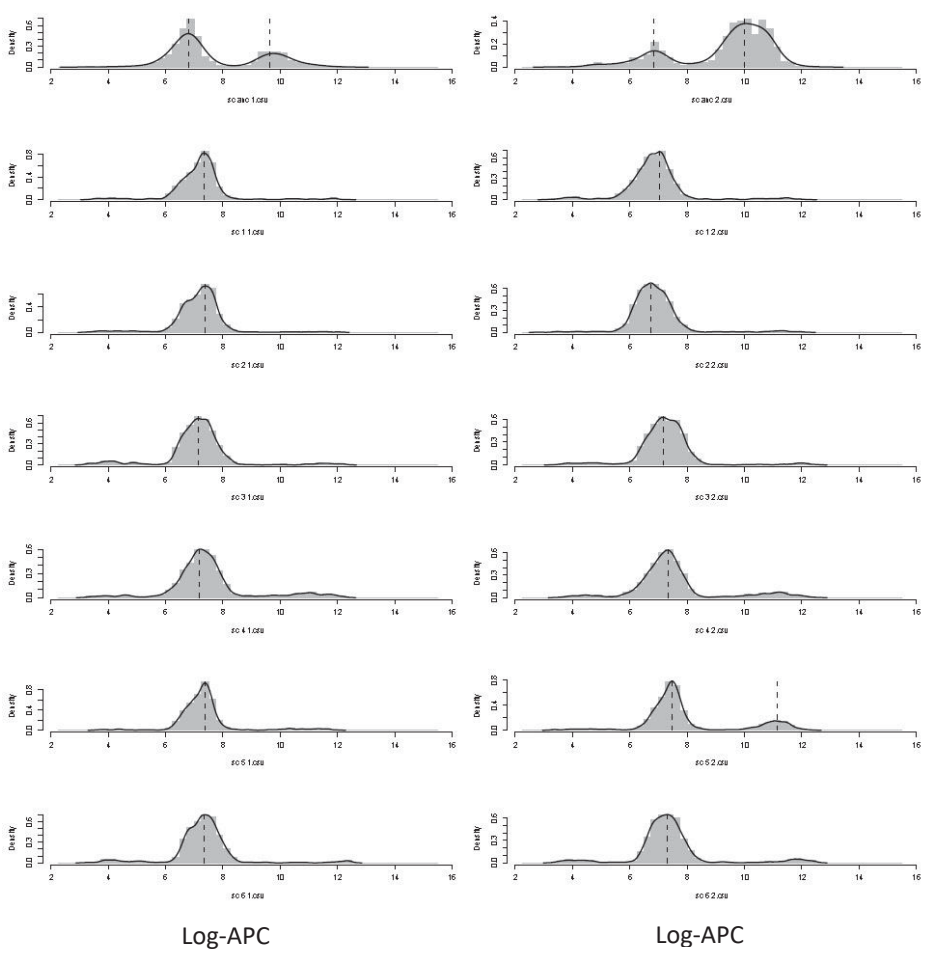

Figure D.10A: DAPI signal measured by flow cytometry. Two replicates for ancestor and evolved lines of SCE2.

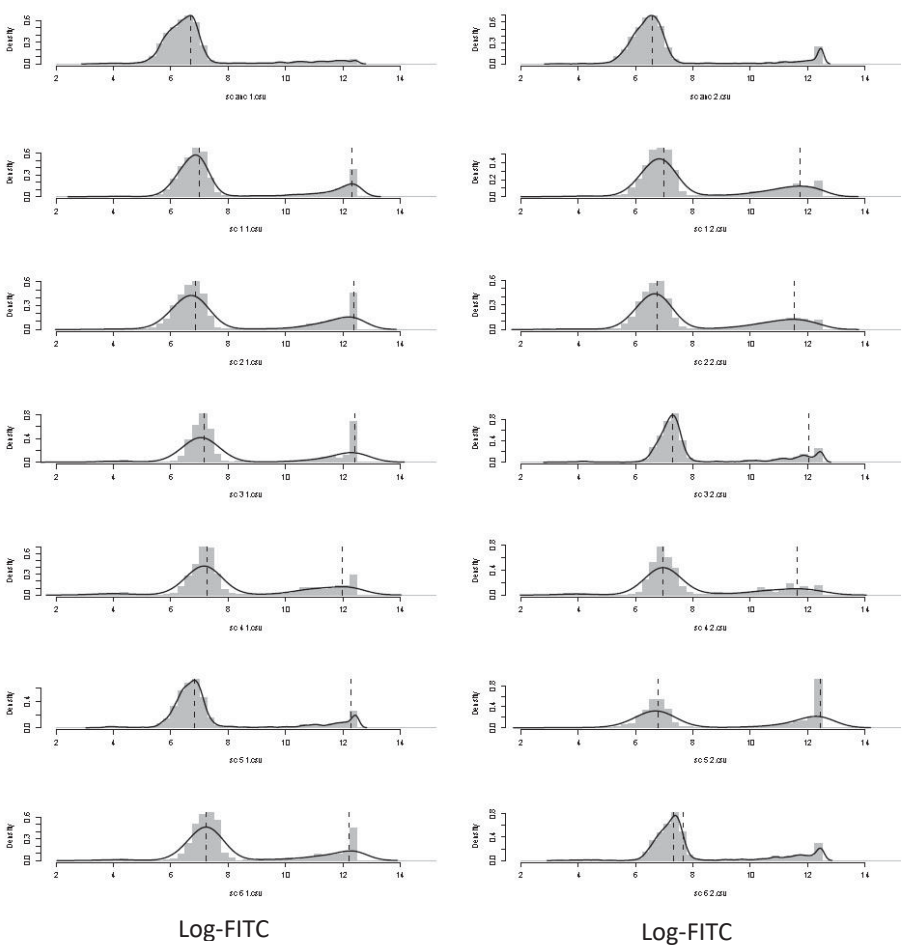

Figure D.10B: SYTOX signal measured by flow cytometry. Two replicates for ancestor and evolved lines of SCE2.

DAPI	SCE2	0	1	2	3	4	5	6
Fraction	Estimate	0.452	**0.491**	**0.497**	**0.461**	**0.383**	**0.392**	**0.434**
lower	T-value		**3.833**	**3.876**	**3.599**	**2.990**	**3.059**	**3.386**
peak	P-value		**0.006**	**0.006**	**0.009**	**0.020**	**0.018**	**0.012**
Position	Estimate	6.824	0.371	0.234	0.324	0.436	**0.605**	**0.508**
of lower	T-value		1.907	1.204	1.666	2.240	**3.110**	**2.610**
peak	P-value		0.098	0.268	0.140	0.060	**0.017**	**0.035**
Position	Estimate	NA	NA	NA	NA	NA	NA	NA
of higher	T-value		NA	NA	NA	NA	NA	NA
peak	P-value		NA	NA	NA	NA	NA	NA
SYTOX	SCE2	0	1	2	3	4	5	6
Fraction	Estimate	0.871	**-0.169**	**-0.186**	**-0.221**	**-0.233**	**-0.187**	**-0.281**
lower	T-value		**-2.457**	**-2.713**	**-3.213**	**-3.391**	**-2.720**	**-4.089**
peak	P-value		**0.044**	**0.030**	**0.015**	**0.012**	**0.030**	**0.005**
Position	Estimate	6.634	**0.357**	0.168	**0.585**	**0.479**	0.168	**0.654**
of lower	T-value		**3.224**	1.519	**5.283**	**4.323**	1.512	**5.905**
peak	P-value		**0.015**	0.173	**0.001**	**0.003**	0.174	**0.001**
Position	Estimate	NA	NA	NA	NA	NA	NA	NA
of higher	T-value		NA	NA	NA	NA	NA	NA
peak	P-value		NA	NA	NA	NA	NA	NA

Table D.10C: SCE2 linear model results for DAPI and SYTOX. This table covers the statistical output for the fraction that is the lowest peak, and the positions of the peaks present as compared to the ancestor. If a peak is missing the results are filled with NA. Statistically significant results are highlighted in bold.

D.11 Cell Tracker

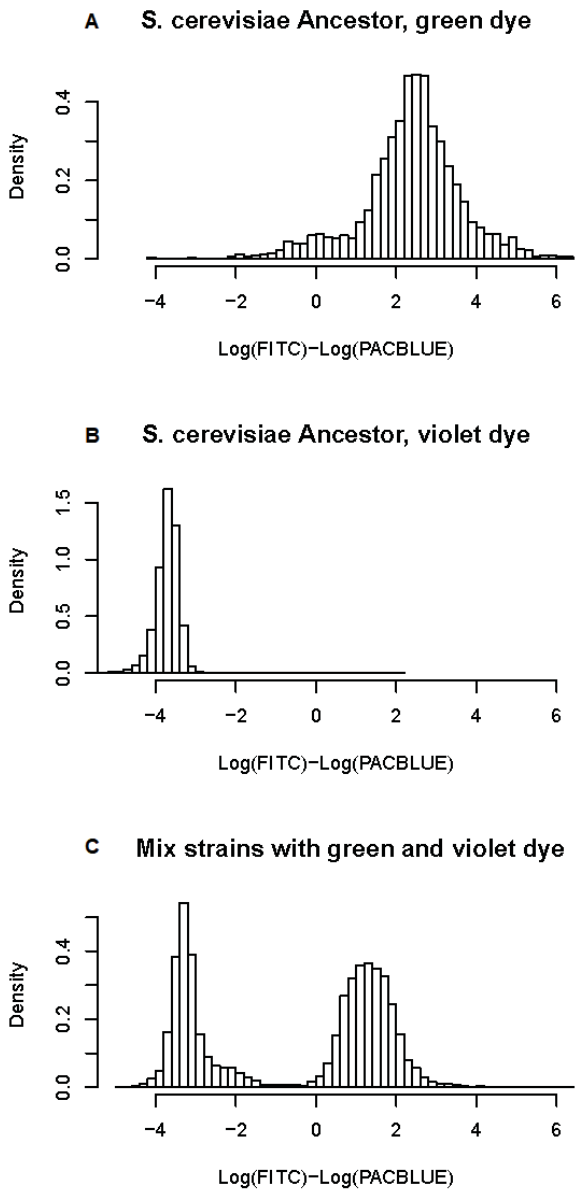

Figure D.11A: Cell Tracker™ controls of both dyes measured by flow cytometry. A) the log transformed signal intensity of *S. cerevisiae* Ancestor stained with Cell Tracker green dye as seen across both the FITC and PACBLUE channels. B) the log transformed signal intensity of *S. cerevisiae* Ancestor stained with Cell Tracker violet dye as seen across both the FITC and PACBLUE channels. C) the log transformed signal intensity of a mixed solution of *S. cerevisiae* Ancestor strains stained with Cell Tracker green dye and violet dye respectively, as seen across both the FITC and PACBLUE channels.

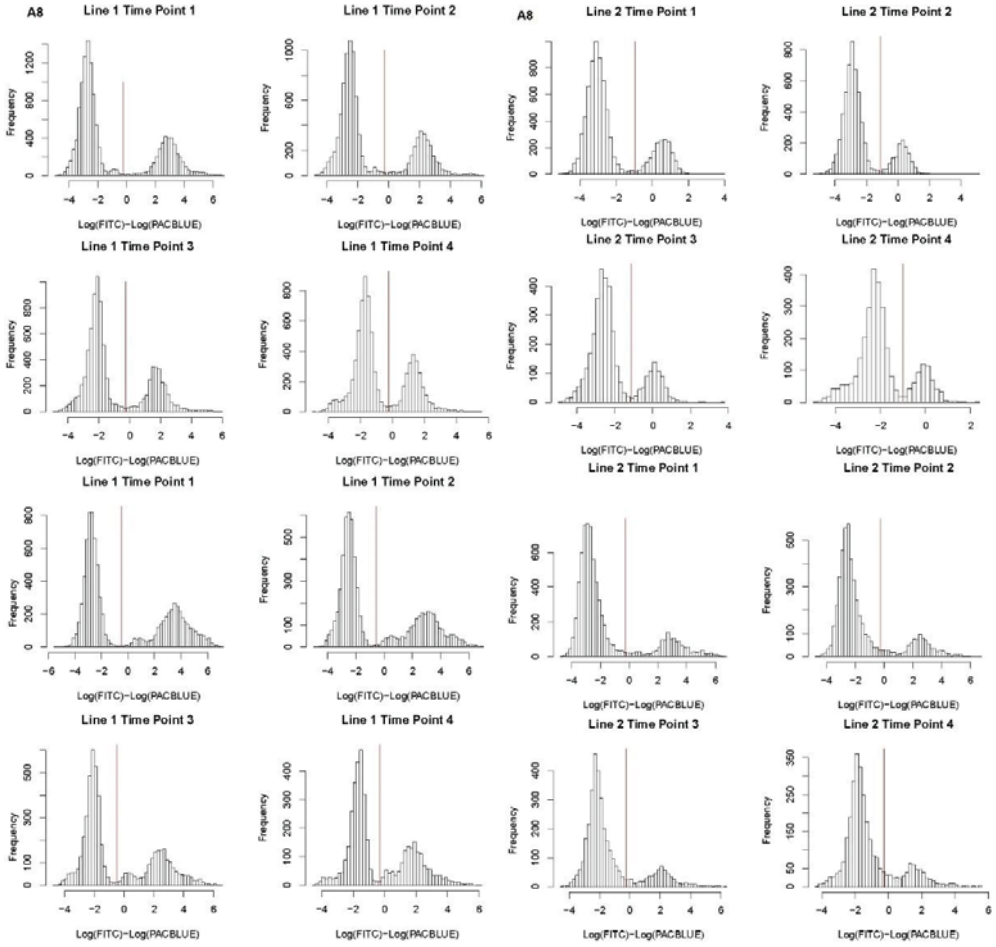

Figure D.11B: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 1 and 2 of A8.

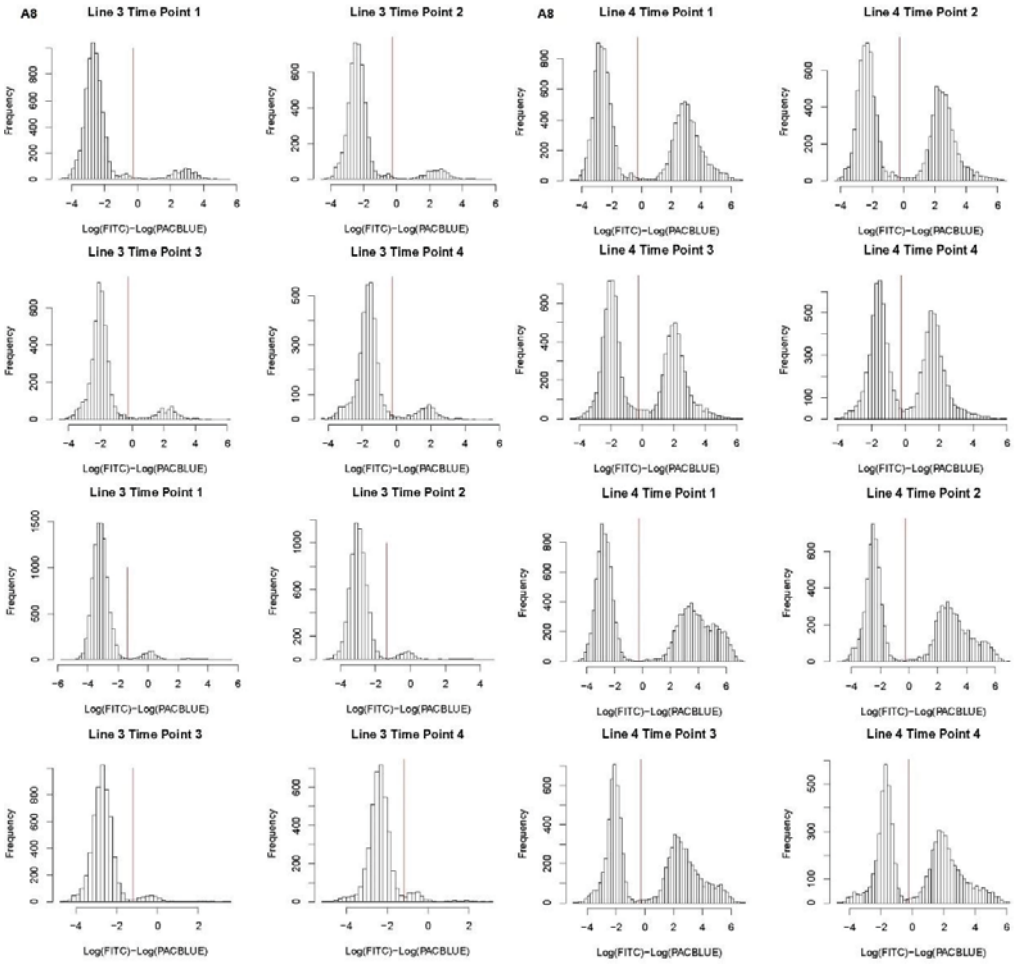

Figure D.11C: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 3 and 4 of A8.

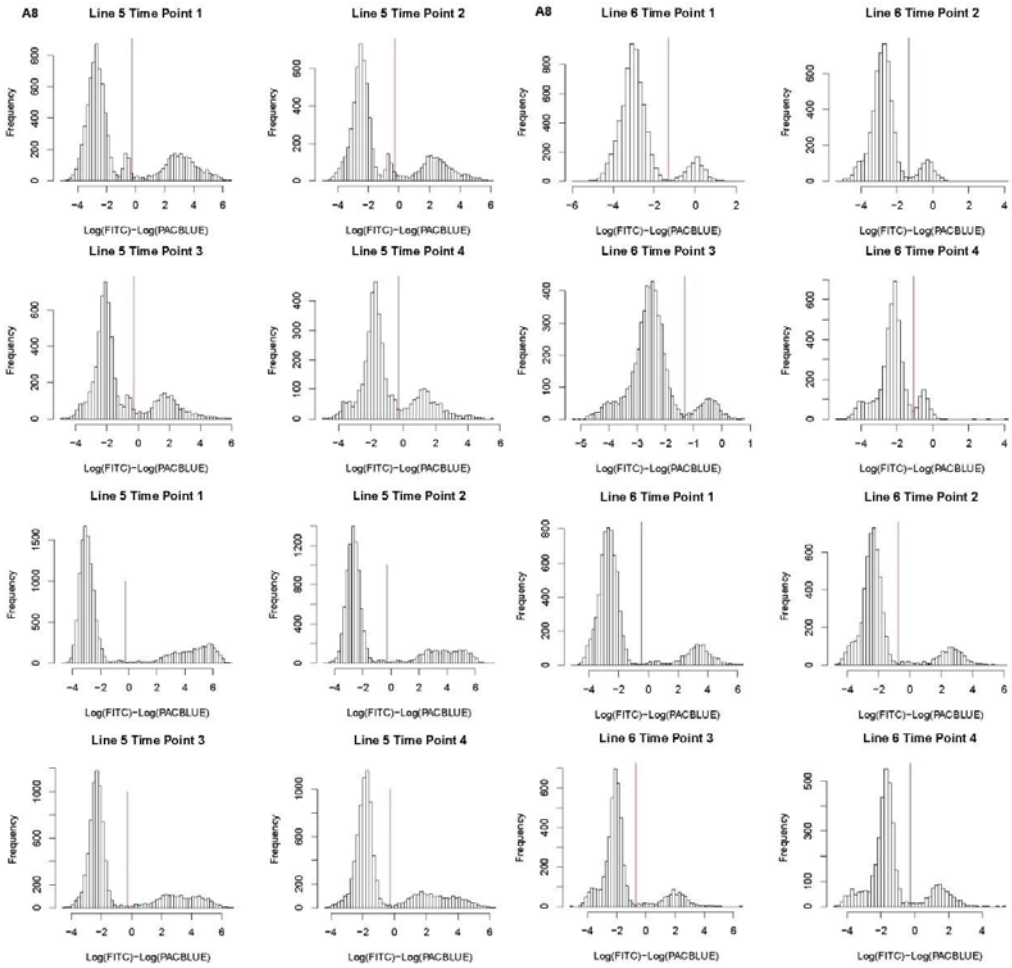

Figure D.11D: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 5 and 6 of A8.

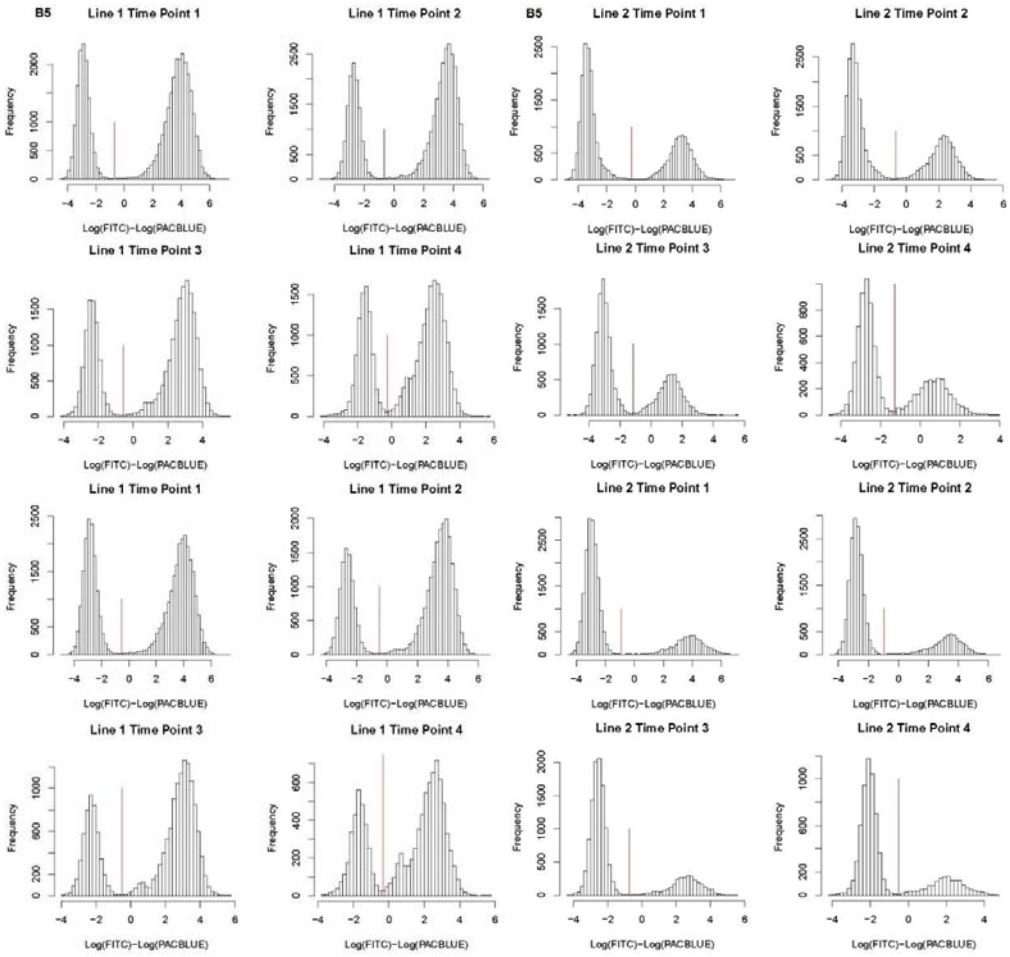

Figure D.11E: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 1 and 2 of B5.

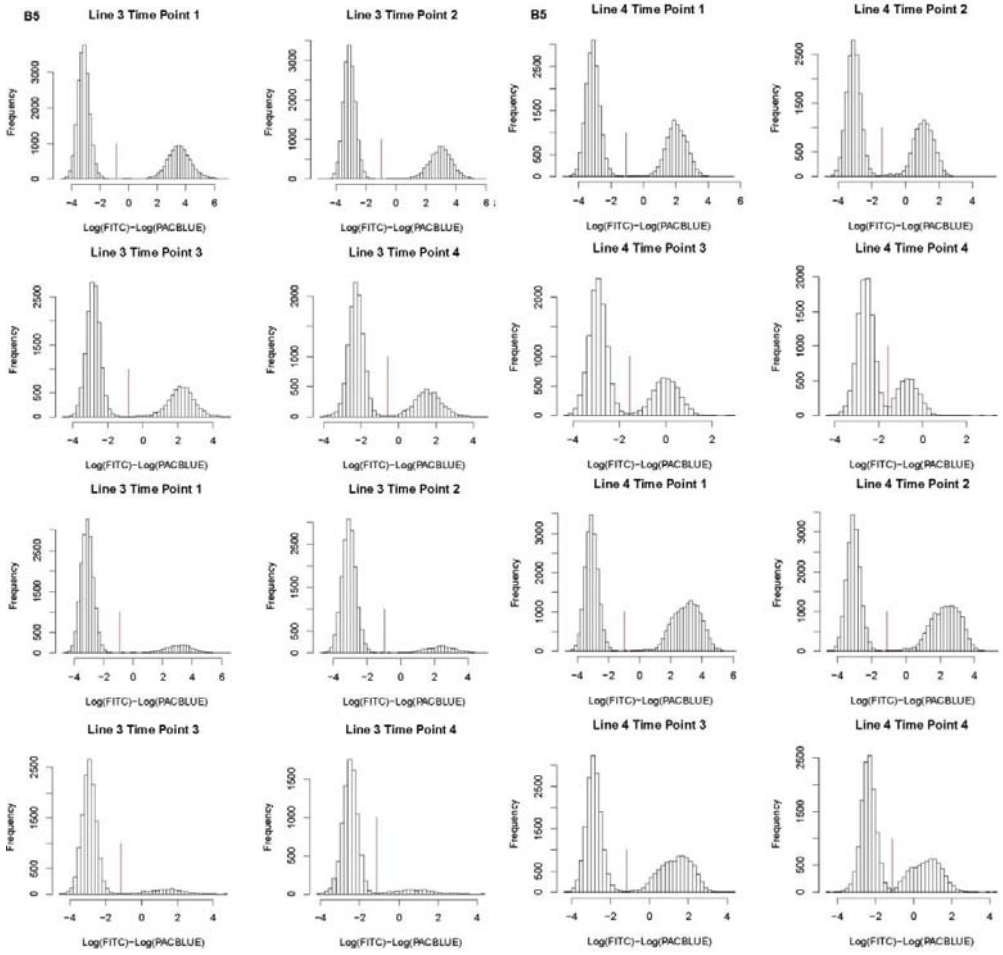

Figure D.11F: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 3 and 4 of B5.

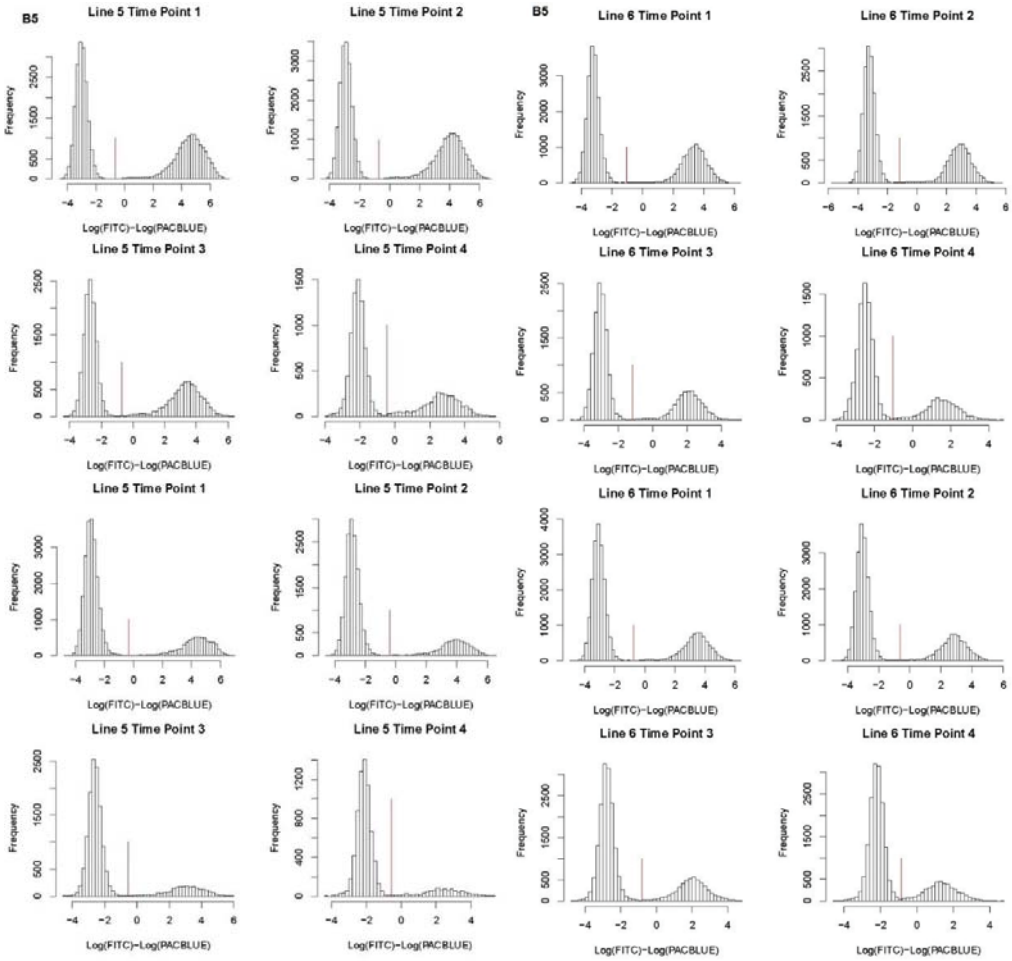

Figure D.11G: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 5 and 6 of B5.

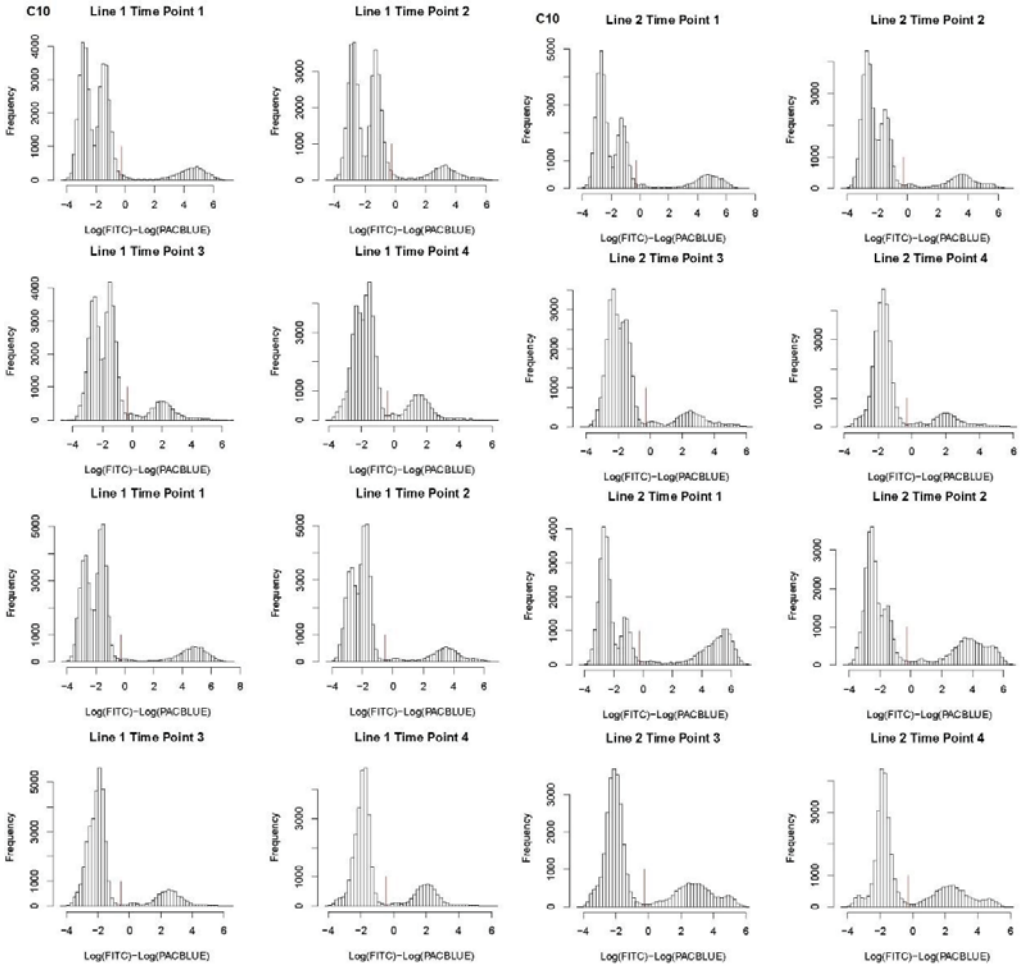

Figure D.11H: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 1 and 2 of C10.

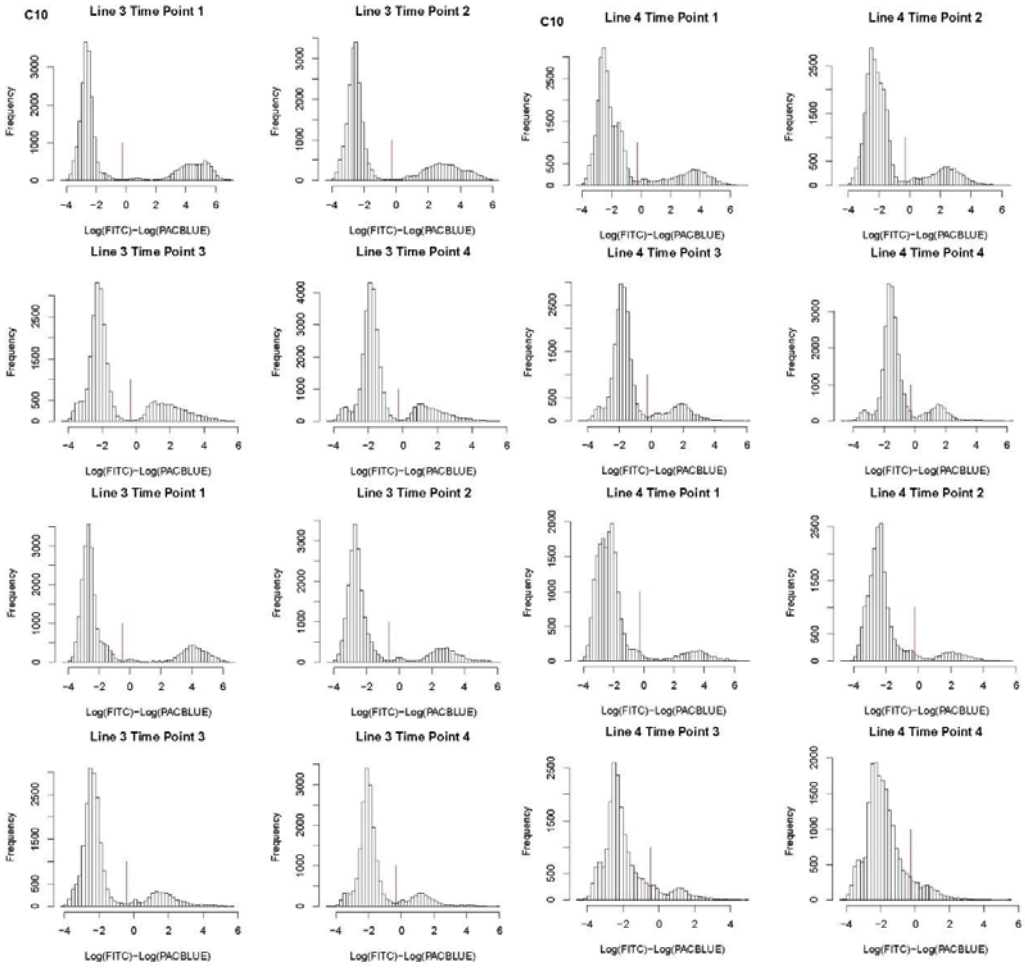

Figure D.11I: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 3 and 4 of C10.

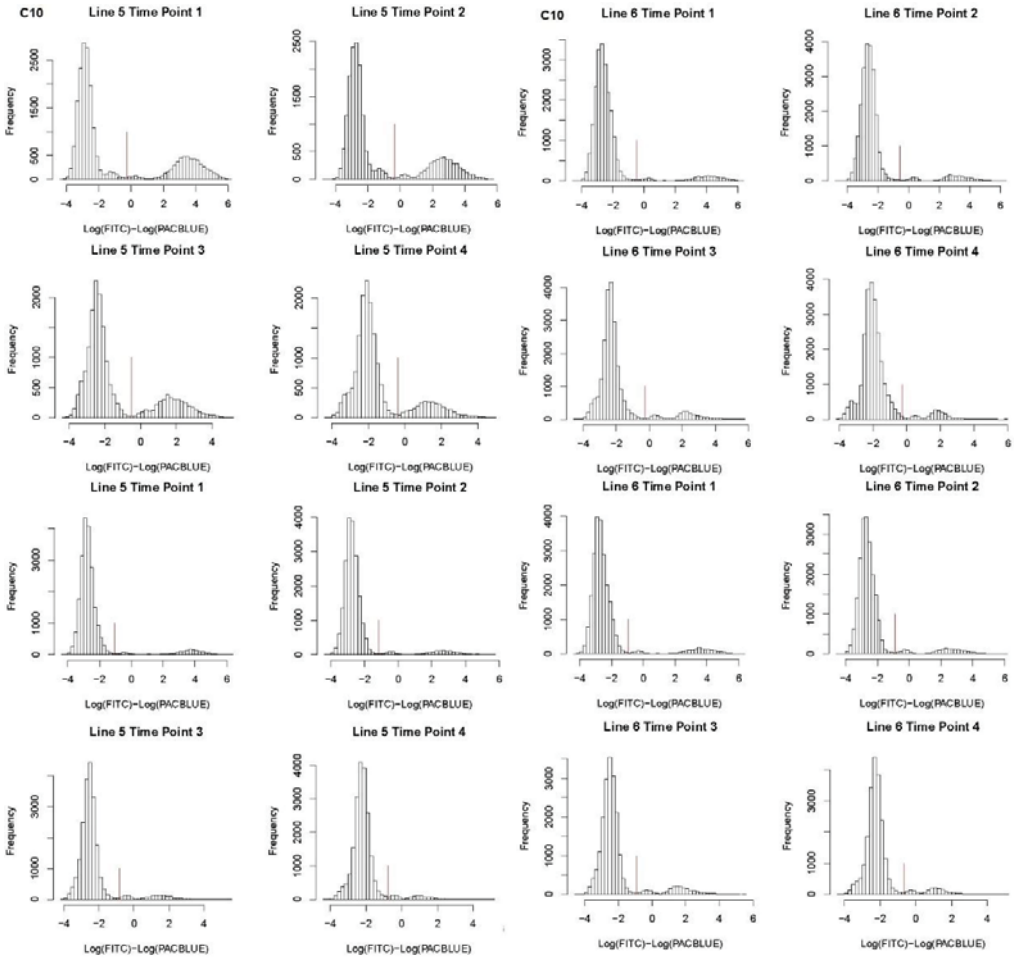

Figure D.11J: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 5 and 6 of C10.

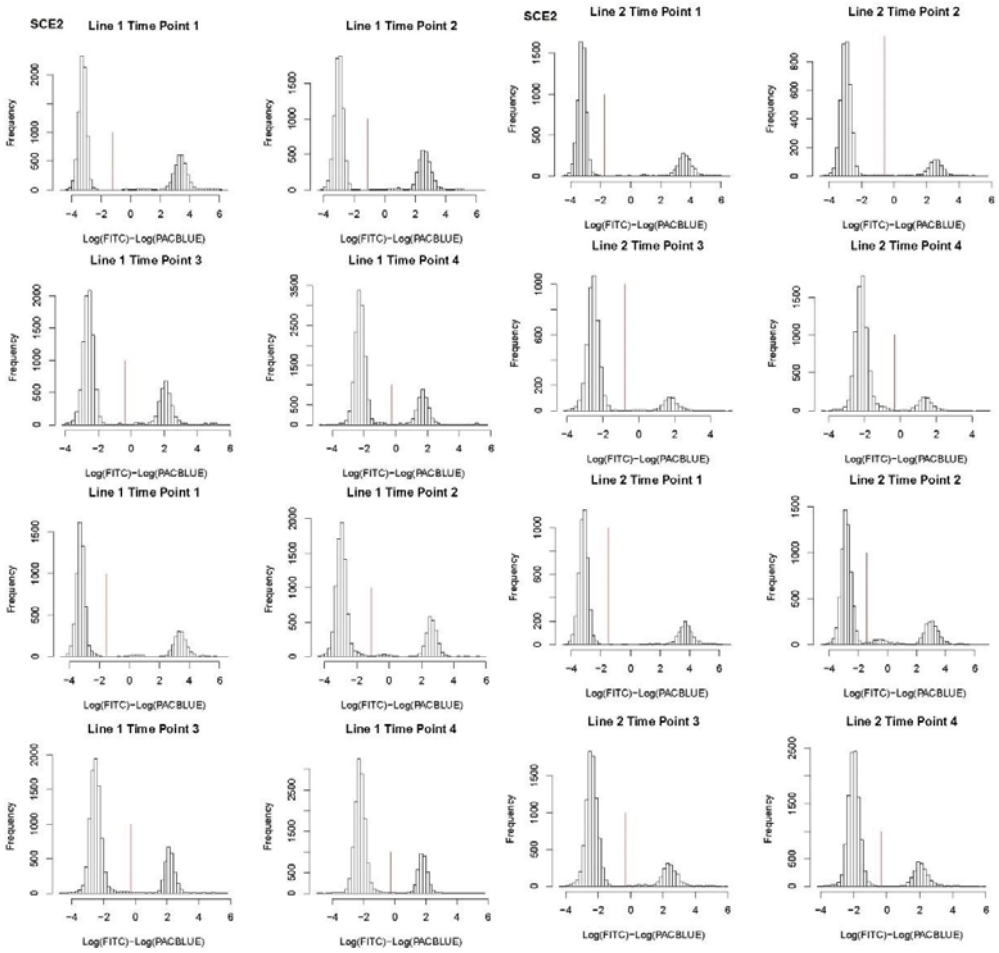

Figure D.11K: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 1 and 2 of SCE2.

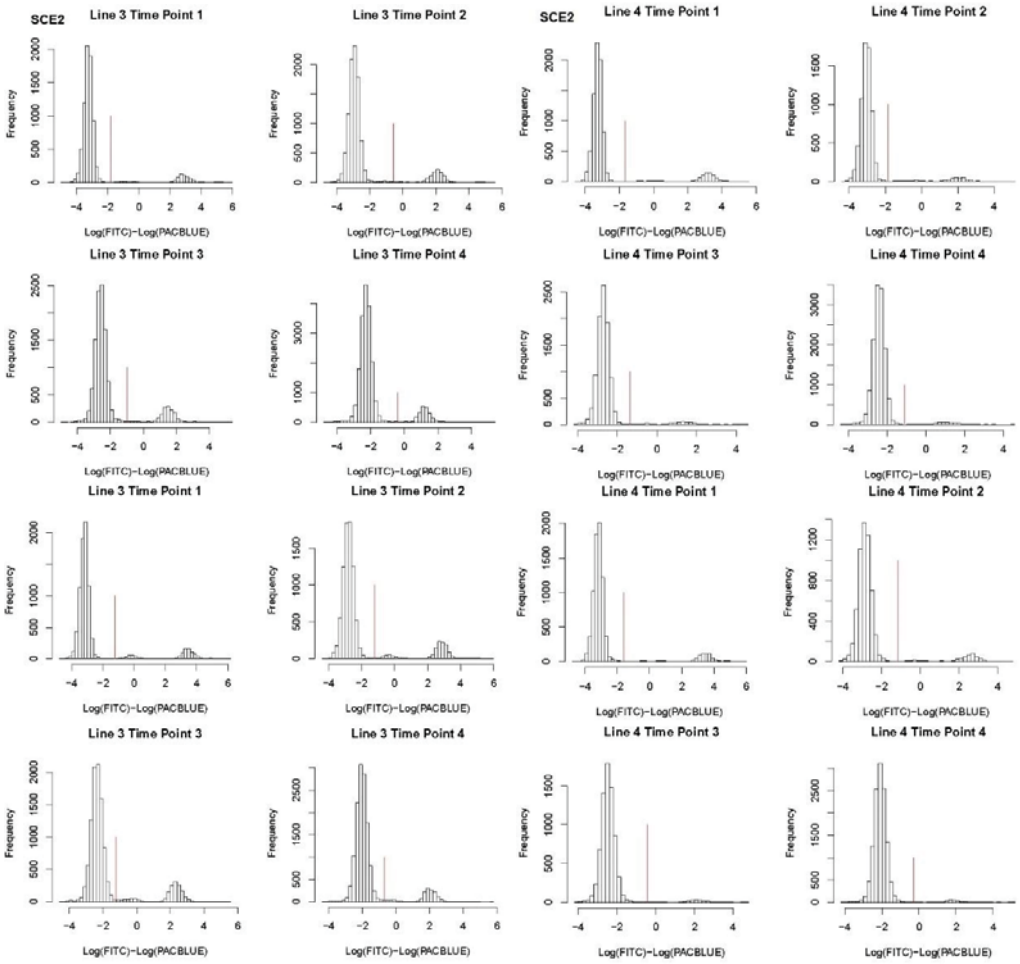

Figure D.11L: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 3 and 4 of SCE2.

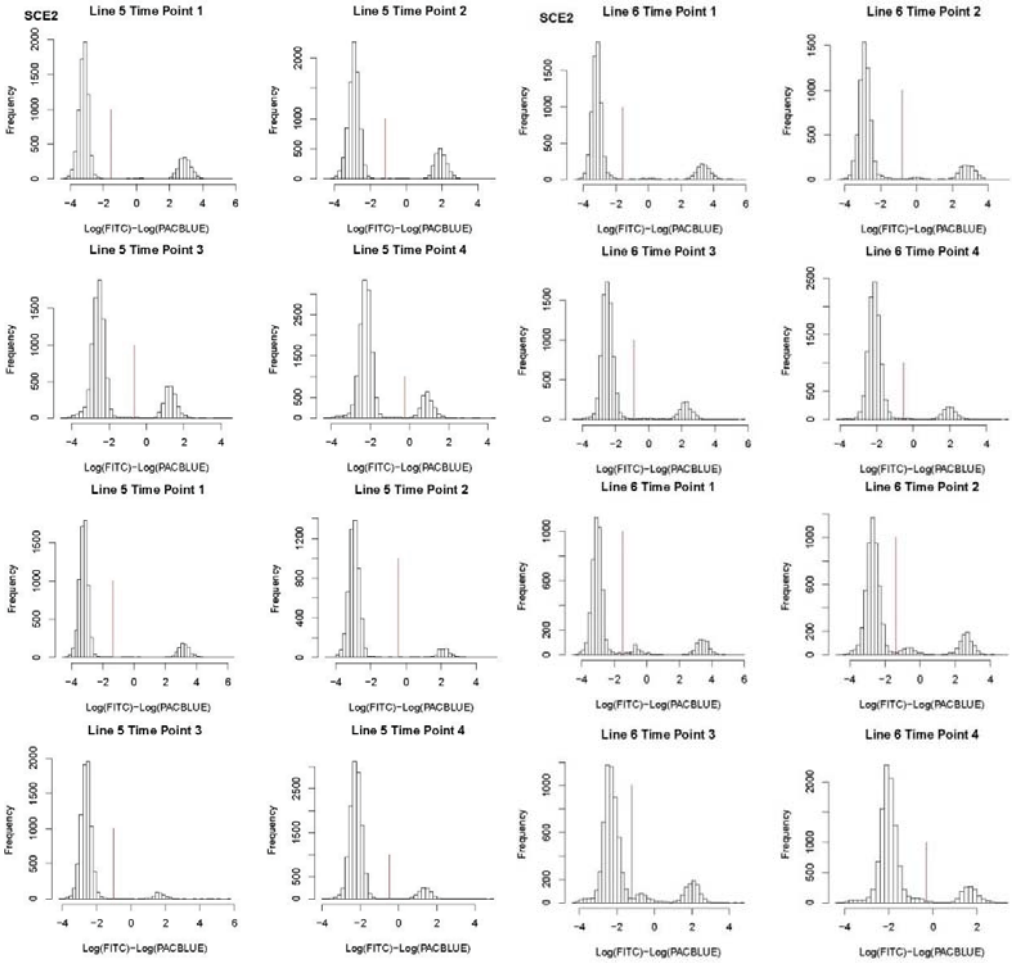

Figure D.11M: Cell Tracker™ signal measured by flow cytometry. Two replicates for ancestor and evolved lines 5 and 6 of SCE2.