Impact of Mutation Rate and Bias on Bacterial Adaptation in Fluctuating Environments

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the MS (by Dissertation) Programme

by

Kushan Lahiri

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

April, 2023

Supervisor: Dr. Deepa Agashe

Certificate

This is to certify that this dissertation entitled Impact of Mutation Rate and Bias on Bacterial Adaptation in Fluctuating Environments towards the partial fulfilment of the MS (by Dissertation) programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Kushan Lahiri at the National Centre for Biological Sciences (NCBS-TIFR), Bangalore, under the supervision of Dr. Deepa Agashe, Associate Professor, National Centre for Biological Sciences, during the academic year 2022-2023.

Dr. Deepa Agashe

Committee:

Dr. Deepa Agashe

Dr. Nishad Matange

Declaration

I hereby declare that the matter embodied in the report entitled Impact of Mutation Rate and Bias on Bacterial Adaptation in Fluctuating Environments are the results of the work carried out by me at the National Centre for Biological Sciences (NCBS-TIFR), Bangalore, under the supervision of Dr. Deepa Agashe and the same has not been submitted elsewhere for any other degree.

Kushan Lahiri

Kushan Lahiri

Date: 25/05/2023

Chapter 1 Table of Contents

List of Tables

List of Figures

Abstract

We use the term ‘mutation spectrum’ to refer to the frequency distribution of the types of mutations that an organism tends to undergo overtime. However, more often than not, this mutation spectrum tends to be somewhat biased towards either end. This phenomenon is called mutation bias. It plays a role in determining several important factors such as predictability and evolution of genetic diversity, and mutational robustness. These factors, in turn, directly influence adaptive evolution in the long run. It has been known for a long time that the occurrence, selection, and accumulation of changes in the genetic material of an organism, i.e., mutations, forms the foundation of evolutionary trajectories. Since the mutation spectrum is a representative of the range of mutations that can occur at any given point, a bias in this spectrum can, therefore, potentially dictate the evolutionary trajectory taken by a given organism under a particular selection pressure. This necessitates a systematic study of evolution from the relatively less explored perspective of mutation biases. Recent studies have demonstrated how a shift in the mutation bias of an organism can lead to a change in the supply of beneficial or deleterious mutations to that organism. Hence, it is important to explore how an organism behaves under various selection pressures when its mutation spectrum is shifted towards either end. The aim of my work was to highlight how the combined effect of mutation bias and mutation rate shapes the evolutionary trajectory of an organism. To that effect, my results show interesting trends which do not completely align with my expectations prior to the experiments. Firstly, in terms of the effect of rate on adaptation in fluctuating environments, reversed-bias mutators did not seem to differ much from each other at any given time point in the evolution in Glucuronate. However, these results were not consistent in Succinate, where there was a noticeable difference in fitness between strains of different mutation rates at day 18. However, in the case of reinforced-bias mutators, this difference in fitness between mutators of different mutation rates was not seen in either medium. Secondly, contrary to my initial expectations, mutation bias was not found to play a strong role in bacterial adaptation in fluctuating environments. These results are in direct contradiction to the expectations which were formed on the basis of distribution of fitness effects obtained in the lab. As a result, this work necessitates further detailed investigations into the mechanistic basis of such adaptation.

Acknowledgements

I would like to thank the National Centre for Biological Sciences for providing me with the opportunity to undertake my M.S. Dissertation Project at its state-of-the-art research facilities and a healthy work environment. I would also like to thank the Department of Biology at IISER Pune for their unwavering support and funding throughout my project. I would like to express my gratitude to my supervisor, Dr. Deepa Agashe, for her guidance and mentorship throughout this project, and the countless occasions on which she has gone out of her way to stand by me and ensure my well-being. I am also extremely thankful to my TAC member, Dr. Nishad Matange, for his continued support and inputs which have only led to growth both personally, and professionally.

I would like to express my gratitude to my mentor, Dr. Mrudula Sane, for showing immense patience in teaching me techniques and rectifying my mistakes in the lab, and being an invaluable friend and a guiding figure to me outside the lab as well. I would like to thank the other members of the Adaptation Lab, namely Shubha, Pratibha, Shyam, and Shivansh, for their valuable inputs and enriching conversations in the lab. I am particularly grateful to Swastik and Shazia for their contributions to my project and my experiments. I would also like to acknowledge the lab kitchen and common facilities at NCBS, without whom, it would not have been possible to complete my project.

Outside the lab, I had the privilege of having incredible support from friends, particularly Anjoom. I would like to acknowledge my former mentors – Susovan, Shikha and Sukanya – for their invaluable guidance. I would like to thank my parents who have supported me through every difficult situation in life. I would like to thank Aryama, who has been the unconditional pillar of strength in my life. Finally, I would like to thank my best friend, Neelangshu, whose memory continues to inspire me to be better in life.

Chapter 1 Introduction

1.1 What are mutations and what is their role in evolution?

Mutations are defined as changes or alterations in the genetic material of an organism. Since the molecules that encode genetic information in all organisms are nucleic acids – Ribonucleic acid (RNA) in RNA viruses, and Deoxyribonucleic acid (DNA) in all other organisms – these alterations manifest themselves as changes made to the composition of these nucleic acid molecules. Within these nucleic acids, the nitrogenous bases – purines and pyrimidines – form the informational moiety, whereas the other components – the pentose sugar and phosphodiester bonds – are required to maintain the structural integrity of these molecules. As a result, when the genetic information contained within nucleic acids are altered through mutations, it is the nitrogenous bases that undergo chemical changes.

We know that the prime driving factor of evolution is natural selection[1]**.** Natural selection is the phenomenon wherein, the fittest or most successful individual or group of individuals within a population in a given environment survives in greater numbers that another individual or group of individuals. As a result of this differential rate of survival amongst different individuals or groups of them within a population, the fitter members of a population, more of whom survive in a given environment, also enjoy a greater chance of reproduction. Consequently, this greater survivability leads to greater chances of reproduction. Moving along in evolutionary time, this leads to a subsequent increase in the proportion of these fitter individuals within a population. In some cases, this process may lead to these fitter individuals completely taking over the niche in which they were competing with the other members of their population.

When this phenomenon is examined at the molecular level, the greater survival and reproduction of these fitter or more successful individuals leads to the greater propagation of their genetic material. This means that with time, as an individual or group of individuals becomes more successful within a group, its genetic material is also established as the more dominant genetic constitution within the population. For

natural selection to act differentially on different individuals, there needs to be pre-existing variation within these individuals at the genetic level.

This is where mutations come into the picture of evolution. As defined earlier, mutations are random changes or alterations in the genetic material of organisms. They may either occur spontaneously, or their chances of occurrence may be enhanced by exposure to environmental factors, such as mutagens. However, what these mutations mean for the genetic constitution of the population is that they lead to an increase in the variation between different individuals at the genetic level. As discussed earlier, this variation in the genetic makeup of different individuals forms the basis for natural selection to act upon. The pre-existing genetic variation between different individuals is limited and arrest the potential of natural selection. However, due to the occurrence of mutations in organisms, their genetic constitutions enjoy a constant source of genetic variations. These new genetic variations, on the other hand, lead to the formation of a bigger platform for natural selection to act on.

1.2 What are mutation spectrum and mutation bias?

As defined earlier, mutations are random changes or alterations to the genetic material. However, all mutations do not occur uniformly. There are different types of mutations that occur in organisms which are classified on the basis of the kind of change they lead to in the host genetic material. Again, there can be multiple criteria for the classification of such mutations. For example, depending on whether the total number of base pairs within a given nucleic acid sequence changes following a mutation, they can be classified into two types. Base substitutions occur when one nitrogenous base within a sequence is replaced or substituted by a different nitrogenous base. On the other hand, insertion-deletion mutations, commonly referred to as “in-dels”, occur when new nitrogenous bases are added (insertions) or existing bases are removed (deletions) from the sequence.

Furthermore, even within these two categories, there can be further subclassifications. For example, base substitutions can occur from purine to purine or pyrimidine to pyrimidine (transitions), or from purine to pyrimidine or vice versa (transversions).

Alternatively, base substitutions can also be further subclassified on the basis of whether the change occurs from an AT pair to a GC pair or vice versa.

The term “mutation spectrum” is used to denote the entirety of mutations occurring within an organism. In addition to accounting for all the different types of mutations occurring within a given classification, a mutation spectrum also includes the frequency of these different mutations. This means that a mutation spectrum takes into account each different type of mutation that occurs within a given classification and how frequently it occurs. As a result, mutation spectra can be used to understand the mutations occurring in a given organism in a very quantitative way.

A close study of the mutation spectra of various organisms shows that most organisms have a biased mutation spectrum[2]. This means that the different types of mutations occur at rates that are different from what a simple rule of probability might dictate. For example, in my work, as well as the work that it builds up on, the transition-transversion (Ts-Tv) mutation of different organisms has been studied in great detail. According to the simple rule of probability, transversions (A→C, A→T) can occur twice as many times as transitions (A→G). Therefore, in a completely unbiased system, the probability of transversions occurring is 2/3 or 0.67. However, it was found that most organisms sample more transitions than transversions. For example, our model organism, K-12 *Escherichia coli* MG1655 shows a transversion bias (probability of transversions occurring) of 0.447, which is lower than that of an unbiased system of 0.67[3]. This means that K-12 *E. coli* MG1655 samples more transitions than what would be expected of it.

1.3 How does mutation bias affect bacterial evolution?

As discussed earlier, mutations are the main supplier of raw materials that are genetic variations, upon which natural selection acts, leading to evolution. However, mutation biases, which have been established in a given organism over a relatively long period of evolutionary time, limit the organism’s access to certain kinds of mutations. As a result, mutation biases have a deterministic role to play in an organism’s evolutionary success in a given environment[4–10].

In 2022, work done by Sane et. al.[11] shed light on the role played by such mutation biases on bacterial evolution. Using the MG1655 substrain of K-12 *E. coli*, the group explored the mutation spectrum of transitions and transversions. In their experiments, they used knockout strains of *E. coli* MG1655 that were generated by knocking out certain DNA repair genes. These DNA repair genes were responsible for proofreading certain kinds of errors during DNA replication using specific biochemical pathways. By knocking out these particular genes, the phenotype obtained was a change in the transversion bias of the host strains. Essentially, these knockouts belonged to one of two categories. The first category included strains whose transversion bias was the opposite of the wild type. This means that they sampled a greater number of transversions than the wild type. On the other hand, the second category included strains whose long-established mutation bias had been reinforced. This means that the strains belonging to the second category sampled a greater number of transitions as compared to transversions, than even the wild type. However, it was not possible to uncouple the phenotype of mutation rate from mutation bias, as the knocking out of these DNA repair genes also led to a higher number of mutations per base pair per generation.

Therefore, by using these reversed-bias and reinforced-bias “mutators” (strains with a higher mutation rate than wild type), long-term mutation accumulation experiments were conducted. The main principle behind mutation accumulation experiments is to not exert any sort of selection pressure whatsoever and then observe the kind of mutations that were sampled and fixed along the way. In order to grow these different strains, different environments were used. The 16 different environments used in this experiment differed from each other only in the source of carbon that was available in the culture medium. After a given number of days, the mutations sampled by reversed-bias mutators were compared with those sampled by the wild type.

This experiment led to three main revelations. Firstly, the reversed-bias mutators sampled a greater proportion of mutations that were beneficial to the host than the wild type. The criterion for fitness here was limited to growth rate in liquid medium. Therefore, a beneficial mutation was one which led to an increase in the growth rate of its host as compared to the wild type. Secondly, the beneficial mutations that were

sampled by reversed-bias mutators were found to be beneficial across a greater number of environments than the beneficial mutations sampled by the wild type. Furthermore, a bioinformatics analysis into the evolutionary history of bacteria revealed that most bacteria undergo multiple reversals in their established mutation biases in the course of their evolutionary history. Therefore, it was concluded from this study that the reversal of long-established mutation biases led to a greater opportunity for adaptation in a given environment. The reason behind this observation was hypothesized to be the new-found access to a previously undersampled mutation space.

1.4 Evolution in fluctuating environments

A substantial amount of work has previously been undertaken in evolution under fluctuating environments[12–18]. A considerable portion of those studies use microscopic organisms for the greater ease of dealing with and evolving them, as well as maintaining lines and stock time points of them. Therefore, this multitude of background literature available in the field gives us valuable clues which can be used to try and predict the outcomes of evolution under fluctuating environments, in different scenarios.

For example, Leroi, et. al. used temperature as an environmental factor while evolving bacteria under fluctuating environments in 1994[12]. Using *Escherichia coli* as a model organism, six separate lines were evolved over 2,000 generations. The fluctuations in the environment occurred temporally. This means that every other day, the temperature at which the *E. coli* were incubate would change from 32°C to 42°C and back to 32°C on the following day. The results of this experiment showed that the lines evolved under fluctuations of temperature had become significantly fitter than their ancestors in this variable temperature regime. Furthermore, it was also found that apart from this variable temperature regime, there had been fitness gains at each of the constant component temperatures. This means that the evolved lines had a higher growth rate than their respective ancestors at both, 32°C and 42°C. However, it was found that despite having adapted to both, the variable fitness regime, and the component temperatures of the fluctuations, the lines hadn’t been able to adapt to the

effect of the sudden change in the environment, or the fluctuation. This is because as compared to their ancestors, the evolved lines saw either no change, or a decrease in their fitness, immediately after a fluctuation or change in temperature. The final conclusion of this work following the comparison of their results commented on the variability amongst the lines. The authors note that there was significant variability amongst the lines that had been evolved under fluctuating environments, each taking a different path toward adaptation.

In another study, Kassen et. al. studied the effects of temporal fluctuations in environment on evolution[19]. Using the unicellular alga, *Chlamydomonas reinhardtii*, experimental evolution was carried out using light as an environmental factor. This work helped shed light on how fluctuating environments might lead to the emergence of different trajectories that fill different niches in an ecosystem. It was noted in this study that by evolving under constant environments – either the presence or the absence of light – specialists were evolved. On the other hand, by evolving *Chlamydomonas* through fluctuations of light and dark periods, the emergence of generalists was observed. These generalists were found to be better than their counterparts which had been evolved in constant environments, as well as resistant to fluctuations of light and darkness.

1.5 Impact of mutation rate and bias on adaptation in fluctuating environments

The main foundation of the work done in this project was formed by the findings of Sane et. al. in 2022[11]. To reiterate, the work attempted to highlight the role played by mutation bias on evolution. In order to do this, “mutators” were generated from MG1655 substrain of K-12 strain of *Escherichia coli* by knocking out DNA repair genes that are responsible for proofreading certain kinds of errors that are experienced during DNA replication. These mutators were either reversed-bias – with a mutation bias opposite to that experienced by the wild type – or reinforce-bias – with a mutation bias strengthened towards the direction of bias experienced by the wild type. These different strains were used in conducting mutation accumulation experiments in 16 different environments, which differed from each other in the carbon source available

in each of them. After growing multiple lines of these reversed-bias and reinforced-bias mutators over a long period of time, the mutations that had accumulated in each of them in the absence of a selection pressure were evaluated by using Next-Generation Sequencing. The distribution of fitness effects (DFE) plots generated for each of the lines in all 16 environments revealed that reversed-bias mutators sampled a greater proportion of beneficial mutations as compared to the wild type. This phenomenon was attributed to the sampling of previously under-sampled mutation spaces by the reversed-bias mutators. Furthermore, it was also found that the beneficial mutations sampled by reversed-bias mutators were beneficial across a greater number of environments than the beneficial mutations sampled by the wild type.

Building up on these two key takeaways of the study, we hypothesized the following:-

1. Owing to the greater supply of beneficial mutations, reversed-bias mutators should adapt to a greater degree in a given environment as compared to a wild type strain of *E. coli*.
2. Since the beneficial mutations sampled by reversed-bias mutators were beneficial across a greater number of environments, they should also adapt to a wider range of environments compared to wild type *E.coli*.

In order to prove this hypothesis, I needed to perform experimental evolution studies on these different mutator strains of *E. coli* in multiple environments at once. However, as pointed out earlier, a bulk of literature existed that highlighted evolutionary scenarios in fluctuating environments. The experimental evolution regimes used in those studies enabled the evolution of model organisms in multiple environments at the same time. Therefore, in the interest of time, I designed an experimental regime that allowed us to do this by fluctuating the environment – media containing different carbon sources – over a temporal scale.

My initial expectations from my experiments were as follows:-

1. Mutation Bias – Mutators with reversed bias will adapt to a greater degree than reinforced-bias mutators. This is because they sample a greater proportion of beneficial mutations than reinforced-bias mutators.

2. Mutation Rate – Mutators with a higher mutation rate will adapt to a greater extent than mutators with an intermediate mutation rate (in comparison to wild type). This is because they will sample a greater number of mutations in total than mutators with an intermediate mutation rate.

By attempting to experimentally highlight the combined effect of mutation rate and bias on bacterial evolution under fluctuating environments, I here try to contribute to a so far relatively underexplored area of investigation. Despite the relatively small number of studies undertaken in this niche area of evolutionary biology, such studies will only grow in relevance with time. This is primarily because of two main reasons.

Firstly, such studies provide a good starting point for undertaking similar investigations in clinical settings. More often than not, multiple antimicrobial drugs are prescribed as part of the treatment regimens for bacterial infections. Such drugs are usually administered in a temporally determined sequence, leading to what can be termed as a fluctuating environment. By understanding the role played by mutation rate and bias in bacterial evolution, better therapeutic practices might be developed to treat bacterial infections[20–24].

Secondly, such studies also hold much ecological relevance. As the climate warms in increasing amounts in the Anthropocene era, many ecological niches, which might be far from the reaches of human exploration, are subjected to increasingly fluctuating environments. Under such circumstances, the development of techniques to build resilience amongst bacterial and other communities in different ecosystems is becoming increasingly relevant in the 21st century. By providing clues into how integral aspects of bacterial evolution – such as mutation rate and bias – I aim to strengthen such attempts to conserve and nurture the biodiversity of our planet.

Chapter 2 Materials and Methods

2.1 Strains and media

I used the sub-strain MG1655 of the strain K-12 of *Escherichia coli* as a model organism for all of our evolution experiments. This strain has been referred to as wild type (WT) in my experiments. Furthermore, whole gene deletions had previously been performed in the lab on *mutH*, *mutY*, *mutT*, *mutS*, *mutL*, and *nth-nei* to generate the corresponding mutators listed below in Table 2.1. Their respective mutation rates and biases, in comparison to WT have been mentioned alongside.

Table 2.1: Strains used for bacterial evolution experiments, and their respective mutation rates and biases.

Strain	Mutation Rate (bp^{-1} gen^{-1})	Mutation Bias
ΔmutT	3.22E-08 (High)	Reversed – 0.997
ΔmutH	2.35E-08 (High)	Reinforced – 0.029
ΔmutY	2.21E-09 (Intermediate)	Reversed – 0.948
Δnth-Δnei	9.79E-10 (Intermediate)	Reinforced – 0.493
ΔmutS	2.76E-08 (High)	Reinforced – 0.025
ΔmutL	2.46E-08 (High)	Reinforced – 0.032

2.2 Limited carbon sources as fluctuating environments

Previous work done in the lab measured the fitness of reversed-bias mutators in media containing one sugar for each medium as the only source of carbon. Here, I have built on that work by analyzing data from the above-mentioned work to choose sugar-enriched media depending upon the number of beneficial mutations available for sampling to strains that have opposite biases. Of the 16 environments tested by Sane et. al., in 9 environments, the reversed-bias mutator sampled a greater proportion of beneficial mutations than the wild type, in two environments, the wild type sampled a greater proportion of beneficial mutations than the reversed-bias mutator, and in the remaining 5 environments, the proportion of beneficial mutations was similar in both

strains[11]. For my experiments, I have built upon this knowledge in choosing the environments for evolution lines.

2.3 "*Everything Everywhere All at Once*" – The pilot experiment

As mentioned in the previous section, the 16 environments previously used by Sane, et. al. could be divided into three categories, depending on the proportion of beneficial mutations sampled by reversed-bias mutators and the wild type[11]. Before initiating long-term evolution experiments, I undertook a small pilot experiment. The motivation behind undertaking this pilot experiment was to not only learn experimental bacterial evolution using *E. coli*, but also to get accustomed to the various challenges that are inherent to such evolution experiments conducted in the laboratory. Furthermore, this experience also helped me make the appropriate choice of environments and the scaling-up process required to undertake the main experiments.

In order to undertake this pilot experiment, I chose four environments from the total set of 16 environments, depending on the behaviour of reversed-bias mutator and the wild type in them. The environments and the corresponding strain behaviour are listed below in Table 2.2.

Table 2.2: Salts of organic acids which were used as the only source of carbon to enrich the M9 minimal medium in the pilot experiment. Media in which the reversed-bias mutator sampled a greater proportion of beneficial mutations than the wild type have been highlighted in shades of green, whereas media in which the wild type sampled a greater proportion of beneficial mutations have been highlighted in shades of red.

Carbon source	Beneficial mutations
Glucuronate	ΔmutY > WT
Succinate	ΔmutY > WT
Gluconate	ΔmutY < WT
Galactose	ΔmutY < WT

The ancestral stocks of the strains used had been stored at -80°C in 30% glycerol. Prior to the start of the pilot experiment, I inoculated 2µl of the glycerol stocks 1ml of LB in 15ml falcon tubes and incubated at 37°C for 14 hours, with the platform shaking

at 180 rotations per minute (rpm). I did this revival period in liquid medium to ensure the viability of cells at the beginning of the evolution. I maintained the evolution lines in 48-well plates. I poured 600µl of media in the wells of the plate and inoculated on the 0^{th} day of the evolution with 6µl of the overnight liquid culture. Following the inoculation, I incubated the 48-well plates for 24 hours at 37°C and 180 rpm. At the end of 24 hours, I transferred 6µl of the liquid cultures into 600µl of the corresponding fresh media in 48-well plates. As a result, I maintained a bottleneck ratio of 1:100 in the evolution lines after every 24 hours. I have maintained this standard method of inoculation and incubation (6µl in 600µl media, and 37°C at 180 rpm) was in all evolution lines, and this has been followed in the pilot experiment, as well as in the subsequent main experiments. This general methodology that was followed during all the experimental evolutions lines has been visually represented in Figure 2.1 below. In the pilot experiment, I maintained four replicates of wild type, and three replicates each of all other strains.

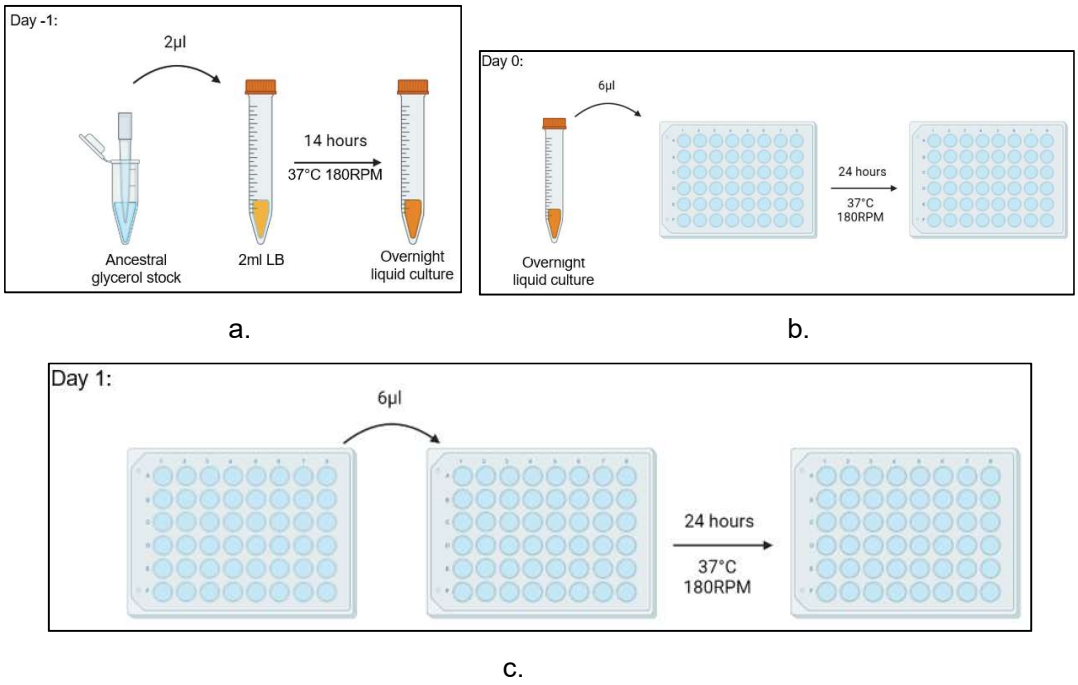


Figure 2.1: a. Day -1 (one day prior to the beginning of experimental evolution), **b.** Day 0 (day of beginning of experimental evolution), and **c.** Day 1 (one day after the beginning of experimental evolution).

For the pilot experiment, I induced fluctuations in the populations at a fixed interval of every 24 hours by changing the carbon source present in the media. However, I carried out these fluctuations in three different regimes. These three regimes have been visually represented in Figure 2.2 below.

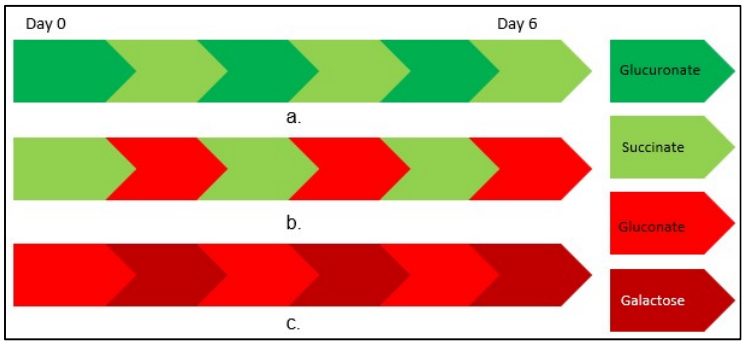


Figure 2.2: a. Regime 1: Daily fluctuations between Glucuronate and Succinate for 6 days, **b.** Regime 2: Daily fluctuations between Succinate and Gluconate for 6 days, **c.** Regime 3: Daily fluctuations between Gluconate and Galactose for 6 days.

After every 3 days of evolution, I collected and stored time points from these evolution lines. I did this by mixing equal volumes of liquid culture from the 48-well plates, and 60% glycerol. As a result, the final concentration of glycerol in these frozen time points or stocks was 30%. After vigorously shaking the mixtures to facilitate proper homogenization, I stored the stocks at -80°C. For conducting the evolution experiments in 48-well plates, I followed a checker-board pattern during inoculation, as shown in Figure 2.3. I did this to check for contamination in the media or cross-contamination across different strains.

T1		H1		Y1		N1		GRN/SCN
	T2		H2		Y2		N2	
T3		H3		Y3		N3		
	T1		H1		Y1		N1	SCN/GLN
T2		H2		Y2		N2		
	T3		H3		Y3		N3	

T1		H1		Y1		N1		GLN/GAL
	T2		H2		Y2		N2	
T3		H3		Y3		N3		
	W1		W2		W3		W4	GRN/SCN
W1		W2		W3		W4		SCN/GLN
	W1		W2		W3		W4	GLN/GAL

Figure 2.3: Plate scheme followed during the pilot experiment. Here, T1 refers to replicate no. 1 of strain ΔmutT, H2 refers to replicate no. 2 of strain ΔmutH, and so on. GRN: Glucuronate, SCN: Succinate, GLN: Gluconate, and GAL: Galactose. GRN/SCN denotes fluctuations occurring between Glucuronate and Succinate.

2.4 Experiment 1: Adaptation to fluctuations between Glucuronate and Succinate

Following the end of the pilot experiment, I ran a bigger set of experimental evolution. The explicit aim of this experiment was to try and determine the effects of mutation rate and mutation bias in bacterial adaptation under fluctuating environments. We know from Sane, et. al. that a reversal of long-established mutation biases leads to the sampling of a greater proportion of beneficial mutations[11]. It was also found in the same study that the beneficial mutations sampled by reversed-bias mutators were beneficial across a greater number of environments than the beneficial mutations sampled by the wild type. Therefore, I hypothesized that reversed-bias mutators would adapt to a greater extent as compared to strains with the same direction of Transversion bias as the wild type. In order to test this hypothesis, I ran an experimental evolution set for a period of 30 days.

For this set of experimental evolution, the strains that I chose were ΔmutT, ΔmutY, WT, ΔmutH, Δnth-Δnei, ΔmutS, and ΔmutL. The environments that I chose for the populations to be fluctuated between were Glucuronate and Succinate. This is because both of these environments belonged to the first category of the 16 environments, wherein, the reversed-bias mutator sampled a greater proportion of beneficial mutations than the wild type. Therefore, during each period spent in each environment, I expected a greater proportion of beneficial mutations to be sampled by the reversed-bias mutators. Furthermore, by fluctuating between both environments belonging to this same category, I expected the difference in extent of adaptation between the reversed-bias and reinforced-bias mutators to be noticeable by the end of the experimental evolution.

In order to conduct this experiment at a scale bigger than that of the pilot experiment, I used a greater number of biological replicates. I used 8 replicates of ΔmutT, ΔmutY, WT, ΔmutH, and Δnth-Δnei. ΔmutS and ΔmutL, both had a high rate of mutation and transversion bias reinforced in the direction of the mutation bias of the wild type, i.e., they were transition biased. Since the same characteristics were exhibited by ΔmutH,

I used only 4 replicates each of ΔmutS and ΔmutL. I did this to reduce redundancy in my results at a later stage.

Apart from following the same protocol for experimental evolution as mentioned above in Section 2.3 for daily transfers and maintaining a 1:100 bottleneck ratio, I altered the frequency of fluctuations in the main experiment. Existing literature suggests that the evolutionary trajectory of populations evolving under fluctuating environments are impacted significantly by the duration and frequency of fluctuations. Therefore, in order to replicate these findings, I followed two experimental regimes to evolve the same populations in Experiment 1. These regimes were as follows:-

1. Regime 1 – The environment is changed from Glucuronate to Succinate and then back to Glucuronate at an interval of every 3 days.
2. Regime 2 – The environment is changed from Glucuronate to Succinate and then back to Glucuronate at an interval of every 6 days.

The experimental design followed in Experiment 1 has been visually represented below in Figure 2.4.

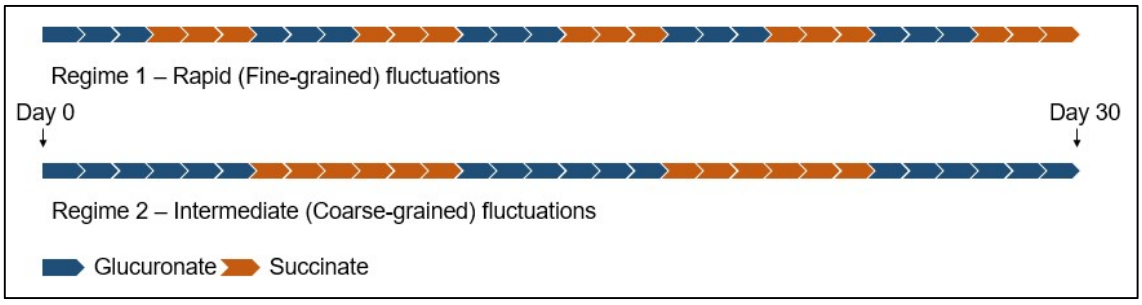


Figure 2.4: Experimental design followed in Experiment 1. The environments that the populations were fluctuated between were Glucuronate and Succinate, which have been represented using different colours. In regime 1, the fluctuations (environment changes) were induced at an interval of 3 days, whereas in regime 2, the fluctuations were induced at an interval of 6 days.

After every 3 days of evolution, I collected and stored time points from these evolution lines. I did this by mixing equal volumes of liquid culture from the 48-well plates, and 60% glycerol. As a result, the final concentration of glycerol in these frozen time points or stocks was 30%. After vigorously shaking the mixtures to facilitate proper homogenization, I stored the stocks at -80°C.

Experiment 1 was a huge experiment in terms of logistics. This is because 8 replicates each of 5 strains and 4 replicates each of 2 strains had to be evolved. I evolved these 48 populations in two separate regimes. Therefore, it led to a total of 96 populations being evolved. Additionally, I made and stored glycerol stocks of time points for both regimes every 3 days, that, if done simultaneously, would have led to 96 each day, every 3 days, for a duration of 30 days of evolution. Consequently, to reduce this logistical challenge as well as the chances of error during experiments, I further divided Experiment 1 into two batches. Batch 1 consisted of 8 replicates each of ΔmutT, ΔmutY and WT and Batch 2 consisted of 8 replicates each of ΔmutH and Δnth-Δnei and 4 replicates each of ΔmutS and ΔmutL. Each of these batches followed the same experimental regime as visually represented earlier in Figure 2.4. The plate scheme followed in Experiment 1 has been visually represented in Figure 2.5 below.

T1		T3		T5		T7	
	T2		T4		T6		T8
Y1		Y3		Y5		Y7	
	Y2		Y4		Y6		Y8
W1		W3		W5		W7	
	W2		W4		W6		W8

H1		H3		H5		H7	
	H2		H4		H6		H8
N1		N3		N5		N7	
	N2		N4		N6		N8
S1		S3		L1		L3	
	S2		S4		L2		L4

Figure 2.5: Plate scheme followed in Experiment 1. As with the pilot experiment, the letters and the respective numbers accompanying them refer to their corresponding strains and replicate numbers. Furthermore, the checkerboard pattern has been followed again with empty wells in order to check for contamination and cross-contamination in the plates.

2.5 Experiment 2: Adaptation to fluctuations between Glucose and Lactose

Experiment 1 consisted of 96 populations of 7 strains evolving in two batches which, in turn, followed two experimental regimes each. For this experiment, the environments that I chose for fluctuations were M9 minimal media enriched with Glucuronate and Succinate. From the first experiment, I found a set of results which I used to shed light on the adaptive behaviour of reversed-bias and reinforced-bias mutators in Glucuronate and Succinate. However, this behaviour could be the result of mutations sampled and accumulated by the mutators in Glucuronate and Succinate that were specific to these environments. Therefore, to find out whether there exists a degree of universality in the results found in Experiment 1, I designed a second experiment using a different set of carbon sources.

For experiment 1, I evolved the populations under fluctuations between Glucuronate and Succinate. I made this choice of environments on the basis of whether reversed-bias mutators sampled a greater proportion of beneficial mutations in these environments than wild type *E. coli*. For the second experiment, I attempted to venture a little further out of this space. This time, I made the choice of environments on the basis of proportion of Synergistic Pleiotropy experienced by reversed-bias mutators and the wild type in each environment.

Synergistic pleiotropy or SP refers to the phenomenon wherein, the effect shown by a particular mutation in a given environment is conserved in its nature in another environment. For example, if a mutation is beneficial across environments A and B, then the phenomenon is called positive synergistic pleiotropy, or positive SP. Alternatively, if a mutation is deleterious across both environments A and B, the phenomenon is called negative synergistic pleiotropy or negative SP.

For making the choice of environments for Experiment 2, I used pre-existing data from work previously done in the lab. I compared the proportion of positive synergistic pleiotropy shown by the reversed-bias mutator and wild type in each environment pair.

Next, I compared the difference between the proportion of positive SP shown by each strain in each environment pair. Finally, I shortlisted environment pairs with the highest positive difference in positive SP proportion between the reversed-bias mutator and wild type for fluctuations in Experiment 2. Finally, I chose the environment pair of Glucose and Lactose owing to strains' behaviour in it matching the ones required for this experiment.

I followed the same protocol for experimental evolution in Experiment 2 as had been followed in the pilot experiment and Experiment 1. Furthermore, I also kept the strains used and the respective numbers of their replicates constant – 8 replicates each of ΔmutT, ΔmutY, WT, ΔmutH and Δnth-Δnei and 4 replicates each of ΔmutS and ΔmutL. Additionally, I also kept the checkerboard pattern shown in Figure 2.5 constant in the 48-well cell culture plates that were used in Experiment 2. I followed the experimental regime corresponding to Regime 1 in Experiment 1 for running Experiment 2 – fluctuations between Glucose and Lactose induced every 3 days. Since I followed only one experimental regime for Experiment 2, I evolved the entire set, all 48 populations, in a single batch in one go. After every 3 days of evolution, I collected and stored time points from these evolution lines. I did this by mixing equal volumes of liquid culture from the 48-well plates, and 60% glycerol. As a result, the final concentration of glycerol in these frozen time points or stocks was 30%. After vigorously shaking the mixtures to facilitate proper homogenization, I stored the stocks at -80°C.

2.6 Fitness measurements: Growth curves to measure extent of adaptation

In order to analyze the results of the experiments undertaken, I took growth rate as a proxy for fitness. Therefore, by calculating the relative increase in growth rate of each population with respect to its own ancestor, I determined the extent of adaptation. In order to achieve this, I set up growth curve readings at 37°C with 180 rpm shaker speed for approximately 16 hours. I used Biotek and Tecan spectrophotometric plate-readers to measure the turbidity of the bacterial cultures at 600nm wavelength of light over 16 hours. I then plotted this data using the app, Curve Fitter and determined the linear growth rate during the exponential phase of the growth curve. I then used the

growth rates of the evolved populations and their respective ancestors to calculate the relative change in fitness using the following formula:

$$Relative\ fitness\ = \frac{Gr_{evolved}}{Gr_{ancestor}} - 1$$

Prior to setting up growth curves, I inoculated 2µl of stored glycerol stocks in 600µl of the same media that would be used in the growth curve. For example, if the growth curve was measured in Glucuronate, I also did the revival in Glucuronate. Following the inoculation, I incubated the cultures were at 37°C and 250rpm for approximately 14 hours. After the completion of the incubation period, I then used these revived liquid cultures to inoculate the 48-well plates used for measuring growth curves.

Each 48-well plate that was used for setting up growth curves contained 3 technical replicates each of both, the evolved population and the ancestral population. Furthermore, each plate also contained 3 wells containing an ancestral wild type population as a positive control, and 3 wells filled with 600µl of the media used in the growth curves but without an inoculum, as a negative control.

Chapter 3 Results

The results below show the adaptive trajectories followed by the populations evolving in Regime 1 (3-day fluctuations) alone. The fitness measurements of Regime 2 (6-day) fluctuations were not possible within the timeframe of this project and have, hence, not been shown as part of the following results.

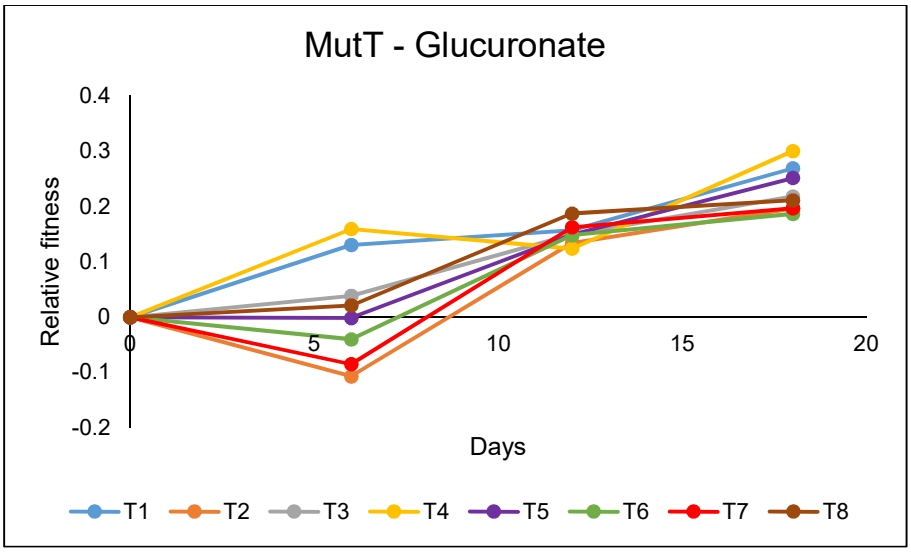
3.1 ∆mutT adapting to 3-day fluctuations

In Figure 3.1, the time series of all 8 biological replicates of ∆mutT has been shown over a period of time over which they were evolved. The fitness measurements were taken at three time points - day 6, day 12, and day 18.

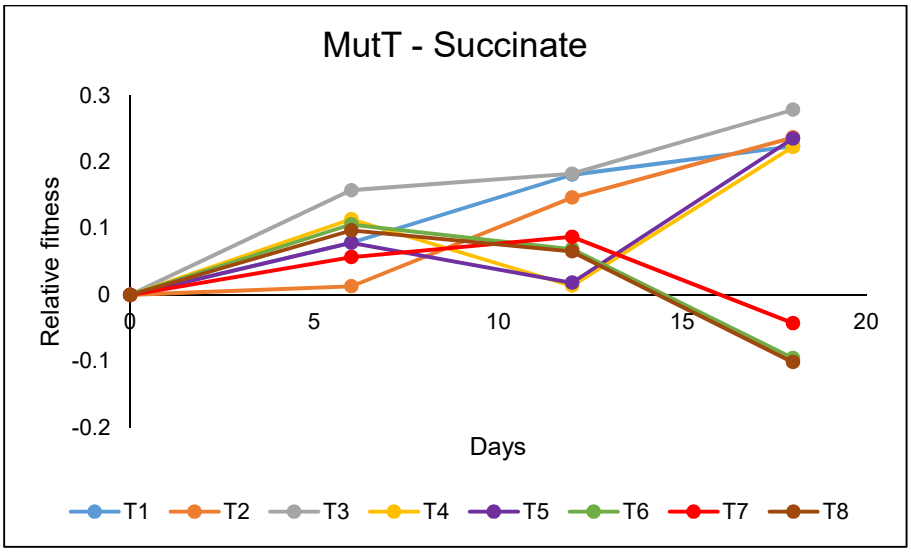
At day 6, the variability in fitness in Glucuronate (Panel a) was the most, with three replicates - T2, T6 and T7 - showing a slower growth rate as compared to their ancestors, after 6 days of evolution. At day 12, this variability was greatly reduced, as the relative fitnesses of all 8 replicates are seen to be clustered between 0.1 and 0.2. Finally, at the day 18 time point, the relative fitness of all 8 replicates of ΔmutT were found to increase slightly. It should be noted that the relative fitnesses of the three replicates showing a decrease in fitness at day 6 - T2, T6 and T7 - were clustered at a slightly lower level than the other replicates at day 18. However, this difference in relative fitness was not significant.

In Panel b of Figure 3.1, it can be seen that in Succinate, the relative fitness of none of the replicates of ∆mutT was less than that of their respective ancestors. However, at day 6, similar to Glucuronate, the fitness of ∆mutT was also very variable in Succinate. At day 12, the fitness in Succinate saw an increase in the degree of variability, as the replicates mostly followed separate trajectories of adaptation. This further gave rise to an even greater degree of variability in the fitness in Succinate at day 18. It should be noted that two replicates – T4 and T5 – followed a somewhat clustered trajectory, with their relative fitness first increasing at day 6, then falling at day 12, and then again recovering at day 18. On the other hand, three other replicates

– T6, T7, and T8 – also followed clustered adaptive trajectories, with all three of them having a lower relative fitness in Succinate than their respective ancestors.



a.



b.

Figure 3.1: The relative fitness of all 8 replicates of ΔmutT in plotted over three time points – day 6, day 12, and day 18. **a.** In Glucuronate **b.** In Succinate.

On comparing the relative fitness of ΔmutT replicates in Succinate, it is noticed that the replicates with a much lower fitness in Succinate at day 18 – T6, T7, and T8 – all experienced very minor increases in their relative fitness in Glucuronate between day 12 and day 18. While these results do not clearly suggest whether there is a role

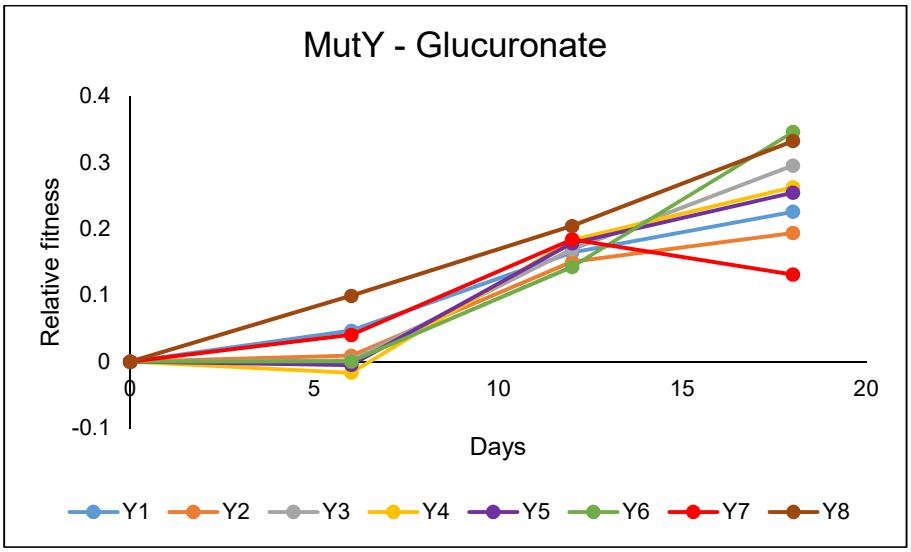
played by tradeoffs in the adaptation of ΔmutT to these two media, the trajectory followed by T6, T7, and T8 might suggest that after 12 days of evolution, that is 2 complete cycles of fluctuation between Glucuronate and Succinate, over the next 6 days of evolution, that is in the third cycle of fluctuation, only a slight increase in fitness in Glucuronate led to a greater drop in fitness in Succinate.

3.2 ΔmutY adapting to 3-day fluctuations

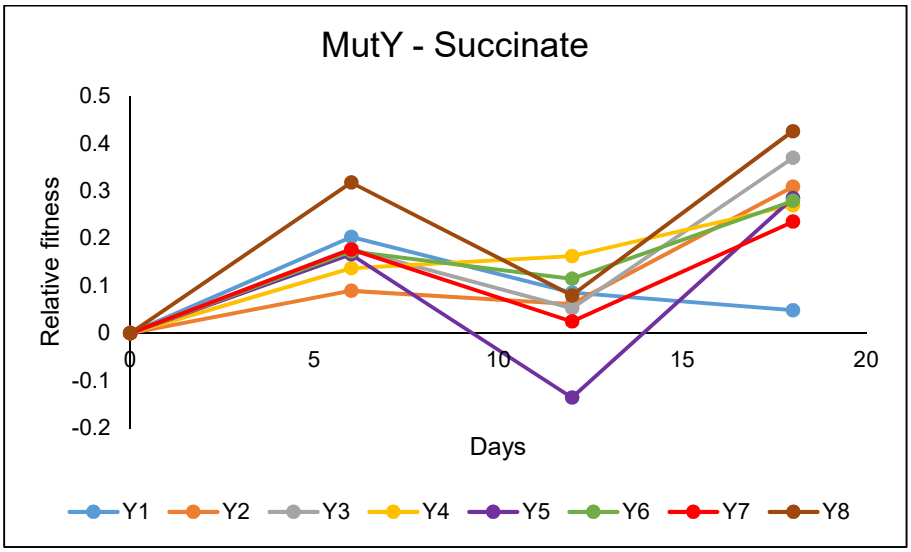
Figure 3.2 shows the relative fitness of all 8 of the biological replicates of ΔmutY over a period of 18 days. The results have been represented in the form of a time series, with time points at day 6, day 12, and day 18.

Panel a of Figure 3.2 shows the relative fitness of ΔmutY in Glucuronate. Similar to what was seen in the case of ΔmutT, the variability in the relative fitness of replicates was greater at day 6 than it was at day 12, and it was found to be the greatest at day 18, the third time point of the time series. However, unlike ΔmutT, the replicates of ΔmutY did not show any major clustering in their trajectories in Glucuronate. Furthermore, Y7 was the only replicate which experienced a reduction in its relative fitness from day 12 to day 18.

Panel b of Figure 3.2 shows the time series of relative fitness of ΔmutY in Succinate. Unlike in Glucuronate, wherein, the trajectories of adaptation were mostly upwards trending, in Succinate, ΔmutY first adapted to Succinate to a greater degree than its ancestor, followed by a dip in its fitness, and finally ending with a recovery in fitness in most replicates. Y4 exhibited an upward trend throughout the period, whereas the downward trend in relative fitness experienced by Y1 in Succinate continued till day 18. Interestingly, Y5 experienced a much greater dip in its relative fitness in Succinate at day 12 than the other replicates, with a growth rate slower than not only its day 6 time point, but also its day 0 ancestor. However, this relative fitness made a noticeable recovery at day 18.



a.



b.

Figure 3.2: The relative fitness of all 8 replicates of ΔmutY in plotted over three time points – day 6, day 12, and day 18. **a.** In Glucuronate **b.** In Succinate.

When the relative fitness of the replicates of ΔmutY in Glucuronate and Succinate are compared, a somewhat antagonistic behaviour can be observed. In Glucuronate, the relative fitness of replicates either deteriorated or increased very marginally at day 6, with respect to their ancestors. At the same time, the relative fitness of replicates in Succinate at day 6 was found to mostly increase at day 6. The only exception to this trend was observed in Y8, which showed an increase in its relative fitness at day 6 in both Glucuronate and Succinate. At the next time point – day 12 – this opposite trend

in trajectory was maintained. In Glucuronate, the relative fitness of replicates, which had seen only minor improvements at day 6, now saw a jump in their fitness at day 12. However, in Succinate, the replicates, which seemed to have made a noticeable improvement in their relative fitness at day 6, were now experiencing either a stagnation in their trajectories, or a deterioration in comparison to their day 6 fitness. It was only at day 18 that this perceived antagonism in the trajectories of ΔmutY replicates was resolved. At day 18, nearly all the replicates were seen to improve their relative fitness with respect to their day 12 time points. Hence, it can be concluded that the replicates of ΔmutY experienced some sort of tradeoffs in fitness while evolving under fluctuations between Glucuronate and Succinate.

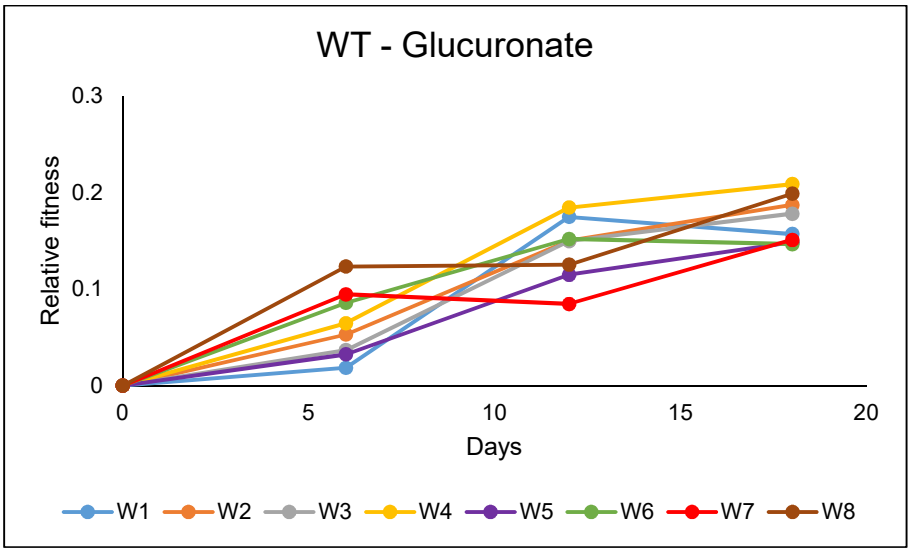
3.3 WT adapting to 3-day fluctuations

Figure 3.3 shows the relative fitness of all 8 of the biological replicates of WT over a period of 18 days. The results have been represented in the form of a time series, with time points at day 6, day 12, and day 18.

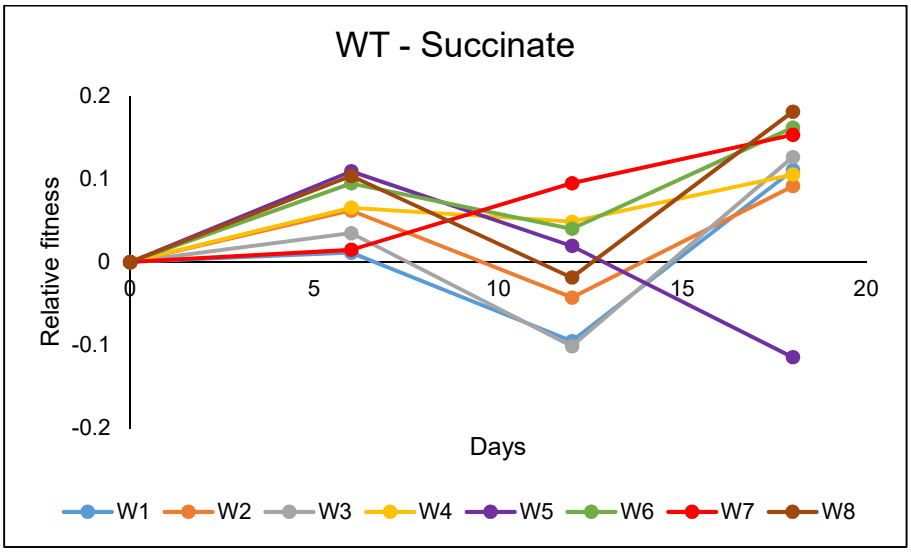
Panel a of Figure 3.3 shows the relative fitness of WT in Glucuronate. At both the day 6 and day 12 time points, the replicates of WT showed a lot of variability in their relative fitness in Glucuronate. However, this variability was found to be slightly reduced at day 18, as their relative fitness in Glucuronate seemed to converge towards a higher point. It should be noted that at day 18, some minor clustering was also observed in the relative fitness. The cluster showing a higher relative fitness consisted of W2, W3, W4, and W8. On the other hand, the cluster showing a lower relative fitness consisted of W1, W5, W6, and W7. The trajectory of the relative fitness in Glucuronate of nearly all replicates were upwards trending throughout the time series. However, two replicates – W7 and W8 – showed an exception to this trend. The relative fitness of both of these replicates in Glucuronate was higher than their day 6 time points. However, in the case of W7, its relative fitness in Glucuronate was lower at day 12 than it was at day 6. On the other hand, W8 seems to have experienced a stagnation from day 6 to day 12, with its relative fitness in Glucuronate having increased only very slightly between these two time points.

Panel b of Figure 3.3 shows the time series of relative fitness of WT in Succinate. Unlike in Glucuronate, wherein, the trajectories of adaptation were mostly upwards trending, in Succinate, WT first adapted to Succinate to a greater degree than its ancestor, followed by a dip in its fitness, and finally ending with a recovery in fitness in most replicates. W7 exhibited an upward trend throughout the period, whereas the downward trend in relative fitness experienced by W5 in Succinate continued till day 18. Interestingly, the relative fitness W1 and W3 seemed clustered at day 12. However, at day 18, the relative fitness of replicates was found clustered in two main groups. The cluster with the slightly higher relative fitness in Succinate at day 18 consisted of W6, W7, and W8. On the other hand, the cluster with the slightly lower fitness in Succinate at day 18 consisted of W1, W2, W3, and W4. Apart from this, the relative fitness of W5 in Succinate dropped lower at day 18 than the fitness of its ancestor at day 0.

When the relative fitness of the replicates of WT in Glucuronate and Succinate are compared, a somewhat antagonistic behaviour can be observed. At day 6, the replicates experienced a comparable improvement in their fitness in both Glucuronate and Succinate. At the next time point – day 12 – an opposite trend in trajectory was observed. In Glucuronate, the relative fitness of replicates continued to rise expect in W7 and W8. However, in Succinate, the replicates, which seemed to have made a noticeable improvement in their relative fitness at day 6, were now experiencing either a stagnation in their trajectories, or a deterioration in comparison to their day 6 fitness. The exception to this observation at day 12 was W7, which continued experiencing an upward trend in relative fitness in Succinate even on day 12. It was only at day 18 that this perceived antagonism in the trajectories of ΔmutY replicates was resolved. At day 18, nearly all the replicates were seen to improve their relative fitness with respect to their day 12 time points. Hence, it can be concluded that the replicates of WT experienced some sort of tradeoffs in fitness while evolving under fluctuations between Glucuronate and Succinate.



a.



b.

Figure 3.3: The relative fitness of all 8 replicates of WT in plotted over three time points – day 6, day 12, and day 18. **a.** In Glucuronate **b.** In Succinate.

3.4 ΔmutH adapting to 3-day fluctuations

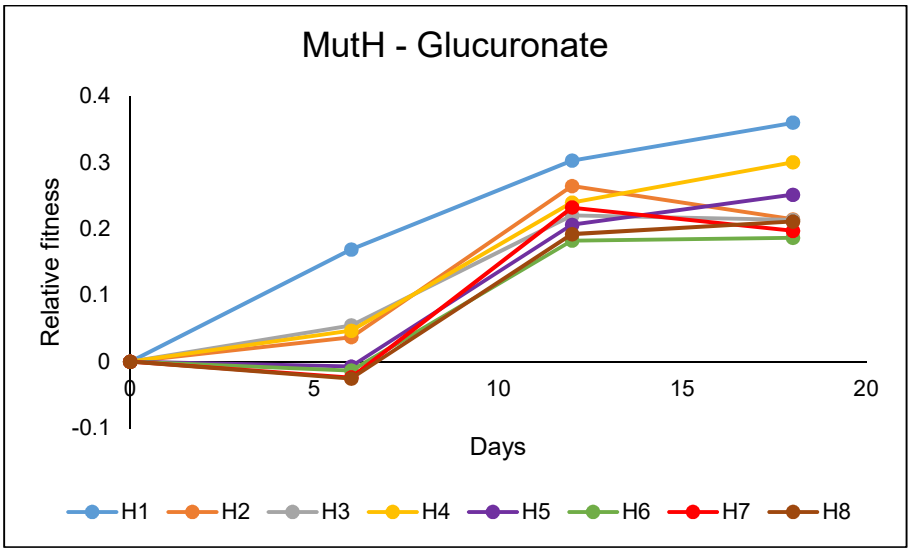
Figure 3.4 shows the relative fitness of all 8 of the biological replicates of ΔmutH over a period of 18 days. The results have been represented in the form of a time series, with time points at day 6, day 12, and day 18.

Panel a of Figure 3.4 shows the relative fitness of ΔmutH in Glucuronate. At all three time points – day 6, day 12, and day 18 – replicates of ΔmutH showed a lot of variability in their relative fitness in Glucuronate. It should be noted that at day 6, some clustering was observed in the relative fitness. The relative fitness of the first cluster was greater than that of the ancestor. This cluster consisted of three replicates – H2, H3, and H4. The relative fitness of the second cluster was less than that of their ancestors. This means that the replicates in the second cluster deteriorated in their fitness in Glucuronate between day 0 and day 6, or after one complete cycle of fluctuation between Glucuronate and Succinate. At the second time point, day 18, a different picture emerged. Both clusters – one where the replicates had improved in their fitness in Glucuronate after 6 days of evolution, and the other where the replicates had deteriorated in their fitness in Glucuronate after 6 days of evolution – seemed to converge in their relative fitness at day 12. This means that the replicates of the second cluster adapted faster between day 6 and day 12, to reach the similar level of adaptive fitness as the replicates in the first cluster. At day 18, there was further change in these trajectories. Out of the seven replicates that had previously been clustered in their relative fitness in Glucuronate, five showed a convergence in their relative fitness at day 18. Within these five replicates, H8 experienced a very slight improvement in its fitness in Glucuronate between day 12 and day 18. On the other hand, all four of the five other replicates deteriorated in their fitness between day 12 and day 18. Two replicates – H4 and H5 – separated from this new cluster at day 18 to continue on an upwards trending trajectory from day 12 to day 18. The only replicate that continued to be an exception to the trends experienced by all other replicates throughout the time series was H1. From day 6 to day 18, H1 experienced an uninterrupted upwards trajectory in its relative fitness in Glucuronate, ultimately reaching a fitness at day 18 that was higher than all other replicates.

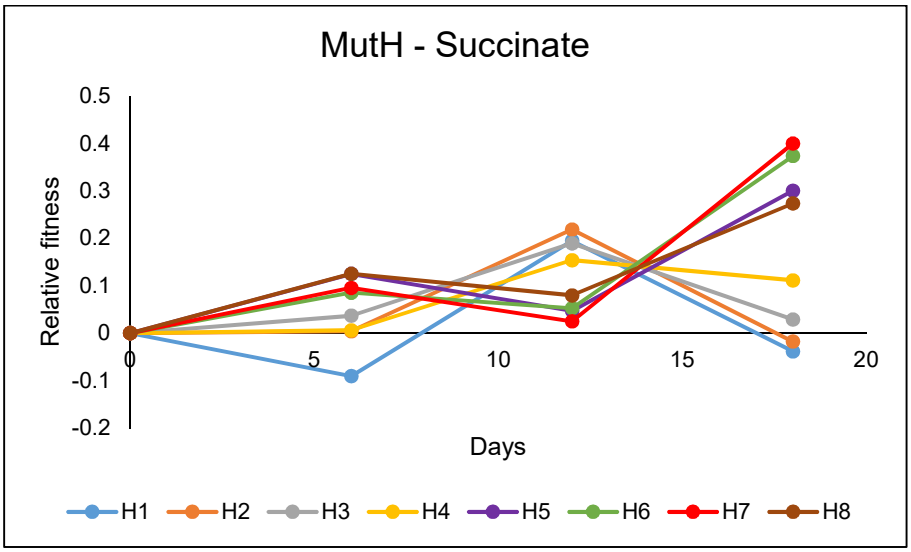
Panel b of Figure 3.4 shows the time series of relative fitness of ΔmutH in Succinate. The trajectory of the relative fitness of the replicates of ΔmutH were the most unusual of all the strains used whose fitnesses were measured. This is because, right from day 6, replicates of ΔmutH experienced a very noticeable clustering effect at all time points. Furthermore, the two clusters observed in the time series also ran antagonistically to each other throughout the series. At day 6, the first cluster – consisting of H5, H6, H7, and H8 – experienced a noticeably increase in their fitness in Succinate with respect

to their ancestors. On the other hand, the second cluster – consisting of H1, H2, H3, and H4 – experienced either negative change or very little positive change in their fitness in Succinate from day 0 to day 6. Moving on to day 12, the first cluster – consisting of H5, H6, H7, and H8 – deteriorated in their fitness in Succinate. On the other hand, the second cluster seen at day 6 – consisting of H1, H2, H3, and H4 – now experienced a jump on their fitness in Succinate and overtook the first cluster in terms of relative fitness in Succinate at day 12. The tables were turned for a second time at the day 18 time point. This time, the first cluster, which had high relative fitness initially at day 6, deteriorated in fitness at day 12, now overtook the second cluster at day 18 to have higher relative fitness in Succinate. On the other hand, the second cluster, which initially had a less advantaged start at day 6, then overtook the first cluster at day 12, now nosedived in fitness, with two replicates – H1 and H2 – being less fit than their ancestors at day 18.

By comparing the time series of relative fitness of ΔmutH replicates in Glucuronate and Succinate, one can argue that some tradeoffs are being experienced by the replicates throughout the duration. For example, the first cluster in the Succinate time series first increased fitness in Succinate, then decreased and then again shot up in Succinate. Whereas in Glucuronate, the same replicates first decreased in fitness, then caught up and then finally decreased at day 18. Similarly, the second cluster followed a similarly antagonistic path, experiencing a dip in the relative fitness in Succinate at times when an improvement in fitness in Glucuronate was seen. Two exceptions to this trend were H1 and H5. H1 exhibited continuous growth in its fitness in Glucuronate, even at day 12, when fitness in Succinate peaked. This suggests that H1 did not experience as strong tradeoffs as the other replicates. Similarly, H5, after undergoing moderate improvement in its fitness in Glucuronate at day 12, continued to see an increase in its fitness in Glucuronate, despite its fitness in Succinate peaking at day 18.



a.



b.

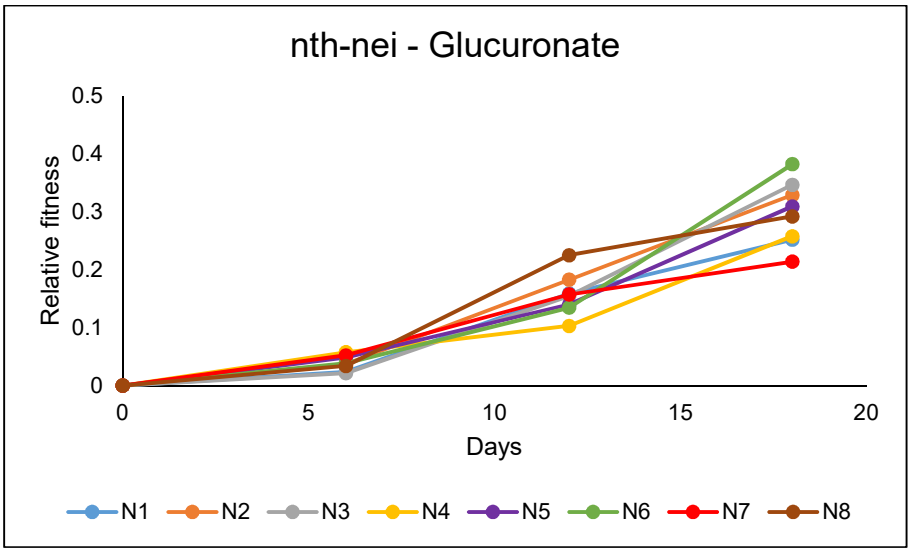
Figure 3.4: The relative fitness of all 8 replicates of ΔmutH in plotted over three time points – day 6, day 12, and day 18. **a.** In Glucuronate **b.** In Succinate.

3.5 Δnth-Δnei adapting to 3-day fluctuations

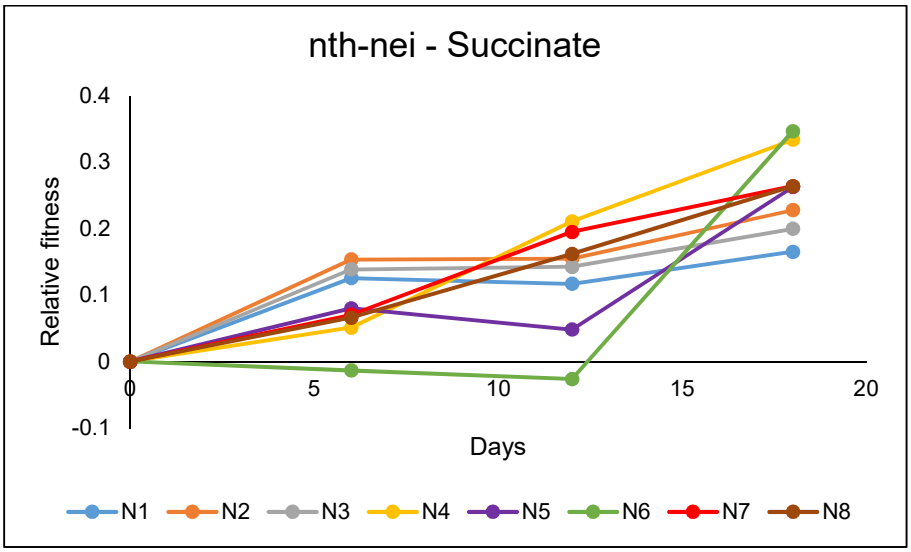
Figure 3.5 shows the relative fitness of all 8 of the biological replicates of Δnth-Δnei over a period of 18 days. The results have been represented in the form of a time series, with time points at day 6, day 12, and day 18.

Panel a of Figure 3.5 shows the relative fitness of ∆nth-∆nei in Glucuronate. At all three time points in the series – day 6, day 12, and day 18 – all the replicates of ∆nth-∆nei showed an upwards trending trajectory. There was very little variability in the relative fitness in Glucuronate at day 6. This led to a very tight clustering of relative fitness of all replicates at this time point. With time, the variability in the relative fitness of replicates of ∆nth-∆nei in Glucuronate increased. However, as mentioned earlier, all replicates continued to grow faster in Glucuronate than their previous time points, with respect to their ancestor.

Panel b of Figure 3.5 shows the relative fitness of ∆nth-∆nei in Glucuronate. At day 6, two main clusters were seen to have emerged, along with one replicate following a different trajectory. The first cluster was formed by N1, N2, and N3, and was seen undergoing a greater increase in fitness in Succinate. The second cluster was formed by N4, N5, N7, and N8, and was seen undergoing an increase in fitness in Succinate, albeit to a lesser degree than the first cluster. In the next time point at day 12, the members of the first cluster – N1, N2, and N3 – were seen undergoing either a stagnation or a deterioration in their fitness in Succinate. Of the replicates forming the second cluster at day 6, N5 was seen undergoing a dip in its fitness in Succinate. However, the rest of the members of that cluster – N4, N7, and N8 – continued in their upwards trajectory, increasing in fitness in Succinate at both, day 12, as well as day 18. The replicate showing the most interesting trajectory in Succinate was N6. At day 6, N6 deteriorated in fitness in Succinate – growing more slowly in Succinate than its ancestor. At day 12, it continued to deteriorate in fitness in Succinate, growing more slowly than even the time point at day 6. However, at day 18, the fitness of N6 jumped up suddenly. After undergoing a massive increase in its fitness between day 12 and day 18, N6 was seen to have a relative fitness in Succinate a little higher than N4, which belonged to the second cluster of replicates and which had been experiencing a continuous increase in its fitness throughout the time series.



a.



b.

Figure 3.5: The relative fitness of all 8 replicates of Δnth-Δnei in plotted over three time points – day 6, day 12, and day 18. **a.** In Glucuronate **b.** In Succinate.

By comparing the time series of the replicates of Δnth-Δnei in Glucuronate and Succinate, comments can be made about the status of tradeoffs in these populations. The second cluster that was seen in Succinate – N4, N7, and N8 – followed a similar continuously upwards trajectory in Succinate as it did in Glucuronate. Therefore, it can be hypothesized that these populations did not experience strong tradeoffs while evolving in a regime fluctuating between Glucuronate and Succinate. Other replicates – N1, N2, N3, and N5 – were found to first increasing in fitness in both, Glucuronate

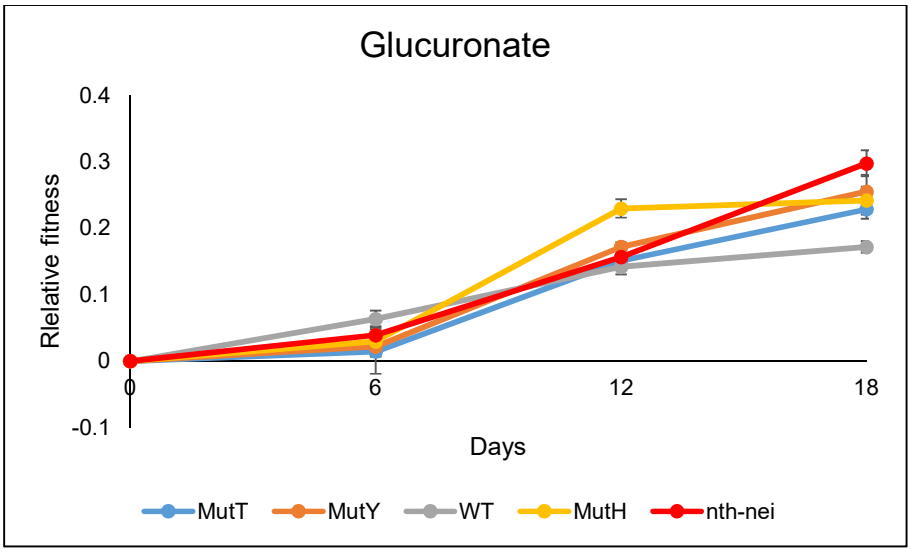
and Succinate. However, despite increasing in fitness in both environments at day 6 and day 18, they underwent a reduction in fitness at day 12 in Succinate while undergoing a continued increase in fitness at the same time point at day 12. Therefore, it can be said that these populations faced some tradeoffs at day 12 while evolving in fluctuating environments between Glucuronate and Succinate. N6, the replicate whose trajectory seemed to stand out in Succinate also experienced tradeoffs. At day 6 and day 12, when N6 was increasing in fitness in Glucuronate, its fitness continued to deteriorate in Succinate. However, its fitness in Succinate shot up suddenly at day 18, when its fitness in Glucuronate also increased. Therefore, it can be argued that N6 faced strong tradeoffs between fitness in Glucuronate and that in Succinate at day 6 and day 12, but these tradeoffs seemed to have been overcome by day 18.

3.6 Strain-wise changes in fitness after fluctuating between Glucuronate and Succinate

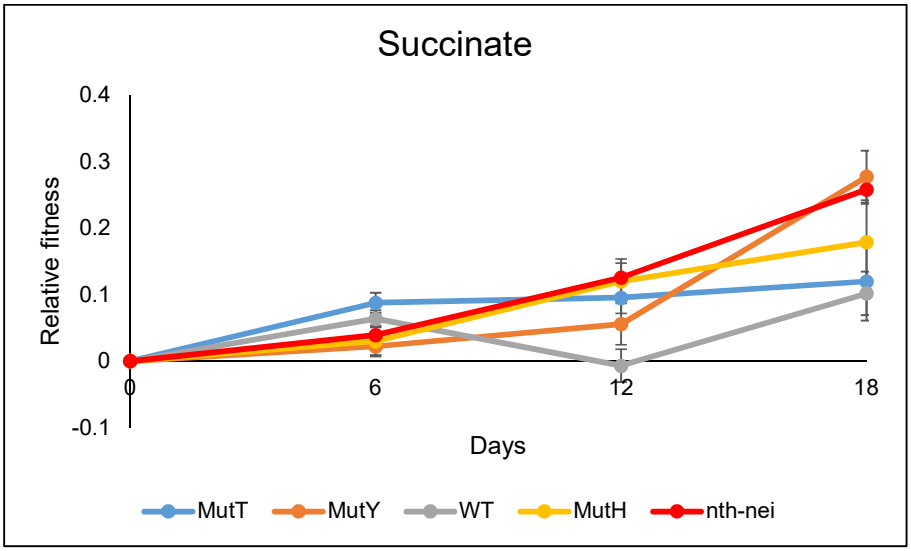
Following the measurement of the growth rate of each evolved population in the 3-day fluctuation regime, I compiled the data for the three time points measured – day 6, day 12, and day 18. The results of this compilation have been represented in Figure 3.6 below. The values used to plot the time series are the strain-level averages of 8 biological replicate populations of each strain.

Panel a of Figure 3.6 shows the relative fitness trajectories of each of the strains used in the experiment, in Glucuronate. At day 6, major clustering occurred in the fitness of all strains in Glucuronate. However, the average fitness of wild type populations was found to be slightly higher than that of the mutators. At day 12, the average relative fitness of most strains was found to be clustered again, albeit at a higher extent than at day 6. However, the average relative fitness of ΔmutH populations was found to be higher than the other strains in Glucuronate at day 12. At day 18, the average relative fitness of all the mutator strains were found to be greater than that of the wild type. Although the relative fitness of Δnth-Δnei was found to be greater than the other mutators, the relative fitness of all the mutators in Glucuronate were found clustered at day 18 as they were not significantly different from each other. What is interesting to note is that despite the fitness of ΔmutH being higher than the other mutators in

Glucuronate at day 12, its trajectory stagnates, leading to a nearly similar average fitness in Glucuronate at day 18. This suggests that ΔmutH could have reached near its adaptive peak in Glucuronate at day 12. As a result, there was no noticeable change in its fitness in Glucuronate at day 18.



a.



b.

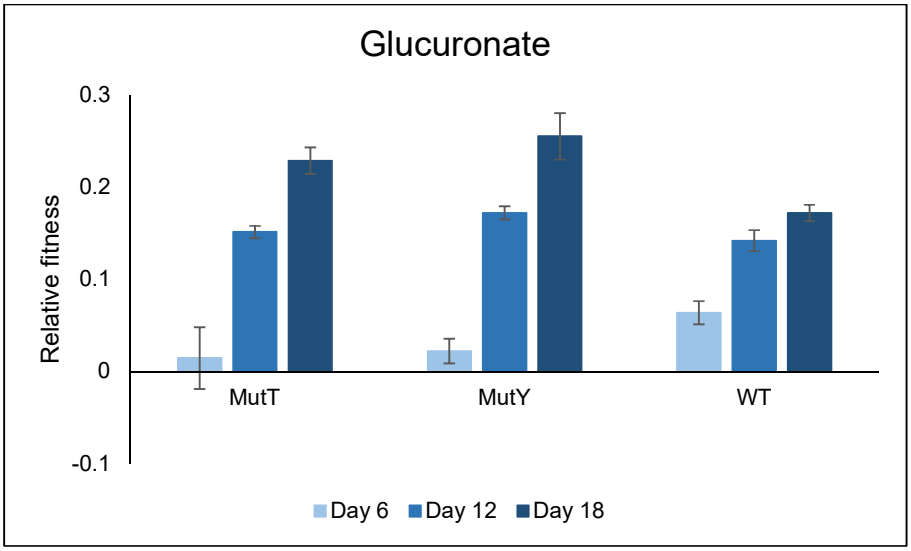
Figure 3.6: Compiled time series of average relative fitness of the strains used in the experiment, in **a:** Glucuronate, and in **b:** Succinate. Each strain contained 8 biological replicate populations and error bars represent standard error.

Panel a of Figure 3.6 shows the relative fitness trajectories of each of the strains used in the experiment, in Succinate. Throughout the time series, variability was seen between strains at all time points, and at later time points, this variability in average relative fitness was seen increasing. At day 6, despite little variability, the average relative fitness of all strains were found clustered. ΔmutT, the mutator with a reversed Tv/Ts bias and high mutation rate, maintained a similar average relative fitness at day 6, day 12 and day 18. This suggests that ΔmutT might have reached near its adaptive peak in Succinate at day 6. The average relative fitness of ΔmutY, the mutator with a reversed Tv/Ts bias was found to increase noticeably in Succinate between day 12 and day 18. This suggests that a high number of beneficial mutations were sampled by it during the third cycle of fluctuation between Glucuronate and Succinate. The average relative fitness in Succinate of both the reinforced-bias mutators – ΔmutH, and Δnth-Δnei – were found to be clustered at both day 6 and day 12. However, at day 18, Δnth-Δnei, the reinforced-bias mutator with intermediate mutation rate, was found to have a higher average relative fitness in Succinate than ΔmutH, the reinforced-bias mutator with a high mutation rate. This suggests that ΔmutH might have had had a lower adaptive peak in Succinate than Δnth-Δnei. The average relative fitness of the wild type in Succinate was found to rise at day 6, but fall around ancestral levels at day 12. However, the average relative fitness of wild type in Succinate at day 18 increased to a level higher than that at day 6. This suggests that the wild type might have been experiencing strong tradeoffs at day 12, which was after the completion of two complete cycles of fluctuation between Glucuronate and Succinate.

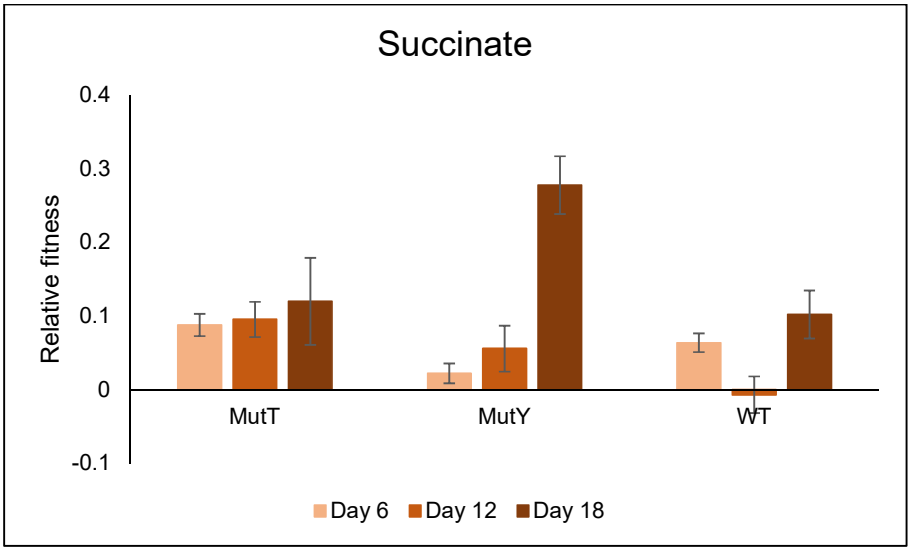
3.7 Effect of mutation rate on bacterial adaptation to fluctuating environments

Figure 3.7 shows the average relative fitness across time of reversed-bias mutators with wild type for comparison. Out of the reversed-bias mutators used for my experiments, ΔmutT had a high mutation rate, and ΔmutY had an intermediate mutation rate. By comparing the average relative fitness as a proxy for extent of adaptation in Glucuronate (Panel a), I saw that at all three time points – day 6, day 12, and day 18 – the extent of adaptation is not significantly different across mutators with the same mutation bias but different mutation rates. However, in the case of Succinate

(Panel b), ΔmutT (high mutation rate, reversed bias) was found to plateau at day 6, whereas the average relative fitness of ΔmutY was found to be significantly higher than both ΔmutT and WT. This suggests that in reversed-bias mutators, there is no significant effect of rate in adaptation to Glucuronate after evolving in fluctuating environments. However, in Succinate, a higher mutation rate was found to be disadvantageous to adaptation. Therefore, it can be hypothesized that the higher mutation rate in ΔmutT led to the accumulation of deleterious mutations in it, leading to a much lower average fitness than ΔmutY.



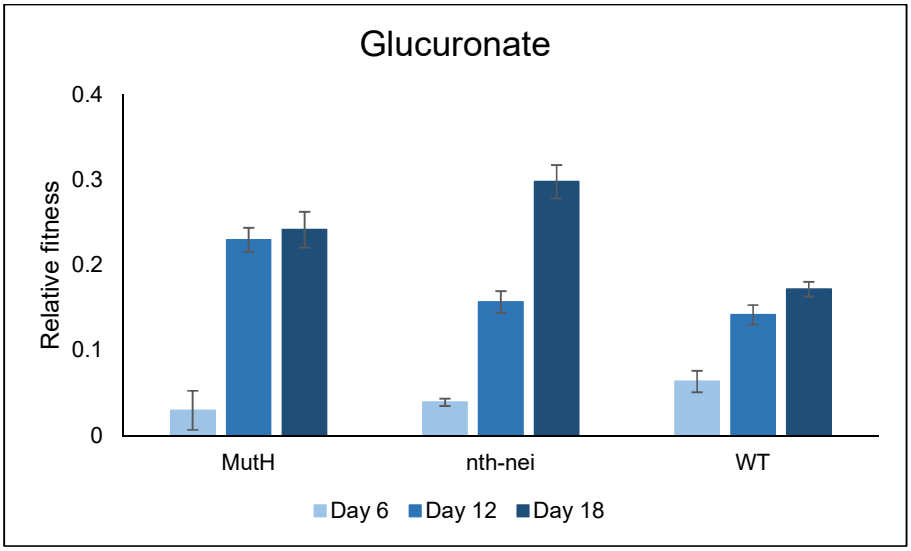
a.



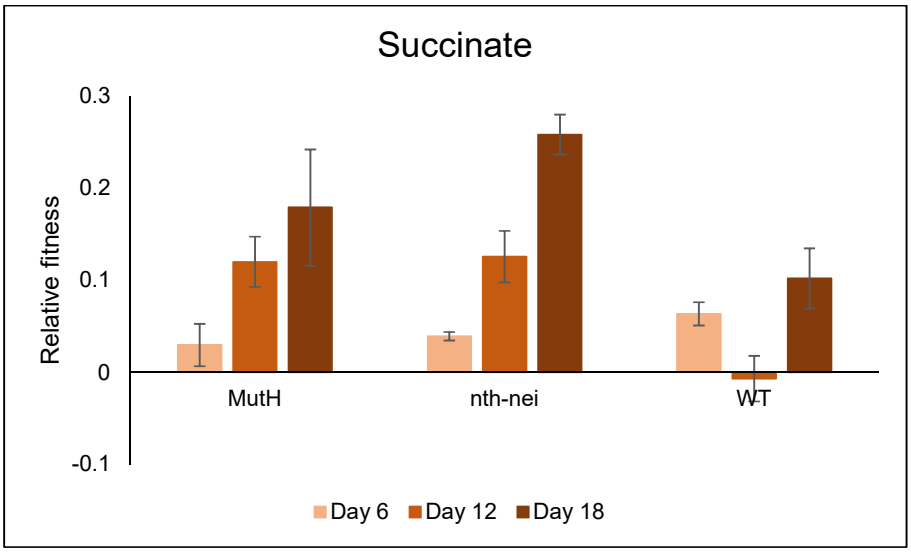
b.

Figure 3.7: Extent of adaptation of reversed-bias mutators compared to wild type in **a.** Glucuronate, and in **b.** Succinate. The number of biological replicates is 8 for each strain and error bars represent standard error.

Figure 3.8 shows the average relative fitness across time of reinforced-bias mutators with wild type for comparison. Out of the reinforced-bias mutators used for my experiments, ΔmutH had a high mutation rate, and Δnth-Δnei had an intermediate mutation rate. By comparing the average relative fitness as a proxy for extent of adaptation in Glucuronate (Panel a), I saw that ΔmutH reached its adaptive peak in Glucuronate at day 12, whereas Δnth-Δnei, which had a relative fitness in Glucuronate significantly lower than ΔmutH at day 12, has a relative fitness significantly higher than ΔmutH at day 18. On the other hand, in Succinate (Panel b), the relative fitness of ΔmutH and Δnth-Δnei were not found to be significantly different at any time point. This suggests that in reinforced-bias mutators, there is no significant effect of rate in adaptation to Succinate after evolving in fluctuating environments. However, in Glucuronate, a higher mutation rate was found to be disadvantageous to adaptation. Therefore, it can be hypothesized that the higher mutation rate in ΔmutH led to the accumulation of deleterious mutations in it, leading to a much lower average fitness than Δnth-Δnei.



a.

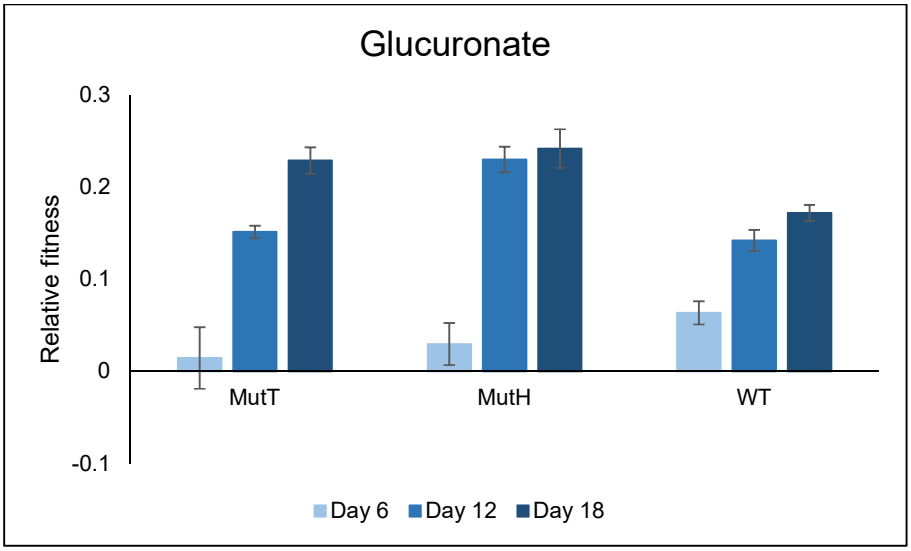


b.

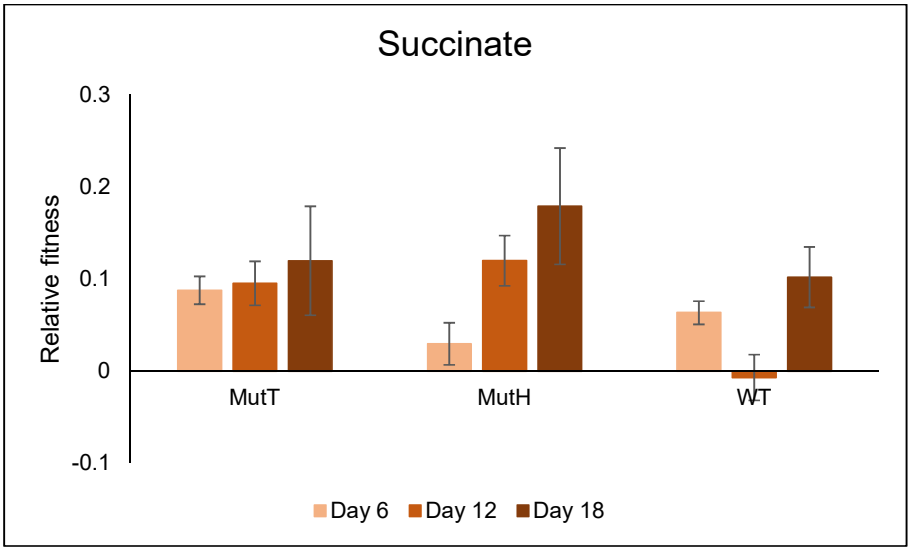
Figure 3.8: Extent of adaptation of reinforced-bias mutators compared to wild type in **a.** Glucuronate, and in **b.** Succinate. The number of biological replicates is 8 for each strain and error bars represent standard error.

3.8 Effect of mutation bias on adaptation to fluctuating environments

Figure 3.9 shows the average relative fitness across time of high-mutation-rate mutators with wild type for comparison. Out of the high-mutation-rate mutators used for my experiments, ΔmutT had a reversed Tv/Ts bias, and ΔmutH had a reinforced Tv/Ts bias. By comparing the average relative fitness as a proxy for extent of adaptation in Glucuronate (Panel a), I saw that ΔmutH has a higher average relative fitness at day 12 than ΔmutT. However, at day 18, there is no significant difference in the ir average relative fitness. This is because ΔmutH appeared to have reached near its adaptive peak at day 12, and did not increase much, whereas ΔmutT experienced a noticeable improvement in its relative fitness in Glucuronate between day 12 and day 18. On the other hand, in Succinate (Panel b), the relative fitness of ΔmutT and ΔmutH were not found to be significantly different at any time point. This suggests that in high-mutation-rate mutators, there is no significant effect of mutation bias in adaptation to Succinate after evolving in fluctuating environments. However, in Glucuronate, a reversed mutation bias was found to be slow down the rate of adaptation.



a.

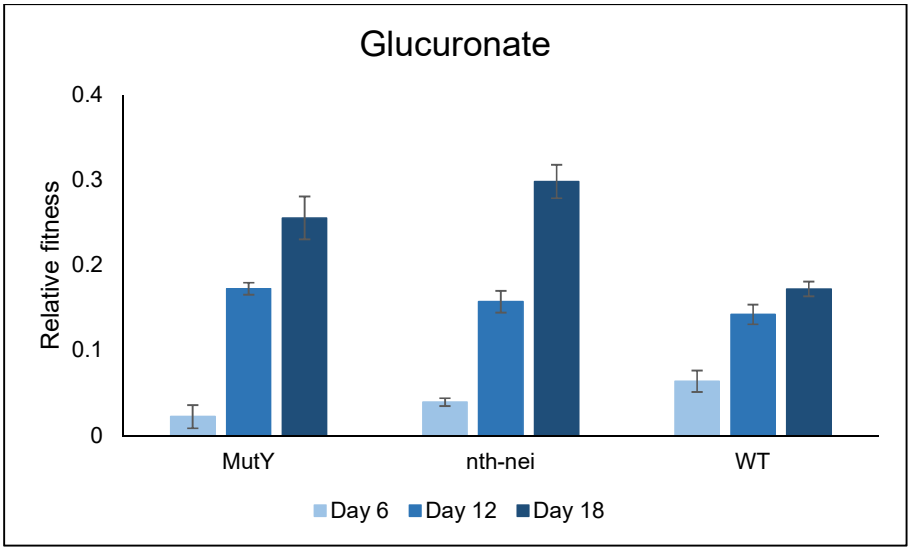


b.

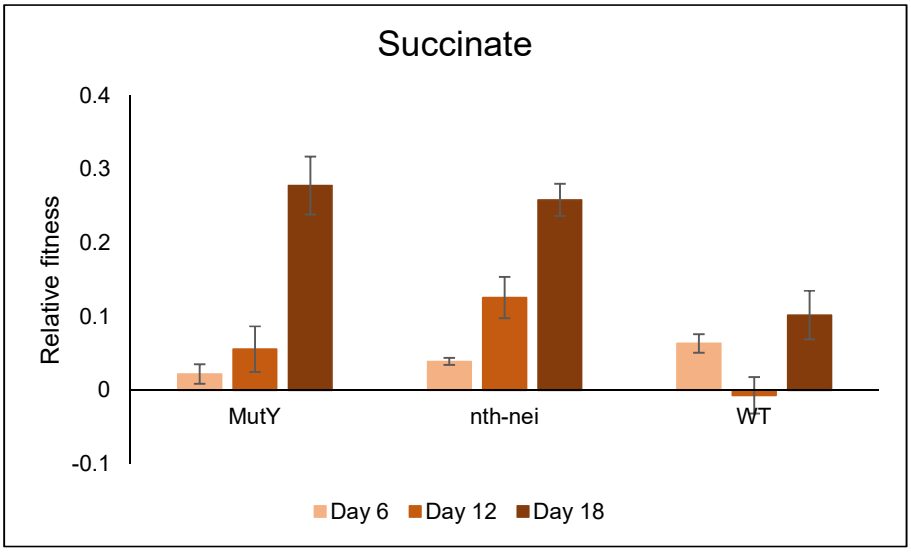
Figure 3.9: Extent of adaptation of high-mutation-rate mutators compared to wild type in **a.** Glucuronate, and in **b.** Succinate. The number of biological replicates is 8 for each strain and error bars represent standard error.

Figure 3.10 shows the average relative fitness across time of intermediate-mutation-rate mutators with wild type for comparison. Out of the intermediate-mutation-rate mutators used for my experiments, ΔmutY had a reversed Tv/Ts bias, and Δnth-Δnei had a reinforced Tv/Ts bias. By comparing the average relative fitness as a proxy for extent of adaptation, I saw that the relative fitness of ΔmutY and Δnth-Δnei were not

found to be significantly different at any time point in either of the environments. This suggests that in intermediate-mutation-rate mutators, there is no significant effect of mutation bias in adaptation after evolving in fluctuating environments.



a.

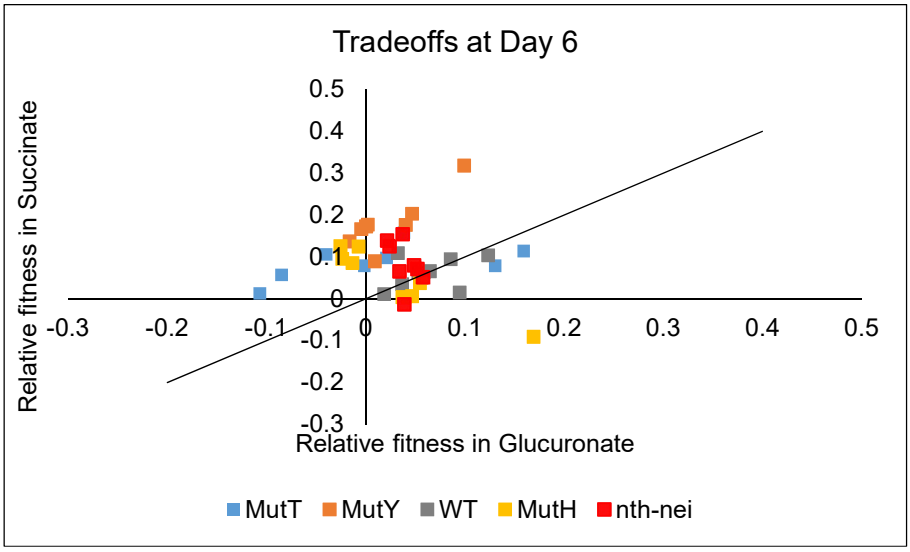


b.

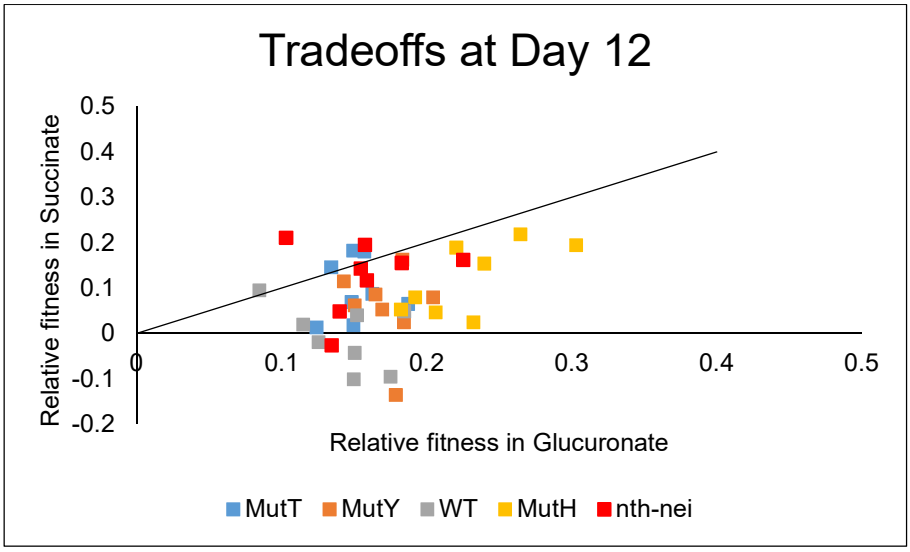
Figure 3.10: Extent of adaptation of intermediate-mutation-rate mutators compared to wild type in **a.** Glucuronate, and in **b.** Succinate. The number of biological replicates is 8 for each strain and error bars represent standard error.

3.9 Tradeoffs in fitness between environments following adaptation to fluctuating environments

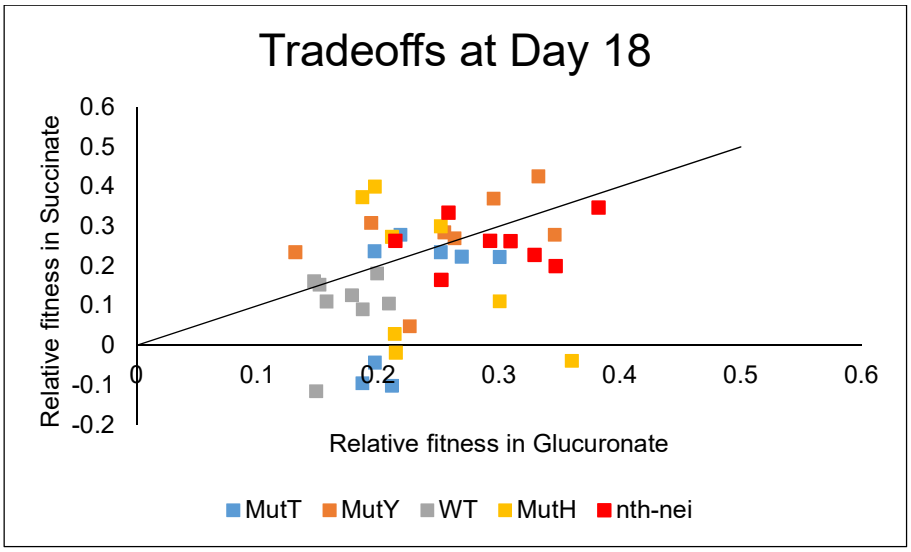
Figure 3.11 shows tradeoffs between changes in fitness in one environment with respect to the other, on a population level. Relative fitness with respect to the ancestor in Succinate has been plotted on the Y axis, whereas the relative fitness in Glucuronate has been plotted on the X axis. At day 6 (Panel a), increase in fitness in Succinate led to decrease in fitness in Glucuronate. At day 12 (Panel b), increase in fitness in Glucuronate led to decrease in fitness in Succinate. At day day 18 (Panel c), tradeoffs were distributed more evenly across environments.



a.



b.



c.

Figure 3.11: Tradeoffs in fitness across environments at **a.** day 6, **b.** day 12, and **c.** day 18.

Chapter 4 Discussion

The aim of this project was to shed light on the impact of mutation rate and bias on bacterial adaptation under fluctuating environments. The background to this work was provided by prior work done in the lab and this project intended to build up on that work in a narrow domain, which was fluctuating environments. To this extent, I have designed the experiments shown above to best highlight this effect using *Escherichia coli* as a model organism. After representing the population-level data for each strain in Figures 3.1 to 3.5, I have compared the strain-level averages in Figure 3.6 in the form of a time series measured in both the environments the given strains had been evolved in – Glucuronate and Succinate.

To answer to my first question, 'What is the effect of mutation rate on bacterial adaptation under fluctuating environments?', I have compared *E. coli* strains of the same Tv/Ts bias, but different mutation rates. In Figure 3.7, I have compared ΔmutT and ΔmutY – both strains with reversed Tv/Ts bias – with wild type as comparison. Here, it is seen that in Glucuronate, there is no noticeable difference in the relative fitness of the two strains at day 6, day 12, or day 18. However, in the case of Succinate, while ΔmutY starts with a lower relative fitness at day 6, catches up with ΔmutT at day 12, and overtakes it by day 18. Similarly in Figure 3.8, we see that in Glucuronate, both, ΔmutH and Δnth-Δnei – reinforced-bias mutators but with different mutation rates – start off at day 6 with similar relative fitness, relative fitness of ΔmutH is higher at day 12 but at day 18, while the relative fitness of ΔmutH seems to have stagnated, that of Δnth-Δnei has increased slightly higher than ΔmutH. Since the results in different environments, as well as in different mutation biases, seem to be so varied, no consistent trend can be observed in terms of the effect of mutation rate on adaptation in fluctuating environments. It should be noted that this comparison is made on the basis of strain-level averages of 8 biological replicates each. While each of these biological replicates have their relative fitness calculated from 3 technical replicates, they each show a great deal of variation, which strain-level average figures fail to capture.

Next, I have attempted to answer the more burning question that my project aimed to shed light on by comparing different strains with comparable mutation rates, but with their Tv/Ts bias towards opposite directions. In Figure 3.9, I have compared the relative fitness of ΔmutT and ΔmutH – both strains of *E. coli* with high rates of mutation but with opposite mutation biases. In Glucuronate, both strains start off with a comparable relative fitness at day 6, but by day 12, ΔmutH is seen to have left behind ΔmutT. However, by day 18, ΔmutH is seen to have stagnated in its relative fitness, whereas ΔmutT is seen to have caught up to the same level of relative fitness. On the other hand, when compared in Succinate-enriched medium, the relative fitness of both ΔmutT and ΔmutH are comparable at all time time points – day 6, day 12, and day 18. On the other hand, in Figure 3.10, it is seen that the relative fitness of ΔmutY and Δnth-Δnei – both strains with an intermediate mutation rate but opposite mutation biases – is comparable across both environments at all time points. Although a very clear trend fails to emerge from these results, it can be said that mutation bias was not found to have a noticeable impact on the adaptive trajectories of *E. coli* when different strains of it were evolved in fluctuating environments.

It should be noted that at the time of writing this thesis, I had been unable to perform any kind of statistical analysis of my results. Therefore, I am unable to comment on the statistical significance of my results confidently. Furthermore, the results that I have focused on evaluating at the end of my experiments were largely focused on the quantitative measurement of growth rate, which is a phenotypic characteristic. Therefore, the lack of cohesive trends in bacterial adaptive trajectories can be demystified to some extent by undertaking genotypic investigations into my time points.

Chapter 5 Conclusions and Future Perspectives

As discussed earlier, the aim of this project was to shed light on the impact of mutation rate and mutation bias on bacterial adaptation while evolving under fluctuating environments. To this extent, the results of the experiments I have performed have helped bring forth some interesting insights. Additionally, they also generate interest in the context of the bigger picture in which these questions were being pursued, in the first place.

A clear trend in the results is lacking in all strains with respect to the effect of both, mutation rate, and mutation bias. This observation, or lack thereof, can be explained using multiple potential scenarios. Firstly, there is the possibility that, at least when evolving under the special circumstances where the surrounding environments are constantly fluctuating, mutation rate and mutation bias do not play as big of a role in determining the adaptive trajectory of bacteria as they do in constant environments. However, this conclusion goes on to directly contradict what was expected from previous work done in the lab, as well as by other groups. Previous work from the lab suggests that reversed-bias mutators should be able to adapt to a greater extent than their reinforced-bias counterparts. Further unpublished genotypic follow-ups on that data further strengthens the case for reversed-bias mutators. However, in my fluctuating-environment experiments, this fails to manifest on a phenotypic level.

The second possibility is that given the sheer number of populations evolving at a given time in my experimental regimes, the variability seen in this populations, in any given strain, is huge. In such a scenario, it just might not be the wisest idea to assess strains but compare populations to populations. The clear trends that seem to be missing on a strain level might then show up at a population level. Thirdly, it is also possible that the time frame of the experiment I conducted was too short for the effects I was expecting to have manifested within my populations. One reason to doubt this possibility is the manifestation of expected results within similar time frames in experimental regimes involving constant environments (not part of this study).

However, given the relatively unexplored domain of evolution under fluctuating environments, this observation might just be one that had not been made earlier, to have been recorded in literature.

Finally, it can also be possible that the experiments undertaken focused too much on the phenotypic aspects of the populations, and missed out on important genotypic adaptations that might have been occurring in the populations. As the different strains that I used in my experiments had been generated by knocking of different DNA repair genes which, in turn, code for enzymes following different pathways, there is a great possibility that on a molecular level, the behaviour of, say, ΔmutT might be so different from that of ΔmutY, that it might be unjustified from an evolutionary perspective, to group them together. While evolving in constant environments, these minute details in the genotype might not have showed up but were possibly brought forward by the additional stress of adapting to a constantly fluctuating environment.

Apart from the points discussed above, I also observed some interesting results were completely unexpected. This came primarily in the form of reductions in relative fitness that were seen in all strains but in different environments. Previously published literature on evolution under fluctuating environments have consistently found an increase in every measure of fitness in populations that had experienced fluctuating environments during their experimental evolution. Therefore, it was very surprising to see a decrease in relative fitness in so many instances in my results. This can potentially be explained using tradeoffs. When a given population is evolved in two different environments in a fluctuating manner, it is possible that increase in the fitness in Environment A might lead to a loss of fitness in Environment B. This can be the result of a phenomenon called Antagonistic Pleiotropy, which has been discussed in detail in previous sections. The population might sample a mutation during its evolution that might grant it a higher fitness in Environment A, but at the cost of its fitness in Environment B. This is particularly true in the case of environments, wherein, the utilization of the two resources occurs in mechanistic pathways that are antagonistic to each other on a molecular level.

However, even when tradeoffs were considered for each population at different time points, no consistent trend emerged. As seen in Figure 11, the tradeoffs are not

consistent with time with respect to the two environments used. At day 6, increase in fitness in Succinate is leading to loss of fitness in Glucuronate, whereas the opposite is seen at day 12. On the other hand, no clear inclination is seen at day 18, where the data points seem to be scattered across the quadrant. I have been unable to explain these tradeoffs with the data I have currently obtained.

As can be judged from the experimental approaches taken in this project, this study has been a very preliminary attempt at studying a phenomenon as complex as the interplay of the effects of mutation rate and bias in an equally complex regime of fluctuating environments. Therefore, this study can be followed up with numerous approaches. The most immediate next step would be to undertake a thorough statistical analysis of my results. Additionally, as mentioned in the earlier sections, I have measured the growth curves of only a small portion of the populations that I had evolved. Preliminary growth curve data from Regime 2 (6-day fluctuations) of Batch 1 (not included) showed interesting differences in fitness measurements in different regimes. An important next step would be to measure the fitness of comparable time points in Regime 2 and compare the data with that obtained from Regime 1. For example, the data obtained from Regime 1 (3-day fluctuations) at day 12 might be directly comparable to the data that can be obtained from Regime 2 (6-day fluctuations) at day 24. This is due to populations in both regimes having undergone the same number of complete Glucuronate-Succinate fluctuations at those time points (two complete cycles). This is true for both, Batch 1, containing ΔmutT, ΔmutY, and WT, and Batch 2, containing ΔmutH, Δnth-Δnei, ΔmutS, and ΔmutL. Furthermore, the fitness of ΔmutS and ΔmutL can be measured independently, and compared to ΔmutH data, owing to the similar phenotypic characteristics of these three strains.

Following the end of the above-mentioned potential follow-up studies, Batch 3 can be taken up for growth curve measurements. This will help shed light on the behaviour of the same populations in a different fluctuation regime – between Glucose and Lactose. This will, in turn, help verify whether my results are specific to the Glucuronate-Succinate pair or represent a more general trend shown by these populations. Finally, the genotypic investigation into these trends can be undertaken by sequencing the different time points from the different experimental regimes, which are currently stored in 30% glycerol stocks.

References

1. VERNON, H.M. (1909). Elemente der exakten Erblichkeitslehre. Nature *81*, 424–424. 10.1038/081424a0.

2. Katju, V., and Bergthorsson, U. (2019). Old Trade, New Tricks: Insights into the Spontaneous Mutation Process from the Partnering of Classical Mutation Accumulation Experiments with High-Throughput Genomic Approaches. Genome Biol Evol *11*, 136–165. 10.1093/gbe/evy252.

3. Foster, P.L., Lee, H., Popodi, E., Townes, J.P., and Tang, H. (2015). Determinants of spontaneous mutation in the bacterium *Escherichia coli* as revealed by whole-genome sequencing. Proceedings of the National Academy of Sciences *112*. 10.1073/pnas.1512136112.

4. Agashe, D., and Shankar, N. (2014). The evolution of bacterial DNA base composition. J Exp Zool B Mol Dev Evol *322*, 517–528. 10.1002/jez.b.22565.

5. Long, H., Sung, W., Kucukyildirim, S., Williams, E., Miller, S.F., Guo, W., Patterson, C., Gregory, C., Strauss, C., Stone, C., et al. (2018). Evolutionary determinants of genome-wide nucleotide composition. Nat Ecol Evol *2*, 237–240. 10.1038/s41559-017-0425-y.

6. Stoltzfus, A., and Yampolsky, L.Y. (2009). Climbing Mount Probable: Mutation as a Cause of Nonrandomness in Evolution. Journal of Heredity *100*, 637–647. 10.1093/jhered/esp048.

7. Streisfeld, M.A., and Rausher, M.D. (2011). POPULATION GENETICS, PLEIOTROPY, AND THE PREFERENTIAL FIXATION OF MUTATIONS DURING ADAPTIVE EVOLUTION. Evolution (N Y) *65*, 629–642. 10.1111/j.1558-5646.2010.01165.x.

8. Rokyta, D.R., Joyce, P., Caudle, S.B., and Wichman, H.A. (2005). An empirical test of the mutational landscape model of adaptation using a single-stranded DNA virus. Nat Genet *37*, 441–444. 10.1038/ng1535.

9. Gomez, K., Bertram, J., and Masel, J. (2020). Mutation bias can shape adaptation in large asexual populations experiencing clonal interference. Proceedings of the Royal Society B: Biological Sciences *287*, 20201503. 10.1098/rspb.2020.1503.

10. Cano, A. V., and Payne, J.L. (2020). Mutation bias interacts with composition bias to influence adaptive evolution. PLoS Comput Biol *16*, e1008296. 10.1371/journal.pcbi.1008296.

11. Sane, M., Diwan, G.D., Bhat, B.A., Wahl, L.M., and Agashe, D. (2022). Shifts in mutation spectra enhance access to beneficial mutations. bioRxiv, 2020.09.05.284158. 10.1101/2020.09.05.284158.

12. Leroi, A.M., Lenski,' And, R.E., and Bennetf, A.F. (1994). EVOLUTIONARY ADAPTATION TO TEMPERATURE. III. ADAPTATION OF ESCHERICHIA COLI TO A TEMPORALLY VARYING ENVIRONMENT.

13. Phillips, K.N., Castillo, G., Wünsche, A., and Cooper, T.F. (2016). Adaptation of *Escherichia coli* to glucose promotes evolvability in lactose. Evolution (N Y) *70*, 465–470. 10.1111/evo.12849.

14. Karve, S.M., Bhave, D., Nevgi, D., and Dey, S. (2016). *Escherichia coli* populations adapt to complex, unpredictable fluctuations by minimizing trade-offs across environments. J Evol Biol *29*, 2545–2555. 10.1111/jeb.12972.

15. Bennett, A.F., Lenski, R.E., and Mittler, J.E. (1992). EVOLUTIONARY ADAPTATION TO TEMPERATURE. I. FITNESS RESPONSES OF *ESCHERICHIA COLI* TO CHANGES IN ITS THERMAL ENVIRONMENT. Evolution (N Y) *46*, 16–30. 10.1111/j.1558-5646.1992.tb01981.x.

16. Reboud, X., and Bell, G. (1997). Experimental evolution in Chiamydomonas. Ill. Evolution of specialist and generalist types in environments that vary in space and time.

17. Hughes, B.S., Cullum, A.J., and Bennett, A.F. (2007). An Experimental Evolutionary Study on Adaptation to Temporally Fluctuating pH in *Escherichia coli*. Physiological and Biochemical Zoology *80*, 406–421. 10.1086/518353.

18. Cooper, T.F., and Lenski, R.E. (2010). Experimental evolution with E. coli in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations. BMC Evol Biol *10*, 11. 10.1186/1471-2148-10-11.

19. Kassen, R., and Bell, G. (1998). Experimental evolution in Chlamydomonas. IV. Selection in environments that vary through time at different scales. Heredity (Edinb) *80*, 732–741. 10.1046/j.1365-2540.1998.00329.x.

20. Couce, A., Guelfo, J.R., and Blázquez, J. (2013). Mutational Spectrum Drives the Rise of Mutator Bacteria. PLoS Genet *9*, e1003167. 10.1371/journal.pgen.1003167.

21. Couce, A., Rodríguez-Rojas, A., and Blázquez, J. (2015). Bypass of genetic constraints during mutator evolution to antibiotic resistance. Proceedings of the Royal Society B: Biological Sciences *282*, 20142698. 10.1098/rspb.2014.2698.

22. Payne, J.L., Menardo, F., Trauner, A., Borrell, S., Gygli, S.M., Loiseau, C., Gagneux, S., and Hall, A.R. (2019). Transition bias influences the evolution of antibiotic resistance in Mycobacterium tuberculosis. PLoS Biol *17*, e3000265. 10.1371/journal.pbio.3000265.

23. Bachar, A., Itzhaki, E., Gleizer, S., Shamshoom, M., Milo, R., and Antonovsky, N. (2020). Point mutations in topoisomerase I alter the mutation spectrum in E. coli and impact the emergence of drug resistance genotypes. Nucleic Acids Res *48*, 761–769. 10.1093/nar/gkz1100.

24. Leighow, S.M., Liu, C., Inam, H., Zhao, B., and Pritchard, J.R. (2020). Multi-scale Predictions of Drug Resistance Epidemiology Identify Design Principles for Rational Drug Design. Cell Rep *30*, 3951-3963.e4. 10.1016/j.celrep.2020.02.108.