Investigating the effects of complex fluctuating environments on the evolution of laboratory populations of *Escherichia coli*

A thesis submitted to a partial fulfilment of the requirement

for the degree of Doctor of Philosophy by

Shraddha Karve

20093044

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CERTIFICATE

Certified that the work incorporated in thesis titled ‘Investigating the effects of complex fluctuating environments on the evolution of laboratory populations of *Escherichia coli*’, submitted by Shraddha Karve was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

Dr. Sutirth Dey

Supervisor

DECLARATION

I declare that this written submission represents my ideas in my own words and where others ideas have been included, I have adequately cited and referred to original sources. I also declare that I have adhered to all principles of academic honesty and integrity and I have not misinterpreted or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above can cause disciplinary action by the institute and evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

Shraddha Karve

20093044

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Contents

SYNOPSIS

Investigating the effects of complex fluctuating environments on the evolution of laboratory populations of *Escherichia coli*

Name: Shraddha Karve

Roll Number: 20093044

Name of Supervisor: Dr. Sutirth Dey

Department: Biology

Date of Registration: 1st of January, 2010

Indian Institute of Science Education and Research (IISER), Pune, India

Introduction

Environmental heterogeneity is an important driver of many ecological and evolutionary processes (Abele 1976, Hiltunen, Laakso et al. 2008, Harrison, Laine et al. 2013, reviewed in Chesson 2000, Lee and Gelembiuk 2008). This heterogeneity can be of different types: spatial/ temporal, exogenous / endogenous, biotic / abiotic, and so on. Temporal fluctuations, specifically, are widespread in occurrence. Environments can vary at different time scales, can be predictable or unpredictable in nature, and many environmental variables can fluctuate together or independent of each other (reviewed in Melbourne, Cornell et al. 2007). Experimental evolution studies conducted under controlled laboratory environments are an excellent tool to understand the evolutionary implications of such heterogeneity. However, in spite of the presence of a large body of theoretical work (Levins 1968, Gavrilets and Scheiner

1993, Whitlock 1996), experimental investigations of environmental heterogeneity are limited in number (Hallsson and Björklund 2012, Condon, Cooper et al. 2014, reviewed in Kassen 2014). Moreover, most of the existing studies look at fluctuations in a single environmental variable like temperature or pH (Hughes, Cullum et al. 2007, Alto, Wasik et al. 2013, Ketola, Mikonranta et al. 2013), whereas in nature, multiple components of the environment can fluctuate simultaneously.

Here I study evolution of laboratory populations of *Escherichia coli* when exposed to an environment which has multiple stressors that fluctuate unpredictably over time. I also investigate the possible mechanisms leading to the observed fitness changes. I further study the effects of history of fluctuating environments on future adaptation events. The primary results are as follows:

Complex, unpredictable fluctuations select for higher fitness in novel environments over short duration of selection (Chapter 2)

Bacterial populations facing unpredictable fluctuations have been shown to improve fitness over all the selection environments (Turner and Elena 2000, Ketola, Mikonranta et al. 2013) or improve in some but not in others (Hughes, Cullum et al. 2007) or show no improvement in fitness (Alto, Wasik et al. 2013). I observed no improvement in fitness in the individual selection stresses after selection in complex, unpredictable fluctuations after ~170 generations of selection. Surprisingly though, populations selected in the fluctuating environments showed improved fitness in the face of multiple novel environments like antibiotics, heavy metals and DNA intercalating agents. The fitness advantage was retained, though decreased in the magnitude, even after deteriorating selection for ~50 generations in novel environments. Moreover, the fitness advantage was evident at the level of individual

bacterial cells. I then investigated the various putative mechanisms that could have led to greater fitness under novel environments.

Efficient energy dependent efflux is the most likely mechanism leading to fitness advantage in novel environments (Chapter 3)

While the fitness outcomes of the different kinds of fluctuations are being experimentally investigated to some extent in microbes (Buckling, Wills et al. 2003, Hughes, Cullum et al. 2007, Ketola, Mikonranta et al. 2013), study of underlying mechanisms is conspicuous by its near absence (but see Coffey and Vignuzzi 2011, Alto, Wasik et al. 2013). I investigated multiple candidate mechanisms which could have led to the observed fitness advantage in the face of novel environments. Results showed that populations facing fluctuating environments evolved enhanced energy dependent efflux ability. Additionally, contrary to theoretical predictions (Gillespie and Turelli 1989, Turelli and Barton 2004), standing variation or the ability to generate variation did not evolve. But this lack of increase in variation could have been due to the comparatively short duration of selection.

Extended exposure to complex, unpredictable fluctuations does not affect the evolvability of the populations (Chapter 4)

If longer duration of exposure to fluctuating environments results in significant increase in standing genetic variation, the latter can in turn lead to increased evolvability i.e. higher speed of adaptation (Falconer, Mackay et al. 1996). I tested this hypothesis after ~560 generations of selection in the fluctuating environment, by exposing the selected populations to a further round of directional selection for ~450 generations in a novel environment. Populations with an evolutionary history of fluctuating environments showed similar rate of

adaptation as the populations with the history of constant selection environment. In spite of the similar rates of adaptation, populations with a history of fluctuations showed superior fitness while adapting to the novel environments, suggesting that history of fluctuations can have long term effects.

Populations facing complex, unpredictable fluctuations for longer duration can take 'no cost' routes to fitness improvement (Chapter 5)

Short duration of selection (~170 generations) was hypothesized to be one reason behind the lack of fitness improvement observed under fluctuating environments. Selection over ~900 generations supported this conjecture, as the selected populations showed adaptation for some of the selection environments which resulted in highest overall mean fitness and lowest variation for the fitness. But more importantly, populations facing complex, unpredictable environment, did not lose fitness in any of the selection environments. Populations selected in a single environmental stress on the other hand, showed a trade-off in at least one of the stresses to which they were not exposed during selection. Fluctuating selection regime thus forced the populations to take the 'no cost' routes to increase in fitness. This could have been a result of simultaneous exposure to multiple selection environments, which is expected to select for variation which is either favourable or neutral in all the selection environments. What role does unpredictability play in this context then, was addressed next.

Predictability does not play a major role in shaping the fitness outcomes over short duration of the selection (Chapter 6)

Selection under predictable fluctuations has been shown to result in improved fitness over all the different environmental values (Turner and Elena 2000, Alto, Wasik et al. 2013, Puentes-

Téllez, Hansen et al. 2013). However, that does not seem to be the case for the unpredictable fluctuations, where fitness may or may not improve over all the selection environments (Hughes, Cullum et al. 2007, Alto, Wasik et al. 2013). I found that the fitness outcomes did not differ between predictable and unpredictable complex environments after ~260 generations of selection.

The results underline the far reaching implications of environmental heterogeneity, both in terms of clinical and evolutionary outcomes. Further empirical work should focus on less explored areas like fluctuations in complex environments and interaction of predictability and complexity, while theoretical line of work needs to generate solid predictions about what kind of mechanism will evolve in the face of environmental heterogeneity.

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Chapter 1. Introduction

In nature, environment varies over space and time and this heterogeneity is thought to be one of the driving features behind the staggering diversity of life on earth (Abele 1976, Hiltunen, Laakso et al. 2008, Stein, Gerstner et al. 2014). Not surprisingly therefore, understanding the causes and consequences of environmental heterogeneity has remained one of the fundamental goals in ecology and evolutionary biology. Environmental heterogeneity can be exogenous (i.e. arising due to external factors) or endogenous, (i.e. factors within the community or population). It can be biotic or abiotic in nature, and temporal, spatial or spatio-temporal in its manifestation. It can also be invader driven where an event of invasion modifies the environment, which in turn affects the invader along with the other life forms. Occurrence of these different kinds of heterogeneities is far from independent. For instance, biological heterogeneities can easily arise through endogenous processes while exogenous heterogeneities arise from both biotic and abiotic factors (reviewed in Melbourne, Cornell et al. 2007).

Of all the kinds of environmental heterogeneities, those in terms of space and time have received the most attention in the literature (Levins 1968, Kassen 2002, Collins 2011, Sæther, Engen et al. 2013). In some cases, both temporal and spatial heterogeneity can lead to similar outcomes in communities, for instance by increasing the invasibility of the community (Melbourne, Cornell et al. 2007). But mostly, outcomes of spatial and temporal variation are very different. Spatial heterogeneity provides a refuge to the different phenotypes, as a result it is expected to maintain or even increase the genetic variation (Korona 1996, Rainey and Travisano 1998). Empirical studies confirm this prediction and populations facing spatial heterogeneity harbour higher amount of genetic variation as compared to the populations facing a constant environment (Reboud and Bell 1997, Rainey and Travisano 1998, Habets, Rozen et al. 2006). They have higher overall fitness across all the patches (as the different

environments across space are called) but for a given patch, their fitness is lower than the native population (Reboud and Bell 1997, Condon, Cooper et al. 2014).

Outcomes of temporal heterogeneity are comparatively more difficult to predict due to factors like the complexity, grain size and predictability of the fluctuations, all of which interact with each other to affect the fitness and diversity in evolving populations. Environments can change within the lifetime of an organism (fine grained) or on timescales of tens of generations (intermediate grained) or over few hundred/thousand generations (coarse grained). Fine grained fluctuations are expected to select for enhanced phenotypic plasticity while coarse grained environments can lead to mutational sweeps in asexual organisms where a mutation favourable in the current environment gets fixed (Ancel 1999, Meyers, Ancel et al. 2005). In asexual organisms, fluctuations at an intermediate scale are a) less likely to select for higher phenotypic plasticity (since the environment remains constant over few generations) and b) less likely to lead to mutational sweeps (if the environment changes sufficiently fast). Outcomes of intermediate grain size are thus difficult to predict and critically depend on the underlying genetic structure (Chevin, Lande et al. 2010). Like grain size, predictability also affects the outcomes of the fluctuations. Environmental fluctuations can be predictable like that of seasonal fluctuations or unpredictable like those induced by some human activities like disposal of domestic sewage. Predictable fluctuations are expected to select for switching phenotypes where rate of switching evolves to match the rate of fluctuations (Acar, Mettetal et al. 2008). Unpredictable fluctuations on the other hand, are likely to favour bet-hedging where phenotype switch is random (Beaumont, Gallie et al. 2009). Unfortunately, very few studies have investigated the effects of complex temporal fluctuations, i.e. environments that vary across multiple variables over time.

Many of the theoretical predictions referred above have been empirically validated. Studies show that predictable fluctuations mostly lead to improvement in the fitness over all the

selection environments faced by the populations (Leroi, Lenski et al. 1994, Turner and Elena 2000, Hughes, Cullum et al. 2007, Coffey and Vignuzzi 2011, Alto, Wasik et al. 2013, Puentes-Téllez, Hansen et al. 2013, Condon, Cooper et al. 2014 but see Buckling, Wills et al. 2003). However, the fitness outcomes of unpredictable fluctuations are more equivocal and include increase (Turner and Elena 2000, Ketola, Mikonranta et al. 2013), no change (Alto, Wasik et al. 2013) or increase with respect to some environments but not others (Hughes, Cullum et al. 2007). Independent of the predictability of the environment, populations facing fluctuating environments minimize the variation in fitness over all the environments when compared with the populations which face only one constant environment (Kassen 2014). One peculiar feature of the populations facing fluctuating environments is the absence of trade-offs across environments i.e. populations gain fitness in some of the environments experienced during selection, without any accompanied fitness loss in the other environments experienced (Turner and Elena 2000, Hughes, Cullum et al. 2007, Condon, Cooper et al. 2014 but see Reboud and Bell 1997). When fitness is assayed for novel environments, along with the selection environments, results show that temporal fluctuations in temperature clearly enhance the fitness in novel biotic environments but not in novel abiotic environments (Ketola, Mikonranta et al. 2013).

In spite of the substantial corpus of empirical studies, several facets of how organisms adapt to complex environments, remain unexplored, particularly in the context of fluctuating environments. Complexity of the environment, both biotic and abiotic, is rarely incorporated in the experimental studies (but see Barrett, MacLean et al. 2005, Cooper and Lenski 2010, Satterwhite and Cooper 2015). Different biotic and abiotic components of the environment interact with each other and fluctuations in any one component of these environments is likely to affect adaptation to the other components. Another underexplored area is that of long term effects of temporal fluctuations. Most of the studies focus on the immediate fitness

effects of fluctuations in the selection or novel environments (Hughes, Cullum et al. 2007, Alto, Wasik et al. 2013, Ketola, Mikonranta et al. 2013). But fluctuating environments are expected to affect the underlying genetic variation (Levins 1968, Gavrilets and Scheiner 1993, Whitlock 1996) and hence are most likely to affect long term evolutionary outcomes. This conjecture is supported in the literature on invasive species which suggests that history of environmental disturbance is positively correlated with the invasive potential (Lee and Gelembiuk 2008). Whether temporal fluctuations affect the evolvability of the populations in longer run remains to be tested experimentally. Another missing link in understanding of temporal fluctuations is information on underlying mechanisms. Very few studies look at the underlying genetic or physiological changes that lead to the observed fitness outcomes of temporal fluctuations, though the trend has been changing recently (Coffey and Vignuzzi 2011, Alto, Wasik et al. 2013). Only with an improved understanding in these areas can we move on to a more inclusive scenario where temporal fluctuations can be studied in conjunction with spatial heterogeneity, community structure and demography.

In my thesis I attempt to fill some of the gaps in this area, focusing on the effects of unpredictable, complex environmental fluctuations in terms of the fitness outcomes, underlying mechanisms as well as the long term evolutionary effects. This kind of a study demands selection experiments lasting large number of generations as well as facility in terms of environmental modifications. This makes fast dividing microorganisms a very suitable experimental system. In addition to this, replicate populations facing different selection regimes can be initiated from the same ancestral population/clone. Large size of a population (~10^9 individuals/ population) and higher mutation rates (as compared to the multicellular organisms) facilitate the accumulation of variation during the course of experiment. Control over initial genetic variation increases the power of the experiment i.e. the outcomes of selection can be attributed to either selection treatment or chance,

eliminating the role of initial genetic variation. Moreover, microbial systems provide a luxury of storing the experimental populations for future assays, almost indefinitely. It is hardly surprising, hence, that majority of the studies on fluctuating environments use microbes as model organisms (Hughes, Cullum et al. 2007, Alto, Wasik et al. 2013, Ketola, Mikonranta et al. 2013, Barrett, MacLean et al. 2005, Cooper and Lenski 2010, Satterwhite and Cooper 2015). With the same vein of reason I chose *Escherichia coli* as the model organism.

I conducted three independent selection experiments. The first selection experiment compared the fitness outcomes of evolution under complex, unpredictably fluctuating environments to those under benign control environments. The results from this selection experiment are presented in chapters 2-4. Along with the fitness outcomes in selection and novel environments, I also investigated the possible mechanisms leading to the observed fitness changes. I also contrasted the evolvability and competitive ability of the populations with a history of fluctuations to those with a history of growth in unchanging, non-stressful environment.

The fifth chapter describes a second selection experiment which looked at the fitness in selection environments in greater detail. Along with the populations facing fluctuating environment, we selected replicate populations under constant exposure of each of the selection environment which constituted the complex, unpredictable fluctuating selection regime. This design allowed us to study the underlying trade off structure as well as the mean and variance for the fitness over all the selection environments. Moreover, fitness assays performed at different stages provide information on how these attributes changed during the evolution.

Results of this second selection experiment clearly outlined the role of complexity in shaping the fitness of the evolving populations, but the role of predictability, or rather the absence of

it, remained unclear. Hence, I used another selection experiment to investigate the fitness effects of predictable *vs* unpredictable fluctuations.

In the last chapter, I highlight the important findings of my thesis and discuss the broader academic and applied implications of the same. I also outline some of the major questions arising out of my results which can potentially act as starting points for future research in the field.

Chapter 2. Complex, unpredictable fluctuations select for higher fitness in novel environments over short duration of selection

1 INTRODUCTION

Environmental heterogeneity affects multiple ecological and evolutionary processes (Chesson 2000, Hiltunen, Laakso et al. 2008, Lee and Gelembiuk 2008, Harrison, Laine et al. 2013). One of the less explored aspects of environmental heterogeneity in this context is that of simultaneous exposure to multiple selection pressures in the form of environmental stressors or resources (but see Barrett, MacLean et al. 2005, Cooper and Lenski 2010) (henceforth, complexity). Previous studies looking at resource complexity show that populations adapt to all the resources which are part of the environment and minimize the variation in the fitness across resources (Barrett, MacLean et al. 2005, Cooper and Lenski 2010). However, these results cannot be intuitively extended to complexity in terms of non-resource variables. Adapting to multiple environmental variables can be a daunting task, especially if some of the variables are negatively correlated with each other (Kassen 2002). Complexity when coupled with unpredictability poses an even greater challenge, since the adaptive landscape is continuously shifting. In spite of its ubiquity in nature, evolutionary outcomes of complex, unpredictable fluctuating environment remain poorly explored. This chapter summarizes the results of fitness outcomes of short term selection in such fluctuating environment.

The selection was carried out for short duration (~ 170 generations). Fitness was assayed in component environments, (i.e. environments that were part of the selection) and novel environments, i.e. environments that were not part of the selection. Selection results in no fitness advantage in component environments but in novel environments. The advantage is retained after deteriorating selection and somewhat after acclimation, at the population level. Results of fitness assessment at the level of individual cells corroborate the population level results.

2 METHODS

2.1 Selection

2.1.1 Selection under a stable environment

This study was conducted on *Escherichia coli* (strain NCIM 5547) populations. We plated this strain on nutrient agar (see Appendix 2.1 for composition), and created a suspension using bacteria from three randomly picked colonies. This suspension was used to initiate six bacterial cultures. Three of these were randomly assigned to a regime wherein they were grown at 37ºC, 150 rpm in 100 ml conical flasks. These three populations were sub-cultured every 24 hours (1ml in 50 ml of nutrient broth) for 30 days and will be henceforth referred to as the S (**S**table-Environment) populations. At the time of every sub-culturing, the optical density (OD_{600}; measured using a Nanodrop^{TM}, Thermo Scientific) was ~0.2, signifying 5.64 generations every 24 hours (Bennett and Lenski 1997). The other three populations were subjected to unpredictably changing complex environments for 30 days (see below) and will henceforth be referred as F (**F**luctuating-Environment) populations. For both these sets of populations, we created glycerol stocks at the time of each sub-culturing.

2.1.2 Selection under complex, randomly changing environments

In a pilot study, we observed the growth of this strain at various concentrations of salt (sodium chloride, NaCl), hydrogen peroxide (H_2O_2) and acidic / basic pH in nutrient broth (NB, see appendix 2.1 for composition). These four conditions are henceforth referred to as *component environments*. Based on these observations, we created 72 arbitrary combinations of NaCl, pH and H_2O_2 values such that in any combination, the magnitude of one component was equal to that found in NB (i.e. pH = 7.0 or [NaCl] = 0.5g% or [H_2O_2] = 0) and that of the other two were individually expected to negatively affect growth. Thus, for example,

combination # 46 denotes a NB containing 2 g% of NaCl + 9.5pH + no H_2O_2 whereas combination # 10 stands for 0.5 g% of NaCl + 9.5 pH + 0.01 M of H_2O_2 (see Appendix 2.2 for all the combinations experienced). Each combination was assigned a tag between 0-71 and a uniform-distribution random number generator was used to obtain a sequence of numbers (with replacement) in that range. Each replicate F population experienced 30 combinations of the components according to this sequence. This ensured that the environments faced by these populations were not only unpredictable, but also complex, i.e. involved three different environmental variables. As a result, unlike the S populations, the F populations did not grow to similar OD_{600} values in every generation. Therefore, we arbitrarily assigned an OD_{600} value ≥ 0.1 as a criterion for sub-culturing to the next environment. The volume used to inoculate the next culture was 1 ml, which translated to approximately 10^8 -10^9 cells. Any F population which did not reach an $OD_{600} \geq 0.1$ was allowed to grow for another 24 hours in the same flask. Pooled over the three replicate populations, this event happened 22 times (out of 90 transfers) during the course of the experiment. If a population failed to cross the threshold even after 48 hrs post-inoculation, 100 µl of glycerol stock from the previous environmental combination was revived in NB and then transferred to the next combination in the selection sequence (pooling over all replicates, this event happened 20 times over the entire experiment). The total exposure to composite environments was kept equal, i.e. each replicate F population experienced 30 composite environments in which they could grow. All other culture conditions were identical to those experienced by the S populations.

2.2 Fitness Estimations

2.2.1 Fitness under component environments

Following a recent study (Ketola, Mikonranta et al. 2013), we estimated fitness as the maximum growth rate over the period of 24 hours. We estimated the fitness (see Appendix 1.1 for details) of S and F populations (after ~170 generations in their respective selection regimes) under four conditions of the component environments i.e. salt, acidic pH, basic pH and H_2O_2. The concentrations used were the extreme values of the selected ranges for these environments (see Appendix 2.3).

2.2.2 Fitness under complex environments

In our study, what the selected populations actually experienced were combinations of stresses (i.e. pH+ salt +H_2O_2), which was designated as complex environments. Therefore, we also estimated the fitness (see Appendix 1.1 for details) of S and F populations (after ~170 generations in their respective selection regimes) under six randomly chosen complex environments faced by F populations during the selection (see Appendix 2.3 for details).

2.2.3 Fitness under novel environments

We assayed the fitness of the S and F populations (after ~170 generations in their respective selection regimes) under four different stressful conditions: presence of two heavy metals (cobalt and zinc), one antibiotic (norfloxacin) and a DNA intercalating agent (ethidium bromide). All these four chemicals are known to reduce the growth rate of *E. coli*, and will be henceforth referred to as *novel environments*. Fitness was estimated (see Appendix 1.1 for details) at concentrations determined by range estimations (see Appendix 2.3 for concentrations). These four conditions were selected because we failed to find any study that

reports a correlated change in fitness in any of them due to selection for any of the component environments (salt, pH or H_2O_2). Thus, it is unlikely that resistance to any of the four novel environments can evolve as a correlated response to direct selection on the component environments (see section 4.3).

2.2.4 Fitness after selection in deteriorating environments

We then investigated whether the fitness advantage of the selected populations over the controls in the novel environments persisted even after deteriorating selection. For this, all F and S populations were further selected separately under continuously increasing concentrations of cobalt, zinc and norfloxacin for 8 days (see Appendix 2.3 for details). Deteriorating selection on ethidium bromide could not be performed for logistical reasons. The maintenance protocol was the same as that for the selection under fluctuating environments (see section 2.1.2). After the 8th day (i.e.~50 generations), populations were stored as glycerol stocks for fitness estimation. Fitness was assayed (see Appendix 1.1 for details) in the 8^{th} day concentration (see Appendix 2.4) of the novel environments.

2.2.5 Fitness after acclimation

Acclimation response of S and F populations was characterized using the same four novel environments as above. 100 µl of glycerol stock of each 30 day selected S and F population was revived overnight in 50 ml NB. 100 µl of this revived suspension was then inoculated into the novel environment (see Appendix 2.3 for concentrations) and allowed to acclimate for 9 hours. Glycerol stocks were stored every 3 hours (to estimate the degree of acclimation) along with OD_{600} measurement on Nanodrop (Thermo, Waltham, MA, USA). The glycerol stocks made after every 3 hour were then used to determine the extent of acclimation. For this

purpose, 100 µl of well mixed glycerol stock was added to the fresh medium with the same novel environment, and OD_{600} was measured using Nanodrop at every two hour interval for 24 hr duration. Fitness was determined as mentioned in protocol I (b). This measure of fitness across time points was used as an estimate of extent of acclimation.

2.2.6 Fitness of the individual bacteria in the novel environments

To estimate the fitness of individual bacterium and characterize the possible heterogeneity within a population, we employed a slide-based observation technique (Lele, Baig et al. 2011). Pilot studies were conducted to determine the sub-lethal concentrations for the four novel environments when the bacteria were grown on slides (see Appendix 2.5 for concentrations). The identity of these novel environments were chosen such that there are no known correlations between the mechanism of stress resistance to them and the three stresses used in the fluctuating selection (see discussion 4.3). Glycerol stock for S or F population was revived overnight in 50 ml Nutrient Broth. This revived culture was used to flood the slide layered with nutrient agar (see Appendix 2.1 for composition) containing one of the novel environment. After the broth had dried off (~ 30 minutes at room temperature under aseptic conditions), the agar surface was covered with a cover slip, excess agar outside the cover slip was removed with the help of a scalpel, and the sides were sealed with the mounting medium DPX (Di-n-butyl phthalate in xylene). The slide was then placed on the stage of a microscope (Primo StarTM, Zeiss, Jena, Germany) which in turn was placed at 37^0 C throughout the observation time.

A suitable field containing 6 to 20 single, well-spaced cells was focused under 100X magnification. For each cell in the field of view, we manually scored the time taken by the cell and its progenies to divide over a period of 240 minutes from the preparation of the slide

i.e. from the time when broth was poured on the agar slide. Two trials were conducted for every replicate population of S and F in every novel environment (2 × 6 × 4 = 48 trials). The yield of each cell was estimated as the number of progenies produced by the cell at the end of 240 minutes. We also measured the 'lag' as the time taken for the first division. Since the cells were not synchronized, the lag estimate is likely to be associated with some amount of error. However, there is no reason to believe that this would affect S and F populations differentially. Moreover, since we measured substantial number of cells per population, such errors arising due to lack of synchronicity should be further ameliorated.

2.3 Statistical analysis

2.3.1 Component, complex and novel assay environments

Pooled data was analyzed using 4-way mixed model ANOVA. Selection (two levels: S and F) and assay environment were fixed factors while replication (three levels) was a random factor nested in selection. Trial was a random factor nested in selection × environment × replicate. Assay environment had four (acidic pH, basic pH, salt, H_2O_2), six (# 13, 22, 49, 51, 54, 68) and four (cobalt, zinc, ethidium bromide, norfloxacin) levels for component, complex and novel assays respectively. To compare the performance of S and F populations in individual environment, we performed 3 way mixed model ANOVA with selection (two levels: S and F) as a fixed factor, replicate (three levels) as a random factor nested in selection and trial as a random factor nested in selection × replicate.

2.3.2 Deteriorating environment

Pooled data was analyzed using 3-way mixed model ANOVA. Selection (two levels: S and F) and assay environment (three levels: cobalt, zinc and norfloxacin) were fixed factors while replication (three levels) was a random factor nested in selection. To compare the performance of S and F populations in every assay environment individually, we performed three different 2 way ANOVA with selection (two levels: S and F) as a fixed factor and replicate (three levels) as a random factor nested in selection.

2.3.3 Acclimation

Pooled data were analyzed using 4-way mixed model ANOVA. Selection (two levels: S and F) duration of acclimation (three levels: 3hr, 6hr and 9hr) and assay environment (four levels: cobalt, zinc, ethidium bromide and norfloxacin) were fixed factors while replication (three levels) was a random factor nested in selection. To compare the performance of S and F populations at every combination of acclimation duration and assay environment, we performed twelve different 2-way ANOVA with selection (two levels: S and F) as a fixed factor and replicate (three levels) as a random factor nested in selection.

2.3.4 Fitness at individual level

The yield and lag data were analyzed separately using mixed model ANOVA with novel assay environment (4 levels: Cobalt, Zinc, Norfloxacin and Streptomycin) and selection (three levels: S and F) as fixed factors and replication (three levels, nested within selection) and trial (two levels, nested in assay environment × selection × replication) as random factors. We also performed the individual mixed model ANOVAs for each of the novel assay environments. For this set of analysis, selection (two levels: S and F) was treated as a fixed factor and replication (three levels, nested within selection) and trial (two levels, nested in

selection × replication) as random factors.

In case of multiple ANOVAs family-wise error rates were controlled through sequential Holm -Šidàk correction of the P values (Abdi 2010). To judge the biological significance of the differences in mean growth rates for F and S populations at different assay environments, we computed Cohen's *d* statistic (Cohen 1988) as a measure of the effect sizes (Sullivan and Feinn 2012). Following existing guidelines (Cohen 1988), we interpreted the effect sizes as small, medium and large for $0.2<d<0.5$, $0.5<d<0.8$ and $d>0.8$, respectively. Cohen's d statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004).

3 RESULTS

3.1 Fitness under component environments:

After ~170 generations of selection, the maximum growth rates of the F populations were significantly greater than the S, when measured under conditions that were components of the fluctuating selection regime, i.e. pH (4.5/10), high salt or H_2O_2 ($F_{1,4}$ = 9.34, p = 0.038, Table 2.1, Fig 2.1 A). However, the effect size for this difference was small (d = 0.2, Table 2.1) which indicated that the difference might be biologically insignificant. There was also a significant main effect of component environment ($F_{3,12}$ = 79.92, p = 3.4E-08) which is not surprising as the different conditions (i.e. pH 4.5, H_2O_2, etc.) are not expected to affect the growth rates similarly. However, the component environment × selection interaction was also significant ($F_{3,12}$ = 3.850, p = 0.038), implying that there were differences in terms of how the growth rates of F and S populations were getting affected by the various conditions. To investigate this in greater detail, we conducted separate ANOVAs for the effect of each condition of the component environment and found that the growth rates of the F and S populations did not differ significantly in any of the stresses (Table 2.2, Fig 2.1 A). The effect sizes of the differences were large for two conditions (pH4.5 and H_2O_2), medium for salt and small for pH10 (Table 2.2). Interestingly, in pH 4.5 although the difference has a large effect size, it is actually the S populations which have higher growth rates than the F. Overall, this set of analysis leads to the conclusion that in spite of a significant main effect, there is no evidence to indicate that the F populations generally performed better than the S under conditions that were components of the fluctuating selection regime.

3.2 Fitness under complex environments

The pattern of fitness differences under complex environments was similar to those under component environments. When data from all six complex environments were analyzed together, there was no significant difference between the growth rates of F and S populations ($F_{1,4}$ = 2.37, p = 0.199, Table 2.1) and the effect size was small (d = 0.34, Fig 2.1 B, Table 2.1). When analyzed separately, the average maximum growth rates of the F populations were always larger than those of S populations (Fig 2.1 B), although none of the differences were significant at the 0.05 level, and only one effect size was large (Table 2.2). Overall, this suggests that the F populations did not show greater fitness in the complex environments.

3.3 Fitness under novel environments:

When analyzed together, the F populations had significantly higher maximum growth rates under the four novel environments that we investigated ($F_{1,4}$ = 1320.48, p = 3.4E-06, Table 2.1, Fig 2.2 A) and the effect size of this difference was large (d = 1.45, Table 2.1). As with component environments, there was a significant effect of the novel environment ($F_{3,12}$ = 20.38, p = 5.3E-05) and a significant novel environment × selection interaction ($F_{3,12}$ = 48.90, p = 5.3E-07). When analyzed separately, F had significantly greater growth rate than S in three (Cobalt, Norfloxacin, Ethidium bromide) of the four novel environments and the effect size in each of these cases was large (Table 2.2, Fig 2.2 A). In the case of zinc, F still had a higher growth rate than S, although the differences were not statistically significant and the effect sizes were small. However, crucially, the S populations never had a larger growth rate than the F populations. This suggests that the F populations have evolved to counter at least three novel environments without becoming worse than the controls in at least one other novel environment.

3.4 Fitness after selection under deteriorating novel environments

After directional selection for ~50 generations in three novel environments, F populations had significantly higher growth rates compared to the S and a medium effect size of the difference ($F_{1,4}$ = 24.68, p = 0.008; d = 0.51, Table 2.1, Fig 2.2 B). When analyzed separately, the difference in growth rates in all three novel environments were significant after Holm-Šidàk correction (p = 0.036, p = 0.039, p = 0.043 for Cobalt, Norfloxacin and Zinc respectively, Table 2.2) and the effect sizes of the differences were large (Table 2.2).

3.5 Fitness after acclimation in novel environments

F populations showed significantly higher fitness than S populations when pooled over all the four stresses for all the three time points ($F_{1,4}$ = 49.61, p = 0.0001; d = 1.07, Table 2.1, Fig 2.3) There was a significant interaction between selection and environment ($F_{3,12}$ = 19.61, p < 0.0001). There was significant effect of novel environment ($F_{3,12}$ = 29.52, p < 0.0001), duration of acclimation ($F_{2,8}$ = 23.54, p = 0.0004) and significant interaction between the two ($F_{6,24}$ = 3.43, p = 0.013). When fitness at every combination of acclimation duration and novel environment was analyzed separately, 4 out of 12 comparisons turned out to be significant after Holm-Sidak correction with large effect sizes (Table 2.2, Fig 2.3). Though F populations had higher mean fitness at every individual comparison, the difference was significant in case of ethidium bromide alone (Fig 2.3). This suggests that F populations have higher post-acclimation fitness in some environments but not in others.

3.6 Fitness of individual bacterial cells

When pooled over all the novel environments, individuals from F populations displayed significantly lower lag time (Fig 2.4 A) and higher yield (Fig 2.4 B) than individuals from S populations, with medium and large effect sizes respectively (Table 2.1). There was a significant effect of the novel environment in both cases ($F_{3, 12} = 66.75$, $p < 0.001$ for lag and $F_{3, 12} = 88.93$, $p < 0.001$ for yield) indicating that the difference in the fitness varies across different novel environments. This is intuitive as all the environments are not expected to affect fitness similarly. When analyzed separately for each novel environment, F populations had significantly and marginally significantly lower lag time in cobalt and streptomycin respectively (Table 2.2 , Fig 2.4 A) and significantly higher yield in cobalt, streptomycin and norfloxacin (Table 2.2, Fig 2.4 B). The effect sizes were large in all these cases (Table 2.2). It is important to note that in all the four novel environments, F populations showed lower lag time and higher yield compared to S populations. Overall, these results demonstrate the growth advantage for individuals of F populations in the four novel environments, corroborating the population level outcomes observed earlier (section 3.3 and 3.4).

Assay	Mean S	Mean F	ANOVA F(1,4)	ANOVA p values	Effect Size±95% CI	Inference
Fitness in Component Environments	0.109	0.121	9.34	0.038	0.20±0.33	Small
Fitness in Complex Environments	0.074	0.088	2.37	0.199	0.34±0.27	Small
Fitness in Novel Environments	0.028	0.058	1320.48	3.4E-06	1.45±0.37	Large
Fitness Post Acclimation	0.014	0.026	49.61	0.002	1.08±0.29	Large
Fitness Post Deteriorating Environments	0.076	0.086	24.68	0.008	0.51±0.54	Medium
Lag of the individual bacterial cells	147.66	118.69	19.20	0.012	0.50±0.18	Medium
Yield of the individual bacterial cells	3.89	6.26	92.94	0.0006	0.94±0.19	Large

Table 2.1 Summary of the main effects of selection in the pooled ANOVAs

Assay	Environment	Mean S	Mean F	ANOVA F(1,4)	p values (Holm-Šidàk corrected)	Effect Size ±95% CI	Inference
Fitness in component environments	pH 10	0.143	0.152	2.20	0.21	0.34±0.66	Small
	pH 4.5	0.0678	0.0455	3.22	0.27	0.82±0.68	Large
	Salt	0.0658	0.0939	4.19	0.29	0.56±0.67	Medium
	H_2O_2	0.161	0.193	6.54	0.23	1.22±0.71	Large
Fitness in complex environments	No. 51	0.037	0.054	4.05	0.38	0.75±0.68	Medium
	No. 54	0.047	0.058	1.16	0.71	0.42±0.66	Small
	No. 68	0.064	0.076	18.65	0.07	0.9±0.69	Large
	No. 49	0.126	0.155	5.67	0.32	0.79±0.68	Medium
	No. 22	0.078	0.084	0.15	0.71	0.29±0.66	Small
	No. 13	0.095	0.102	0.23	0.88	0.34±0.66	Small
Fitness in novel environments	Cobalt	0.019	0.079	308.37	2.4E-04	2.71±0.90	Large
	Norfloxacin	0.024	0.070	68.02	1.3E-03	3.34±1.01	Large
	EtBr	0.037	0.055	111.88	2.3E-03	1.14±0.70	Large
	Zinc	0.030	0.029	0.63	0.47	0.20±0.65	Small
Fitness post acclimation	Cobalt 3hr	0.01	0.015	0.5	0.767	0.63±0.95	Medium
	Cobalt 6hr	0.018	0.02	0.47	0.531	0.58±0.94	Medium
	Cobalt 9hr	0.018	0.03	14.87	0.135	2.92±1.33	Large
	Zinc 3hr	0.008	0.015	2.57	0.705	1.1±0.99	Large
	Zinc 6hr	0.01	0.016	11.48	0.18	2.4±1.21	Large
	Zinc 9hr	0.009	0.016	51.26	0.021	3.99±1.6	Large
	Norflox 3hr	0.024	0.028	0.609	0.859	0.49±0.94	Small
	Norflox 6hr	0.028	0.032	0.94	0.913	0.79±0.96	Medium
	Norflox 9hr	0.034	0.037	0.7	0.908	0.39±0.93	Small
	EtBr 3hr	0.001	0.029	40.42	0.027	3.16±1.39	Large
	EtBr 6hr	0.001	0.033	47.97	0.02	3.78±2.14	Large
	EtBr 9hr	0.001	0.035	417.5	0.01	7.59±2.64	Large
Fitness post selection in deteriorating environments	Cobalt	0.083	0.093	9.56	0.03	2.08±1.14	Large
	Norfloxacin	0.092	0.104	17.81	0.03	1.91±1.11	Large
	Zinc	0.054	0.060	13.15	0.04	1.20±1.00	Large
Lag of the individual bacterial cells	Cobalt	4.877	7.759	43.33	0.008	1.79±0.43	Large
	Norfloxacin	5.396	7.479	40.77	0.006	1.15±0.42	Large
	Zinc	1.897	3.306	6.47	0.064	0.69±0.34	Medium
	Streptomycin	4.000	7.519	99.53	0.002	1.77±0.42	Large
Yield of the individual bacterial cells	Cobalt	119.070	77.000	34.53	0.017	1.46±0.36	Large
	Norfloxacin	118.854	108.208	0.99	0.375	0.26±0.4	Small
	Zinc	206.059	185.056	1.62	0.47	0.42±0.33	Small
	Streptomycin	131.667	84.278	14.93	0.053	1.22±0.4	Large

Table 2.2 Summary of the main effect of selection in the ANOVAs under individual environments

	Assay Environment	Mean C	Mean F	ANOVA *p* values	Effect Size±95% CI	Inference
Growth rate	24^0C	0.31	0.34	<0.001	0.43±0.25	Small
	31^0C	0.46	0.51	<0.001	0.53±0.25	Medium
	38^0C	0.37	0.41	<0.001	0.61±0.25	Medium
Yield	24^0C	0.086	0.089	<0.001	0.21±0.25	Small
	31^0C	0.1	0.11	0.478	0.12±0.25	Small
	38^0C	0.075	0.079	<0.001	0.45±0.25	Small

Table 2.3 Effect size computed for the growth rates and yield measured in the three temperature environments from Figure 1 of Ketola et al 2013 (Ketola, Mikonranta et al. 2013, Ketola, Mikonranta et al. 2013). Means and ANOVA p-values are as reported in that paper

4 DISCUSSION

4.1 On measurement of fitness

Most experimental evolution studies in microbes measure fitness in either of two ways. The first is a measure of fitness in terms of growth rate or yield (Holder and Bull 2001, Ketola, Mikonranta et al. 2013). The second involves measuring competitive fitness by mixing the evolved strains with the ancestors and scoring their relative densities after a period of growth (Travisano, Vasi et al. 1995, Silander, Tenaillon et al. 2007). It is sometimes argued that the second method is more preferable as it also includes a measure of the competitive ability and hence gives an estimate of the magnitude of adaptation that has occurred in the selected populations over the course of the experiment (Kassen 2014).

By definition, measuring competitive fitness equates evolutionary change with change in competitive ability and thus equates evolution with the ability of one genotype to replace another. It is, therefore, a narrow definition of fitness in the context of a correspondingly narrow and strict concept of evolution. However, the present study employs a broader notion of evolution as change through time within a species (Losos, Baum et al. 2013) and fitness as a measure of number of offspring in a given unit of time (i.e. yield) or any trait that affects that number (i.e. lag time). This is because we intuitively find no reason to expect that exposure to randomly fluctuating environments would lead to a change in competitive ability. Furthermore, we explicitly aimed to study fitness at the level of individual bacterium, which also enabled us to investigate phenomenon like population-based resistance. The notion of competitive fitness is not congruous with this aim and hence is not used here. To summarize, our concept of fitness is similar to the usage some (Holder and Bull 2001, Ketola, Mikonranta et al. 2013) and may or may not correspond with a change in competitive fitness as used in some other studies (Travisano, Vasi et al. 1995, Silander, Tenaillon et al. 2007).

4.2 No significant adaptation to the component or complex environments

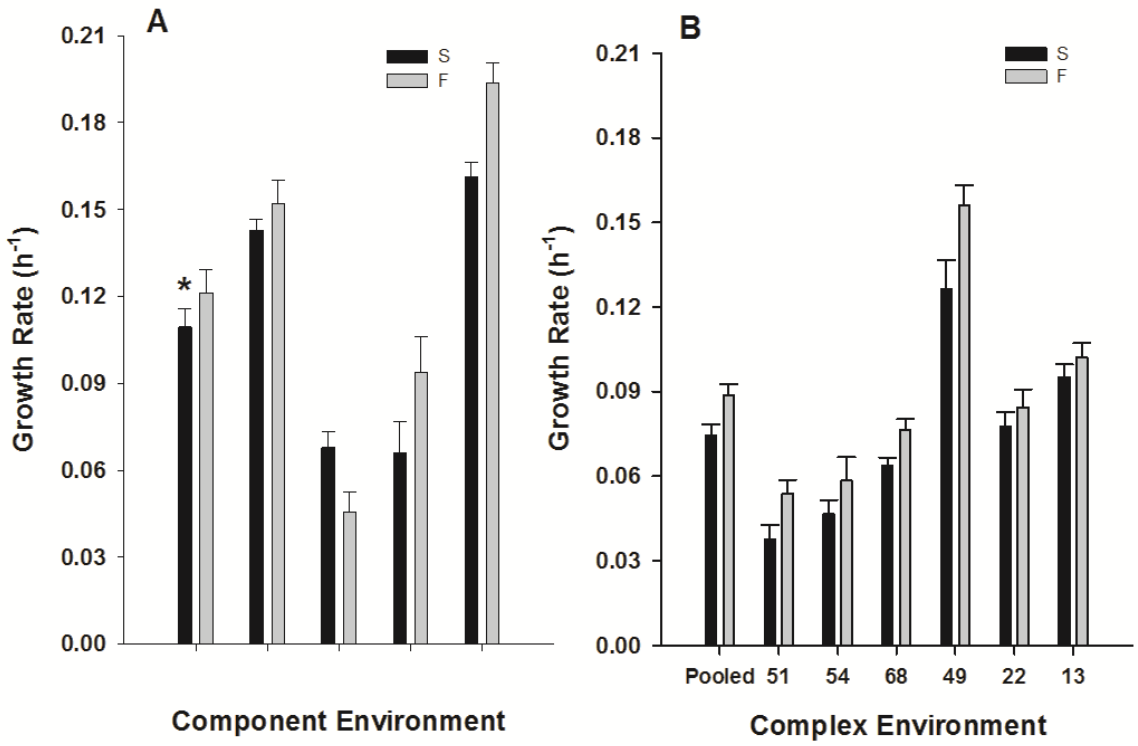

Figure 2.1: Mean (±SE) fitness in component and complex environments. A. Component environments. In the first comparison of the pooled means over all four component environments, the selected (F) populations show significantly higher fitness than the control (S) populations. The next four comparisons are for the individual component environments with no significant difference (individual ANOVAs) in the fitness of F and S populations. **B.** Complex environments. There was no significant difference between the fitness of the F and S populations, either pooled over the six complex environments, or separately in each environment. Fitness was measured as maximum slope of the growth trajectory over 24 hours.

* denotes $p < 0.05$.

When fluctuations in the selection environment are predictable, organisms often evolve to have higher fitness in the component environments (i.e. conditions experienced during the selection process) (Leroi, Lenski et al. 1994, Turner and Elena 2000, Hughes, Cullum et al. 2007, Coffey and Vignuzzi 2011, Alto, Wasik et al. 2013, Puentes-Téllez, Hansen et al. 2013, Condon, Cooper et al. 2014), although some studies report no change in fitness relative to the ancestors or controls (Buckling, Wills et al. 2003). However, when the selection environment changes unpredictably, fitness in the component environments can increase (Turner and Elena 2000, Ketola, Mikonranta et al. 2013), show no change (Alto, Wasik et al. 2013), increase w.r.t some life-history traits and decrease w.r.t others (Hallsson and Björklund 2012) or increase in some of the environments but not all (Hughes, Cullum et al. 2007). In this study, although we observed an overall statistically significant increase in fitness under component environments (Fig 2.1 A), the magnitude of this increase was not biologically meaningful (Table 2.1), particularly on short time-scales. In terms of complex environments (i.e. the kind of environments actually faced during selection), the fitness of the selected populations did not differ significantly from the controls and the magnitude of the effect size was low.

There can be multiple (and non-exclusive) reasons for the above observations. Firstly, it is known that the response to selection depends on the rate at which the environment is changing (Venail, Kaltz et al. 2011) and some evolutionary outcomes are possible only when the environments change relatively slowly (Lindsey, Gallie et al. 2013). In our experiment, the environment changed every 24 hours i.e. every ~6 generations, which might be too fast for the bacteria to adapt. Secondly, earlier studies on effects of unpredictable environmental fluctuations have mostly been in the context of simple conditions like temperature (Hallsson and Björklund 2012, Alto, Wasik et al. 2013, Ketola, Mikonranta et al. 2013) or pH (Hughes, Cullum et al. 2007). However, our study looked at unpredictable combinations of multiple

values of three conditions (pH, salt and H_2O_2) leading to a total of 72 possible environments, out of which 24 were actually experienced during the course of this study (Appendix 2.2). This means that, on an average, our bacteria were forced to adapt to a novel combination almost every day. When the direction and target of selection changes stochastically, alleles that experience positive selection in a given environment might end up being neutral or negatively selected in the next environment. If the changes in environment happen sufficiently fast (as was the case in our populations), alleles with positive effects on fitness may not get sufficient time to get fixed before the environment changes. As a result of this, at least in the short run, a population might keep on evolving continuously, without really improving in terms of fitness in any of the component environments.

Another reason for the insignificant change in growth rate in component and complex environments might be the relatively short duration of the selection experiment (30 days, ~170 generations). This appears to contradict a recent study demonstrating that within three weeks of selecting *Serratia* populations under randomly fluctuating temperatures, the population growth rates can increase significantly (Ketola, Mikonranta et al. 2013). However, when we estimated the effect sizes from the fitness data of the previous study (Ketola, Mikonranta et al. 2013a), it was found that the Cohen's *d* for the differences in fitness of the control and the selected were small to medium (Table 2.3) and never large. It is evidently difficult to compare effect sizes across model organisms. However, if we adopt the same criteria for interpreting Cohen's *d* statistics for both studies, then the conclusions are identical: selection for short durations leads to statistically significant increase in fitness under component environments, but the magnitude of change is small. Interestingly, when *E. coli* are subjected to long term selection (~2000 generations), the magnitude of increase in fitness under unpredictable environments is typically less than that under predictably fluctuating environments (Hughes, Cullum et al. 2007). This suggests that somehow,

stochastic fluctuations hinder the evolutionary increase of fitness more than deterministic fluctuations, which is consistent with similar experiments on viruses (Alto, Wasik et al. 2013).

To summarize, given the fast rate of change of environment, large number of possible states that selection environment could take and the relatively short duration of the selection experiment, it is not surprising that the F populations in general failed to increase fitness in the component environments. By that line of reasoning, there was no reason to expect any changes in fitness under novel environments either. However, that was not the case.

4.3 Short-term and long-term increase in fitness under novel environments

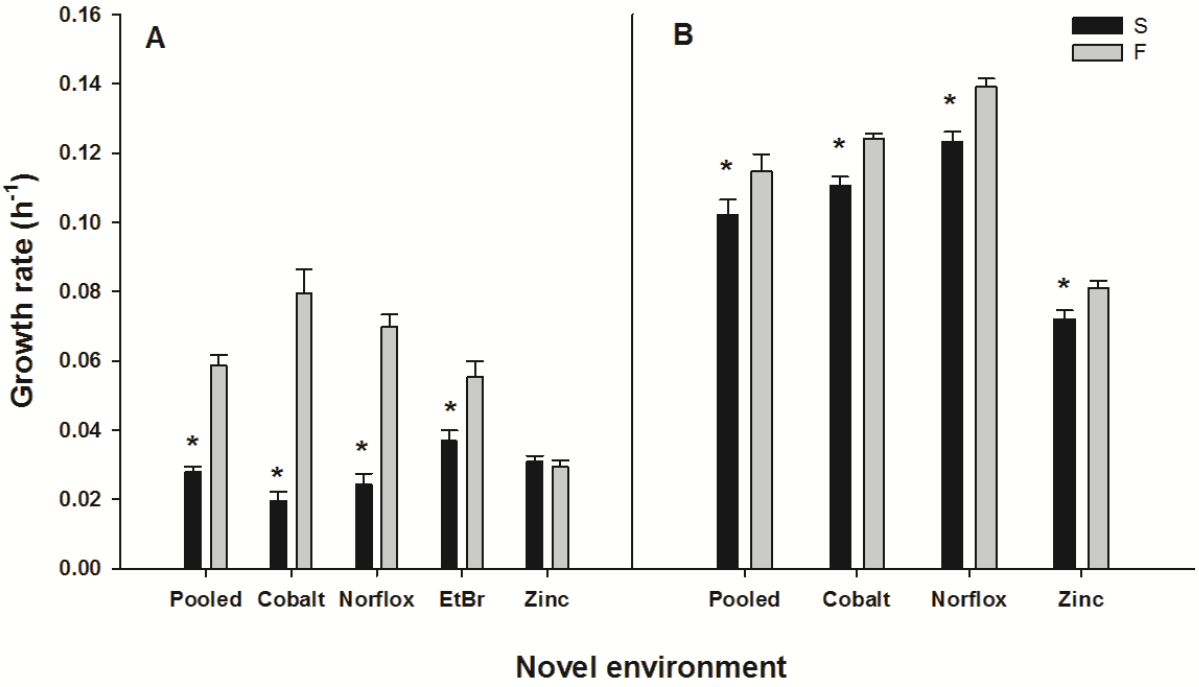

Figure 2.2: A. Mean (±SE) fitness in novel environments. Pooled over the four novel environments, F populations show significantly higher fitness than S populations. When compared separately for each novel environment, F populations show significantly higher fitness in cobalt, norfloxacin and ethidium bromide and similar fitness in zinc. **B. Mean (±SE) fitness in novel environments after facing deteriorating environments.** Pooled over the three novel environments, populations selected under fluctuating environments (F) show significantly higher fitness than the control (S) populations. When compared separately for each novel environment, the F populations still had significantly higher growth rate than the S populations. This indicates that the fitness advantages of the F populations were retained after ~50 generations of deteriorating selection.
* denotes $p < 0.05$ (after Holm-Šidàk correction in the case of comparisons under individual environments).

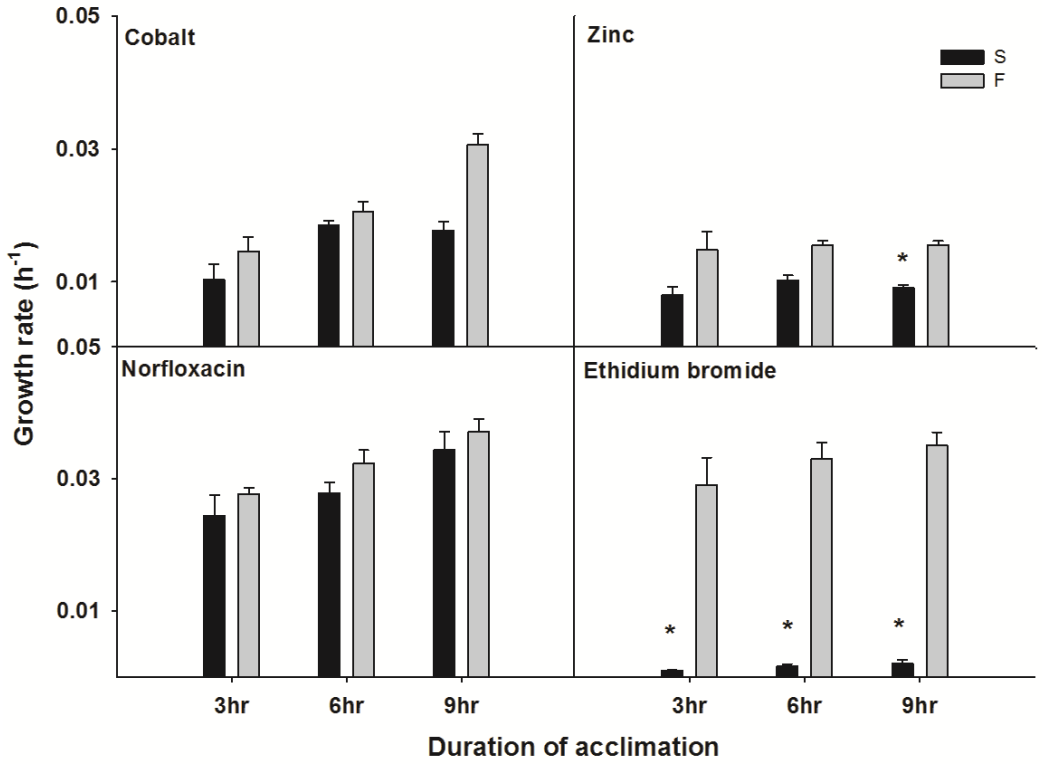

Figure 2.3: Mean (±SE) fitness in novel environments after acclimation. F and S populations compared separately for the combination of each novel environment and duration of acclimation. F populations show significantly higher growth rate than the S populations in four out twelve instances.

* denotes $p < 0.05$ (after Holm-Šidàk correction in the case of comparisons under individual environments).

When exposed to novel environments, the F populations had significantly higher fitness in three of the four environments and suffered no disadvantage compared to the controls in the remaining one (Fig 2.2 A, Table 2.2). This suggests that selection under fluctuating environments can increase the ability of populations to face completely novel stresses, which agrees with the results of a previous study (Ketola, Mikonranta et al. 2013). This is also consistent with observations from the literature on invasive species that organisms inhabiting disturbed habitats are often able to cope better with novel environments, thus becoming better invaders (reviewed in Lee and Gelembiuk 2008).

The simplest explanation for the better performance of the F populations under novel environment could be the presence of cross-tolerance / correlated response to selection for pH, salt and H_2O_2. However, we failed to locate any study in the literature that has observed an increased growth rate under any of our novel stresses as a result of selection for any of the three stresses that we used as components for the fluctuating environment. This is perhaps not surprising since the four novel stresses that we used had very different modes of action. For example, while norfloxacin is a DNA gyrase and topoisomerase IV inhibitor (Drlica and Zhao 1997), ethidium bromide is a DNA intercalating agent which can cause frame-shift mutations (Singer, Lawlor et al. 1999). Cobalt affects the activity of iron-sulfur enzymes (Ranquet, Ollagnier-de-Choudens et al. 2007) but zinc acts primarily by preventing manganese uptake by cells (McDevitt, Ogunniyi et al. 2011). Although, in principle, it is possible that the F populations had evolved four different mechanisms to fight each of these stresses through correlated response(s), the likelihood of such an event was low, particularly given the relatively short duration of the selection. While this lack of evidence from the literature cannot be construed as a proof, it at least forced us to explore the possibility of other mechanisms that might be at work to confer a growth advantage to the F populations upon exposure to novel environments.

Some theoretical studies suggest that environmental fluctuations spanning across a few generations (as in our study) can promote the maintenance of genetic variation in a population (Turelli and Barton 2004), presumably leading to advantages under novel conditions. A different modelling approach predicts that when environments fluctuate rapidly, organisms of intermediate fitness are selected, which can tolerate a multitude of conditions, but none too well (Meyers, Ancel et al. 2005). Intuitively, it is expected that any mechanism which allows a population to attain higher growth rates upon first exposure to a novel stress, will also be beneficial under acclimation and directional selection for that stress. This is because a higher growth rate during the initial stages of exposure to a stress allows the bacterial population to undergo more divisions and hence an increased probability of acquiring a suitable beneficial mutation which can then spread in the population. Thus, the F populations were expected to have greater fitness after the acclimation as well under deteriorating selection in deteriorating environments. After ~50 generations, the F populations retained an overall higher growth rate than the S populations in all three novel environments (Fig 2.2 B, Table 2.2). However, the magnitude of difference in fitness between F and S populations reduced after 50 generations of deteriorating selection (see Fig 2.2). This observation is consistent with a scenario where the selected populations have more standing genetic variation but do not have an increased mutation rate. Greater standing genetic variation makes the existence of a favorable mutation in the population more likely, thus providing an early growth advantage. On the other hand, an increased mutation rate in the F populations is expected to increase the difference in fitness vis-à-vis the controls, at least in the short run (Wagner 1981), which was not found to be the case.

Surprisingly, F populations did not show significantly higher mean growth rate than S populations after acclimation (Fig 2.3). Mean growth rate of F populations was always

greater than S populations but out of 12 such comparisons, only 4 were statistically significant with large effect size (Table 2.2, Fig 2.3). Moreover, the growth rates after acclimation are lower than those estimated at first exposure to the novel environment. This discrepancy is probably due to the lack of revival step before the growth rate estimation. Growth rate estimation was performed by inoculating the part of glycerol stock into the novel environment, which could have affected the growth rates of S and F populations. In spite of higher mean growth rates, F populations do not perform significantly better in the face of novel environment in post acclimation conditions.

4.4 Higher fitness of individuals in novel environments

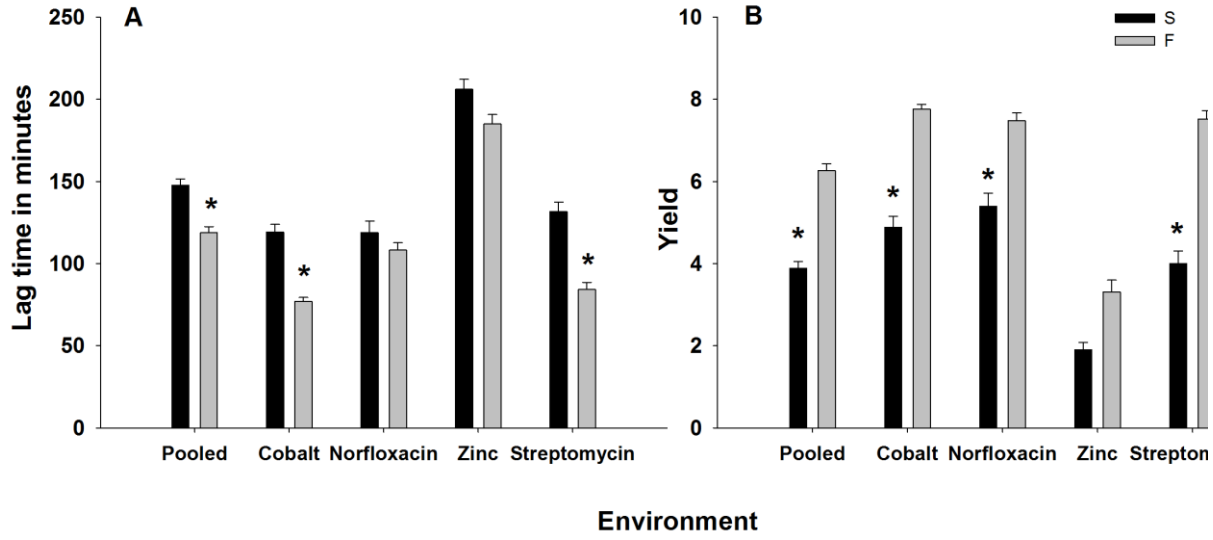

Figure 2.4: Fitness of individual bacterial cells. A. Mean (±SE) lag time is significantly lower for F populations than S populations when pooled over four novel environments. When compared separately for each novel environment, F populations show significantly lower lag time in cobalt and streptomycin and similar lag time in norfloxacin and zinc. **B.** Mean (±SE) yield is significantly higher for F populations than S populations when pooled over four novel environments. When compared separately for each novel environment, F populations show significantly higher yield for cobalt, norfloxacin and streptomycin and similar yield for zinc.

* denotes $p < 0.05$ (after Holm-Šidàk correction in the case of comparisons under individual environments).

For all the four novel environments, the lag times were lower for F populations and yields were higher (Fig 2.4) although the differences were not statistically significant for each comparison (Table 2.2). This corroborates similar observations at the population level (Fig 2.2).

Increased fitness in multiple novel environments can come about in at least two major ways: an increased rate of generating new variation or the existence of larger amount of standing variation. If the first case were true, then one would not expect the progenies of all individuals of the F populations to acquire the favorable mutations at the same time in a novel environment. If the F populations had increased standing variation which was contributing to their enhanced fitness under novel environments, then again one would expect that most individuals would fail to grow and the progenies of only few individuals would primarily contribute to the final population size. However, we found no outliers in terms of contributions to the final size of the population (see Fig 3.2 and section 4.2 in chapter 3 for details) which suggests that whatever the mechanism that had evolved, was benefitting all the existing F individuals similarly. This observation does not fit with either increased rate of generation of variation or increased standing genetic variation.

5 SUMMARY

The results of this chapter show that F populations do not improve fitness under component or complex environments but show improved fitness in the novel environmental backgrounds. Fitness advantage is retained over two different time scales of exposure to the novel environments as well as at the level of individuals. Next chapter looks at the possible mechanisms which could have led to the observed patterns of fitness advantage in the various novel environments.

Chapter 3. Efficient energy dependent efflux is the most likely mechanism leading to fitness advantage in novel environments

1 INTRODUCTION

One previous study (Ketola, Mikonranta et al. 2013) and the previous chapter suggest that fluctuating environments can select for higher fitness in novel environments. The parsimonious explanation for such an increase could be a correlated response to fitness improvements in the selection environments. In such a case, common mechanism employed for fighting both the selection and novel environments is the most likely candidate for the mechanistic basis of the observed improvement in fitness. But contradictory to this premise, previous results show that F populations show fitness advantage in the face of novel environments without any similar outcome in the component environments. More importantly, the novel environments tested, are diverse in their mechanism of action. Norfloxacin is a DNA gyrase and topoisomerase IV inhibitor (Drlica and Zhao 1997), ethidium bromide is a DNA-intercalating agent which can cause frameshift mutations (Singer, Lawlor et al. 1999). Cobalt affects the activity of iron–sulphur enzymes (Ranquet, Ollagnier-de-Choudens et al. 2007), but zinc acts primarily by preventing manganese uptake by cells (McDevitt, Ogunniyi et al. 2011). Taking these observations into account, here we investigated two variation based mechanisms which can confer fitness advantage in the face of any novel environment, correlated or not.

First one is the ability to generate greater genetic variation. This can be achieved by increasing the rate of generation of variation, which has been suggested to be one of the primary mechanisms by which evolvability can evolve (reviewed in Pigliucci 2008). Populations selected under constant glucose limitation have been previously shown to evolve the ability to generate higher amount of variation, *aka* the hypermutator phenotype (Sniegowski, Gerrish et al. 1997). Owing to faulty mismatch repair, hypermutators show 2 to 3 orders of magnitude increase in the mutation rate and can help the corresponding genotype to acquire favourable variations faster. The hypemutator phenotype can then hitchhike with

the favourable variation generated. The second possible route to obtain greater amount of genetic variation is by increasing the standing variation in the population. Theoretical studies predict that fluctuating environments can select for higher standing variation (Gillespie and Turelli 1989, Turelli and Barton 2004) which, in turn, can increase the variation for fitness. We checked this possibility by looking at the distribution of fitness for individual cells in the novel environments. Additionally, we also compared the phenotypic variation across F and S populations as a meaningful proxy of standing genetic variation

Next we investigated a system specific mechanism of energy dependent efflux which is known to play a role in resistance to heavy metals and antibiotics (Li and Nikaido 2009, Nikaido and Pagès 2012). In addition to F and S populations, we also estimated the efflux rate for populations exposed to constant concentration of component environments (see section 2.5 for details).

Differential efflux abilities between F and S populations prompted us to investigate whether the fitness advantage in the face of novel environments can be extended to cold temperature where efflux is not likely to play any role. We also checked whether fitness advantage of F populations decays with increased duration of relaxation i.e. growth in NB for 5 to 6 generations.

We found that F populations do not differ from S populations in their variation based attributes. Instead they have evolved a systemic response of higher energy dependent efflux which can confer fitness advantage in multiple novel environments.

2 METHODS

2.1 Mutation rate measurement& Sequencing mutS and mutL genes

For the presence of hypermutators, we estimated the mutation rates (see Appendix 1.2 for details) of S and F populations in presence of antibiotic rifampicin. Additionally, we sequenced the candidate genes from mismatch repair pathway (see Appendix 1.3 for details), mutS and mutL, for the presence of mutations.

2.2 Population based resistance in novel environments

Population-based resistance occurs when a small fraction of the individuals synthesize a chemical which is then available to the other individuals of the population. However, as in our assay for individual-level fitness (see section 2.2.6 of chapter 2), when the bacteria are immobilized over an agar surface at extremely low densities for short durations, exchange of such chemicals become almost impossible. Thus, only those bacteria can resist the stresses which are able to synthesize the stress-fighting chemical on their own. If such bacteria are an extremely small fraction of the population, then they are expected to show up as outliers in the growth rate assay (see discussion for further elaboration).

Most formal tests of outlier detection assume the underlying data to be normally distributed (Barnett and Lewis 1994). Since our yield data did not meet this assumption, we used plots of the cumulative yield percentage to check for outliers. For this, we computed the percentage contribution of each parental bacteria to the final yield, arranged the values from both trials in ascending order and plotted the cumulative percentage yield against the percentage of the parental cells. In this plot, any cell(s) with disproportionate contribution to the overall yield can be easily identified by a sharp upward inflection towards the right of the graph.

2.3 Assay for phenotypic variation

We assayed the phenotypic variation in the population using GEN III MicroPlate^{TM} (Catalog no. 1030 Biolog, Hayward, CA, USA). Each of these plates contains 94 separate substrates of which 71 can be utilized as carbon sources while 23 can act as growth inhibitors. The presence or absence of growth is indicated with the help of tetrazolium redox dye where intensity of purple color is proportional to the amount of growth.

From each of the F and S populations, we obtained 8 clones by streaking the glycerol stock on a Nutrient Agar plate and incubating overnight at 37^0C. Thus a total of 48 clones were isolated over the three S and three F populations. Every clone was then characterized for the 94 different phenotypes on the Biolog plate using standard protocol (see Appendix 3.1 for details). An ancestral clone was processed in the same way to obtain the ancestral phenotypic profile.

Following a previous study (Cooper and Lenski 2000), we measured absorbance of the plates at 590 nm using a microplate reader (SynergyHT BioTek, Winooski, VT, USA). For the 23 wells with inhibitory compounds, considering the recommendations of the product manual, we scored optical densities that were 50% or more of the corresponding positive control as 1 (i.e. no inhibition) and others as 0 (inhibition). Similarly, for the 71 wells with substrate utilization test, optical density that was ≥200% of the corresponding negative control was scored as 1 (i.e. utilized) while the others were scored as 0 (i.e. not-utilized). These binary scores were then used to determine standing phenotypic variation as well as the differences from the ancestral phenotypic profile. 33 phenotypes showed no variation in S and F (i.e. all individuals in S and F were either 0 or 1) and were ignored. For estimating standing phenotypic variation over the remaining 61 phenotypes, we computed the sum score of every

replicate population over the eight clones. These values, ranging from 0 to 8, denote the variation within every population for that phenotype. It should be noted here that in some of the 94 substrates, absence of growth (i.e. 0) was the dominant phenotype while for the other substrates, the presence of growth (i.e. 1) was the dominant one. We were not interested in the qualitative nature of the phenotype (1 or 0) and wanted to analyze the variation over the entire set of 94 phenotypes. Therefore, we mapped phenotypic variation values of 5, 6, 7 and 8 to 3, 2, 1 and 0 respectively. In other words, a population in which three clones showed no growth (i.e. 3 zero values) and five clones showed growth (i.e. 5 values of 1), was deemed to have the same phenotypic variation for a given phenotype as a population which had five non-growers and three growers for a different phenotype. These mappings work only across phenotypes and fail if there are differences between the three replicates of S or F for the same phenotype. However, only three such cases were found in S populations and none at all in the F populations. The interpretations of our statistical analysis did not change with or without these points and hence we have retained these three data points.

For estimating the phenotypic divergence from the ancestor, we recorded the number of clones displaying phenotype that was different from the ancestral one, for all the F and S populations.

2.4 Efflux measurement

Energy dependent efflux of 30 day selected F and S populations was measured (see Appendix 1.4 for details).

2.5 Efflux activity under selection for adaptation to constant environment

Median values for each of the stress variable used in selection (Salt 4g%, pH5, pH9.5 and 0.012M H_2O_2) were chosen for selection in constant environment. *Escherichia coli* (strain NCIM 5547) was revived overnight in Nutrient Broth. This revived culture was used to initiate three replicate populations in each of the selection environments and Nutrient Broth, a total of 15 populations. Culturing conditions and transfer volume was as mentioned in section 2.1.1 in chapter 2. The selection lasted for seven days (i.e ~40 generations), without any extinction events, after which the populations were stored as glycerol stocks. These stocks were then used for efflux measurement (see Appendix 1.4 for details).

2.6 Growth rate estimation in cold environment

Based on range estimations with the ancestor, 22^0C was chosen as an assay temperature for fitness assessment of S and F populations in the face of broad novel stress (Bárria, Malecki et al. 2013). 100 µl of 30 day selected S and F populations were revived in 50 ml NB each. 4 µl of each of the revived culture was inoculated in 2 ml of NB in triplicates in a 24 welled plate. The plate was then incubated at 22^0C for 24 hr and fitness was assayed (see Appendix 1.1 for details).

2.7 Effect of duration of relaxation on the response to novel environments

The fitness of F populations in novel environments after 24 hr were compared to the fitness after 48 hr to check whether the fitness advantage of F populations stem from plastic mechanisms. These could be the carry over responses activated during selection in the

fluctuating stressful environment. If not inherited stably, such responses can deteriorate with the increase in the duration of relaxation.

Fitness in novel environments after 48 hr were determined with one additional overnight passage in NB before the fitness assessment. After the second day growth in NB, fitness in novel environments was assayed (see Appendix 1.1 for details) and these fitness were compared with those after 24 hr (data represented in Fig 2.2 A).

2.8 Statistical analysis

2.8.1 Mutation rate estimation

Estimated mutation rate was analyzed using 1-way ANOVA with selection (two levels: S and F) as a fixed factor.

2.8.2 Phenotypic variation

The phenotypic variations were analyzed by a two way ANOVA with phenotype (61 levels) and selection (2 levels: S and F) as fixed factors while the differences from the ancestor was analyzed using a two way ANOVA with phenotype (61 levels for Phenotypes) and selection (2 levels: S and F) as fixed factors.

2.8.3 Efflux

Efflux measurement after 30 days of selection was analyzed using 2-way ANOVA with selection (two levels: S and F) as a fixed factor and replicate (three levels) as a random factor nested in selection. For the efflux measurements after the selection in the constant

environments, the average of the three efflux measurements for each population was used for analyzing all the environments together. The pooled data was analyzed using 1-way ANOVA where Selection (five levels: Salt, pH5, pH9.5, H_2O_2 and NB) was a fixed factor. For analyzing each stress separately, we performed four separate 2-way mixed model ANOVAs where selection (two levels: selected and control) and replicates (three levels, nested in selection) were treated as fixed and random factors respectively.

2.8.4 Growth rate in cold environment

Fitness estimates in cold temperature were analyzed using a 3-way mixed model ANOVA with selection (two levels: S and F) as a fixed factor, replicate (three levels) as a random factor nested in selection and trial (two levels) as a random factor nested in selection × replication.

2.8.5 Effect of duration of relaxation on the response to novel environments

Fitness estimates were analyzed using 4-way mixed model ANOVA with duration of relaxation (two levels: 24 hr and 48 hr) and assay environment (four levels: cobalt, ethidium bromide, norfloxacin and zinc) as fixed factors, replicate (three levels) as a random factor and trial (two levels) as random factor nested in duration of selection × assay environment × replicate. We compared the effect of duration of relaxation in each of the assay environment using 3-way mixed model ANOVA with duration of relaxation (two levels: 24hr and 48 hr) as a fixed factor, replicate (three levels) as a random factor and trial (two levels) as a random factor nested in duration of selection × replicate. Family-wise error rates were controlled through sequential Holm-Šidàk correction of *p* values.

To judge the biological significance of the differences in mutation rate or efflux potential or growth rate of F and S populations, we computed Cohen's *d* statistic (Cohen 1988) as a measure of the effect sizes (Sullivan and Feinn 2012). Following existing guidelines (Cohen 1988), we interpreted the effect sizes as small, medium and large for $0.2<d<0.5$, $0.5<d<0.8$ and $d>0.8$, respectively. Cohen's d statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004).

3 RESULTS

3.1 Mutation rates and presence of hypermutator

Although the F populations had higher mutation rates than the S populations with a large effect size (d = 1.65, Table 3.1), this difference was not statistically significant ($F_{1,4}$ = 4.112, p = 0.11; Table 3.1, Fig 3.1). More importantly, the mutation rates were of the order of 10^{-8} to 10^{-9} which suggests that the ability of the F populations to face novel environments is not attributable to the evolution of hypermutators (i.e. alleles that cause 100-1000 fold increase in mutation rate) (Denamur and Matic 2006).

mutS and mutL genes were sequenced for one clone each from every S and F population. When these sequences were aligned with the library sequence for the same genes, no gaps or insertions were found except an insertion of adenine residue at 540^{th} position in mutS gene of one of the replicate F population. Similar event of insertion have been shown to result in a hypermutator phenotype in laboratory populations adapting to glucose limited environment (Shaver, Dombrowski et al. 2002). Populations with hypermutator phenotype show 100 to 1000 times increase in the mutation rates (Sniegowski, Gerrish et al. 1997). However, such increase in mutation rate was not observed in this case (Fig 3.1). Results are summarized in Table 3.2. Overall, we concluded that there were no major mutations that can be classified as a hypernutator.

3.2 Population based resistance in the novel environments

Inspection of the data suggested that there were no individual cells whose progeny contributed disproportionately to the final population size. This can also be seen from the plot

of the cumulative percentage yield of the cells, where the F populations showed a linear trend in three out of the four novel environments (Fig 3.2). Only in zinc, there was a small departure from the linearity (Fig 3.2 D). However, even then ~20% of the cells contributing to ~40-60% of the observed yield and hence, there was nothing to suggest the presence of a small number of outliers that contributed disproportionately to the growth. Interestingly, zinc was the only novel environment where F populations did not display a fitness advantage in terms of yield or lag (see discussion), thus ruling out the possibility of a few individuals conferring fitness advantage to the entire population.

3.3 Phenotypic variation

For 33 out of the 94 substrates tested, no variation was found i.e. all the 48 clones of S and F gave the same phenotype. In the remaining 61 substrates, at least 1 out of the 48 clones (8 clones each for three S and three F populations) gave a different phenotype. ANOVA on the phenotypic distances showed a significant main effect of phenotype ($F_{60, 244} = 3.69$, $p << 0.001$) suggesting some phenotypes explained more variation than others. This is intuitive as one does not expect similar number of variation for 61 traits over six populations. However, more crucially, there was no significant difference for the phenotypic variation across S and F populations, with a low effect size for the difference (Table 3.1, Fig 3.3 A). Thus, we conclude that there was no evidence of an increased phenotypic variation in the F populations.

61 out of 94 phenotypes (not the same 61 as above though) showed at least one clone that was phenotypically different from the ancestor. Although, averaged over the 61 phenotypes, the S populations showed greater divergence which was marginally statistically significant (Fig 3.3 B) the corresponding effect size of the difference was low (Table 3.1). More

crucially, there was no phenotype for which all S or F populations were different from the ancestor. Barring two cases, no consistent pattern was observed in terms of acquiring or losing a phenotypic trait. 43 out of all the 48 clones tested acquired the ability to utilize methyl pyruvate while 39 became capable of utilizing β-methyl- D-glucoside. Although prior studies indicate that the ability to catabolize methyl pyruvate (Timonen, Jørgensen et al. 1998) and β-methyl- D-glucoside (Perkins and Nicholson 2008) often evolves under different kinds of stresses, the reason for the same remains unknown. Since, both S and F populations acquired the ability to utilize these compounds it is possible that there is some fitness advantage of these two phenotypes in nutrient broth. Crucially, there were no clear patterns in terms of phenotypic divergence from the ancestor, indicating that the variation accumulated is likely to be either neutral or have very weak effect on fitness. Apart from one replicate population of S in which all the individuals tested had lost the ability to utilize D-raffinose and pectin as a carbon source, there was not a single population in S or F in which all eight individuals had diverged from the ancestor. This suggests that the observed phenotypic variation is unlikely to be a result of a strong and /or directional selection pressure on one of the phenotypes. The divergence from ancestral phenotype varied significantly across different phenotypes ($F_{60, 244} = 18.82$, $p << 0.001$) with a significant interaction with selection ($F_{60, 244} = 2.98$, $p << 0.001$). Both these results are intuitive since one neither expects similar levels of divergence over 61 substrates nor similar patterns of divergence in S and F populations.

3.4 Efflux rates

The F populations had significantly higher activity of the ATP dependent efflux pumps with a large effect size of the difference ($F_{1,4}= 34.65$, $p = 0.004$; $d = 3.79$, Table 3.1, Fig 3.4). This indicates the evolution of an increased rate of efflux of toxic materials from inside the cells,

which can explain the ability of the F populations to maintain a higher growth rate in novel environments.

The observed increase in efflux activity could have been either due to exposure to unpredictable fluctuations in the environments, or merely due to the component stresses themselves. To distinguish between these two possibilities, we compared the efflux activities of populations directionally selected under the four component stresses (acidic pH, basic pH, salt and H_2O_2). When analyzed together, there was no main effect of selection in the ANOVA ($F_{4,10}$= 0.577, p = 0.686) suggesting that efflux activity did not differ significantly among the populations subjected to the four stresses and those evolved under NB. When we analyzed the efflux activities separately for each stress, none of the differences turned out to be statistically significant at the 0.05 level (Table 3.3). In terms of the effect size of the difference, acidic pH (d = 0.29) and basic pH (d = 0.19) showed small effect sizes. In the case of salt, although the effect size was large (d = 1.13), the salt-selected populations actually had lower efflux activity than the controls. Only in case of H_2O_2, the selected populations had a larger efflux activity, and the effect size of the difference was large (d = 1.91). However, given that we failed to get a significant difference either in the pooled ANOVA or the individual one for H_2O_2, it is difficult to state that there was a significant change in efflux due to H_2O_2. Overall, these results suggest that exposure to unchanging component environments could not have been responsible for the observed increase in efflux activity of the F populations (see section 4.4 for discussion).

3.5 Fitness in cold environment

F populations displayed higher mean fitness than S populations with large effect size but the difference was not statistically significant ($F_{1,4}$ = 4.88, p = 0.092, d = 1.3, Table 3.1, Fig 3.5).

3.6 Effect of duration of relaxation on response to novel environments

Fitness differed marginally between 24 hr and 48 hr of relaxation when pooled over all the novel environments, but the effect size of the difference was small ($F_{1,2}$ = 17.97, p = 0.051, d = 0.13, Table 3.4, Fig 3.6). Surprisingly though, the fitness increased after 48 hrs of relaxation. There was a significant effect of novel environment ($F_{3,6}$ = 126.38, p <0.0001) along with the significant interaction with duration of relaxation ($F_{3,6}$ = 114.19, p <0.0001). When each of the novel environments was analyzed separately, the fitness differed significantly with large effect size between 24 hr and 48 hr of relaxation (Table 3.4, Fig 3.6). However, the direction of difference was not the same for the four novel environments. F populations displayed higher fitness after 48 hr compared to 24 hr of relaxation in cobalt and zinc while this was reversed in presence of norfloxacin and ethidium bromide. Overall, these results indicate that fitness of F populations in novel environments does not decrease with increasing the duration of relaxation.

Assay	Mean S	Mean F	ANOVA F (df effect, df error)	ANOVA p values	Effect Size±95% CI	Inference
Mutation Rate	1.0E-09	6.4E-09	4.11 (1,4)	0.11	1.65±1.85	Large
Efflux potential	0.204	0.364	34.65 (1,4)	0.004	3.79±1.55	Large
Fitness in cold environment	0.03	0.046	4.88 (1,4)	0.092	1.3±0.72	Large
Phenotypic variation	0.61	0.51	1.51 (1,244)	0.22	0.11±0.2	Small
Phenotypic divergence from ancestor	0.97	0.79	3.98 (1,244)	0.047	0.1±0.2	Small

Table 3.1 Summary of the main effects of selection

Gene	Product length (bp)	Identity	Read length (bp)	Mismatch	Gaps	Comment
mutS	2562	S	2435	4	1	(At the very end)
		S	2415	0	0	
		S	2434	1	0	(T → A at the beginning)
		F	1816	0	0	(Primer 1 did not work)
		F	2411	0	0	
		F	2302	50	1	(Insertion of 'A' near 540th base)
mutL	1850	S	1848	0	0	
		S	1848	7	0	(Between 660 and 1210 bp)
		S	1848	0	0	
		F	1153	0	0	
		F	1848	2	0	(Both G→A, between 850 and 1200)
		F	1515	1	0	(C→G, at the beginning)

Table 3.2 Details of mismatch and gaps for the sequenced mutS and mutL gene from S and F populations

Selection environment	Mean Control	Mean Selected	ANOVA F(1,4)	ANOVA *p* values	Effect Size±95% CI	Inference
Salt	0.648	0.49	1.52	0.285	1.13±0.99	Large
pH 5	0.551	0.507	0.1	0.765	0.29±0.93	Small
pH 9.5	0.59	0.622	0.04	0.851	0.19±0.93	Small
H_2O_2	0.458	0.626	4.74	0.095	1.91±1.11	Large

Note that no Holm-Šidàk correction was done since even the lowest *p*-value was not significant at the 0.05 level.

Table 3.3 Results of individual ANOVA show no difference in the energy dependent efflux of control and any of the selected populations when analyzed separately

Assay environment	Mean after 24 hr of relaxation	Mean after 48 hr of relaxation	ANOVA F (1, 2)	ANOVA / Holm-Šidàk p values	Effect Size±95% CI	Inference
Pooled	0.059	0.064	17.99	0.052	0.13±0.33	Small
Cobalt	0.079	0.047	56.51	0.017	1.37±0.72	Large
Norfloxacin	0.07	0.1	83.1	0.023	1.07±0.7	Large
Ethidium bromide	0.055	0.09	262.2	0.011	1.01±0.69	Large
Zinc	0.03	0.011	681.5	0.006	2.41±0.85	Large

Table 3.4 Effect of duration of relaxation on the response to novel environments along with the results of individual ANOVA in each of the novel environment.

4 **DISCUSSION**

4.1 No significant change in mutation rates

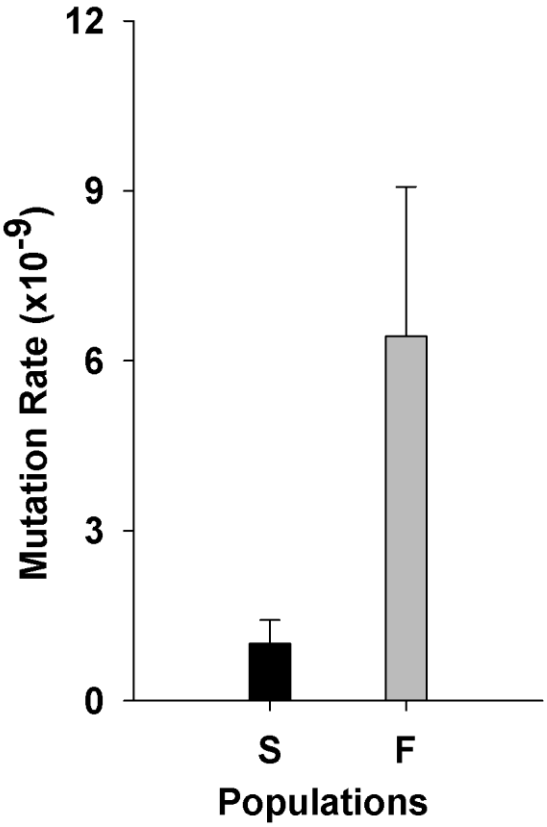

Figure 3.1: Mean (±SE) mutation rate for S and F populations estimated in the Rifampicin background. Although the F populations had slightly increased mutation rates than the S populations, the difference was not statistically significant. More importantly, the mutation rates are of the order of 10^{-9}, which indicates that hypermutators did not evolve in either of the populations.

Fluctuating environments are expected to favor an increase in the rate of generation of genetic variation through an increase in mutation rates of the populations (Leigh Jr 1970, Ishii, Matsuda et al. 1989). Although the average mutation rate of our F populations was larger than that of the S (Fig 3.1), the difference was not statistically significant. There can be multiple (not mutually exclusive) reasons for this observation. Firstly, ~170 generations of selection might have been insufficient for the mutation rates of the selected populations to have diverged enough to be statistically distinguishable from those of the controls. The fact that the magnitude of the difference is ~6 folds and the effect size of the differences is large (Table 3.1) is consistent with the notion that mutation rates in the selected populations are increasing. Secondly, constitutively-expressed mutator alleles are typically thought to spread in a population by hitch-hiking with a beneficial allele (Sniegowski, Gerrish et al. 1997, Taddei, Radman et al. 1997, Gentile, Yu et al. 2011), although see (Torres-Barceló, Cabot et al. 2013). However, as in our experiments, if the direction of selection keeps on changing very often, then it is unlikely that a particular mutation would be favorable for long. This will also make it difficult for an attached mutator allele to hitch-hike to fixation with the selected mutation. In spite of this reduced chances of retaining the mutator allele, F populations could benefit from the increased mutation rate and hence increased variation, over short time scales. The results of sequencing mutS allele revealed an insertion of adenine residue near 540^{th} base pair in one of the replicate F populations. Such frameshift mutation can potentially hamper the mismatch repair mechanism and increase the mutation rate (Sniegowski, Gerrish et al. 1997, Shaver, Dombrowski et al. 2002). In line with this possibility, the same replicate F population with the insertion showed an order of magnitude increase in the mutation rate, 1.1×10^{-8}, as opposed to 1.3×10^{-9} and 7.8×10^{-9} of the other two replicates. But when we looked at the growth rates estimates, cobalt was the only novel environment in which the replicate carrying the insertion mutation in mutS outperformed the other two replicates.

Thus, although it is difficult to pin-point the reason, the chief result here is that the difference between the mutation rates of the selected and the control populations was relatively small and not statistically significant. In other words, there is no evidence to suggest that fluctuating environments select for large increases in mutation rate. Coupled with the relatively short period of selection (~170 generations) it is unlikely that the observed growth rate differences in the novel environments are due to the selected populations generating or accumulating genetic variation faster than the control populations. This prompted us to look at another variation based mechanism that can lead to broad based stress resistance.

4.2 No evidence for population-based resistance

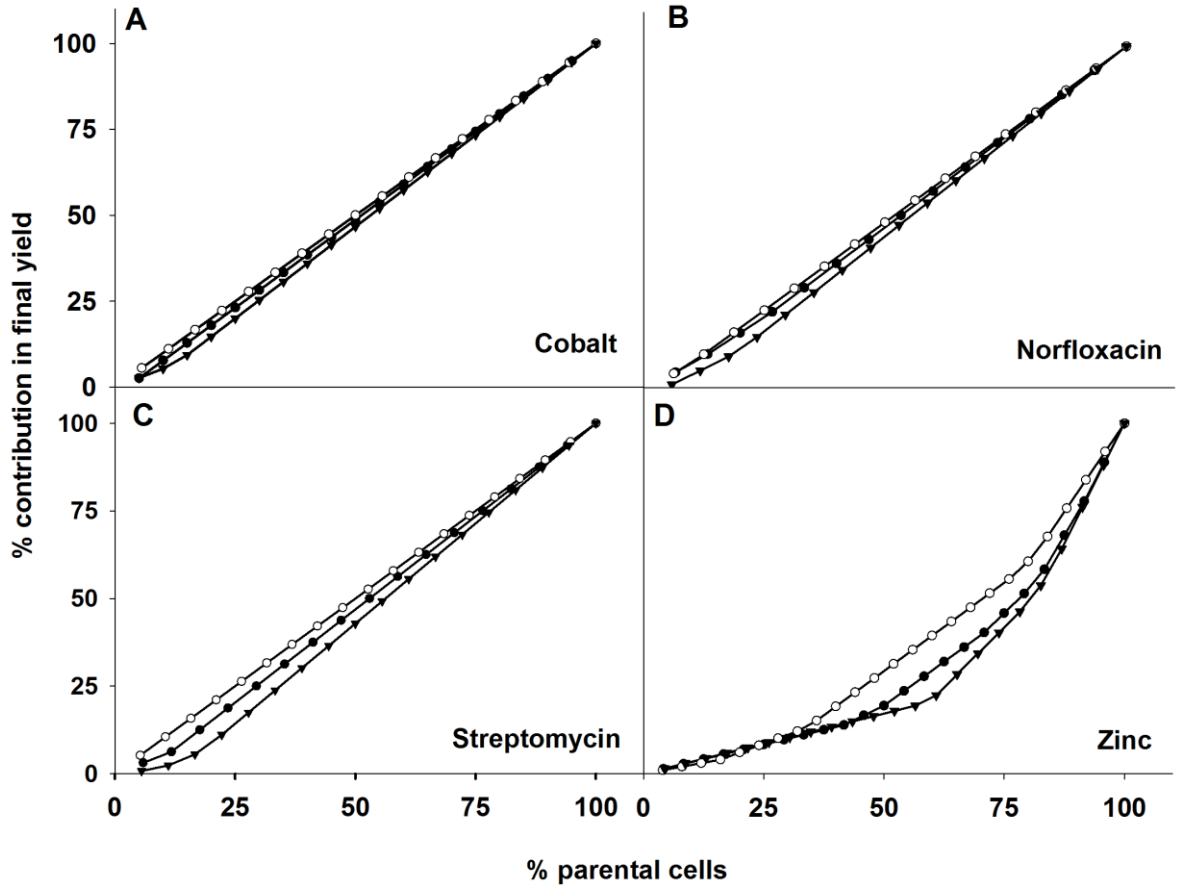

Figure 3.2: Population-based resistance in F populations. The cumulative percentage contribution of parental bacteria to the final yield is plotted for three replicate F populations in four novel environments. Each line in a figure stands for a replicate population of F. A. Cobalt, B. Norfloxacin, C. Streptomycin, D. Zinc. In this kind of a graph, the presence of outliers is detected as a sharp inflection towards the right, which was not observed. This indicates that no individual cells contributed disproportionately to the total yield.

When the magnitude and direction of selection fluctuates continuously, traits that are favorable under one set of conditions, might become neutral or even deleterious when the environment changes. This can lead to a scenario where a population is continuously changing with each shift in environment, without really evolving to greater fitness. One way by which a population can escape such a stasis is through the evolution of cooperation which allows subsets of the population to specialize in countering particular stresses and then confer resistance to the population as a whole (West, Griffin et al. 2007). For example, it has been shown that in populations of the bacteria *Pseudomonas aeruginosa*, the proportion of individuals that synthesize the iron-scavenging siderophore pyoverdin, changes based on the kind of competition and genetic relatedness (Griffin, West et al. 2004). Similarly, when *E. coli* populations are challenged with antibiotics, a very small percentage (0.1 - 1%) of the individuals secret excess amounts of indole to the external environment, which then allows the entire population to become antibiotic resistant (Lee, Molla et al. 2010). Since only a small fraction of the population needs to evolve the resistant mechanism for a given stress, in principle, this mechanism allows different subsets of the population to evolve resistance to different stresses. This should increase the population level variation in terms of the ability to resist diverse stresses, and hence increase fitness in different kinds of novel environments. Given that antibiotics were among the novel environments that we studied, population-based resistance was a possible explanation for the fitness advantages of F populations. Our assay for individual fitness was expected to detect the resistant subset as outliers with exceptionally high yield. This is because immobilization of cells at extremely low density over an agar surface limits the diffusion of extracellular metabolites over long distances and only those cells which synthesize the resistant factors can resist the stresses. However, we did not find any outliers in terms of the yield and, except in the case of zinc, all the plots of cumulative yield were linear (Fig 3.2). Even in the case of zinc, where there was a slight departure from

linearity, at the point of inflection, ~20% of the parents contribute to ~40-60% of the yield. Overall, the conclusions are unambiguous, the observed increase in yield of the F populations were not attributable to a small fraction of the population.

The above result could have arisen in at least two other ways. It was possible that the F populations do exhibit population-based resistance, but we had managed to sample only those bacteria that conferred resistance to the population. The chances of such an event happening are probably negligible since, as stated already, the fraction of bacteria that confer the population-wide resistance is typically very low (Lee, Molla et al. 2010). As we had sampled around 12- 40 bacteria out of ~2×10^8(over two trials) for each F population, it is highly unlikely that only individuals with altruistic capacities were sampled. In fact, the second possibility was far more likely, namely that we had sampled only those bacteria that did not confer any resistance to the population. In principle, this could also explain the absence of outliers in the F populations in terms of overall yield. However, in that case, we could not have observed an increase in the yield when compared to the S populations. Since the F populations did show a significantly larger yield compared to S populations (see Fig 2.4 from chapter 2), we conclude that whatever mechanism was responsible for it, was not present only in a small number of individuals.

There can be multiple, non-exclusive reasons for which population-based resistance failed to evolve in our F populations. Our F populations were sub-cultured every 24 hours with 1/50 of the existing population forming an inoculum for the next generation (see section 2.1.2 from chapter 2). It is difficult for population-based resistance to evolve in such a system due to a high chance of losing the resistant cells (which are in very low frequency) during each sub-culture. Moreover, it is known that when the environment changes, the production of the chemical that benefits the whole population can be costly for the producer cell (Lee, Molla et al. 2010). Thus, in our F populations, there could have been a strong selection against the

resistant cells, each time the environment changed. Taken together, perhaps it is not surprising that population-based resistance did not evolve in our F populations.

4.3 Fluctuating selection does not increase standing variation

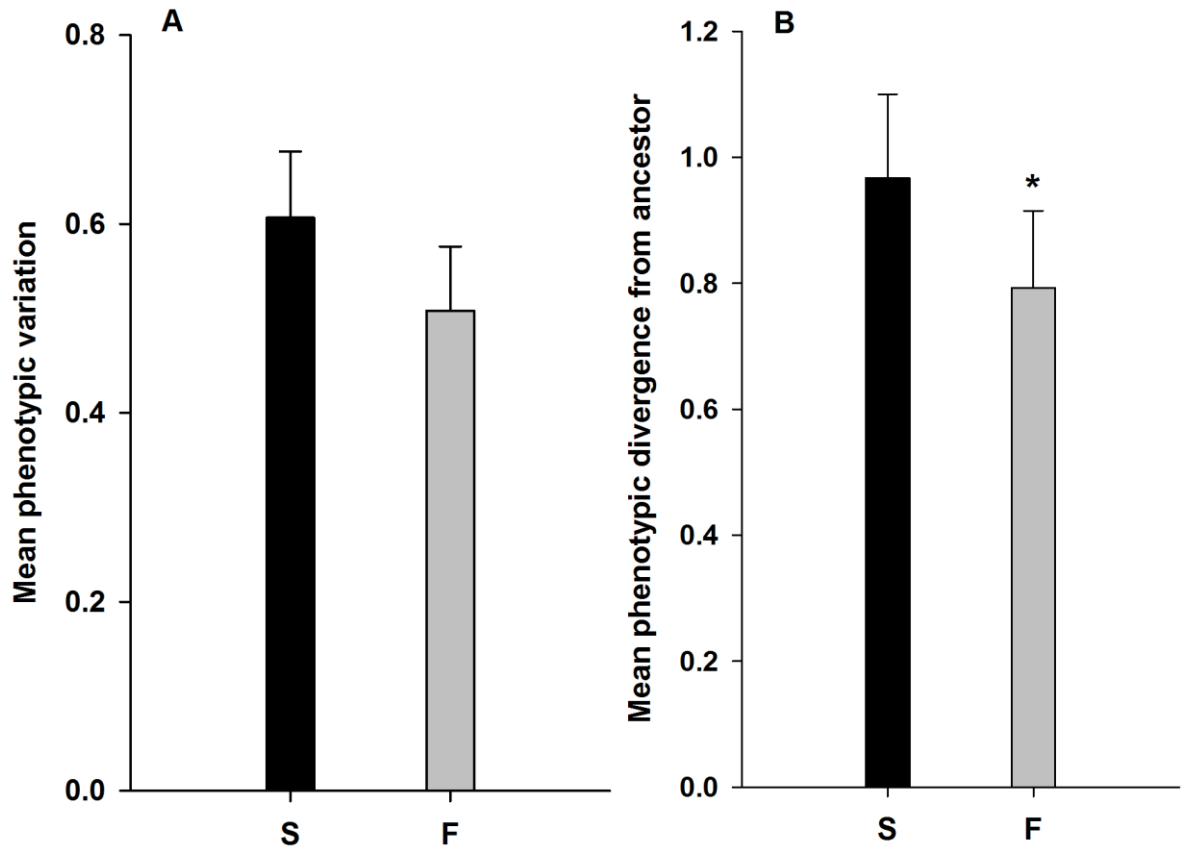

Figure 3.3: Phenotypic variation and divergence from ancestors. A. Mean (±SE) phenotypic variation for S and F populations. **B**. Mean (±SE) phenotypic divergence from the ancestors. The S populations show slightly higher variation and divergence albeit with small effect sizes.

* denotes $p < 0.05$.

Populations with greater standing variation are expected to respond faster to selection pressures compared to those with increased mutation rates. This is because with standing variation, the population need not wait for a beneficial mutation and such mutations are typically at a slightly higher frequency than those that arise *de novo* after exposure to the selection pressure (Barrett and Schluter 2008). Furthermore, theoretical studies show that fluctuating environments are expected to promote standing variation in the populations (Gillespie and Turelli 1989, Turelli and Barton 2004). Taken together, the greater fitness of the F populations in novel environments can be potentially explained if such populations have greater standing variation. We note here that a larger standing variation does not automatically guarantee that a population would be better able to face novel environments, it merely increases its chances for the same. However, it is difficult to visualize how large standing variation can be maintained when the direction of selection is changing very often (Via and Lande 1987). One way out of this problem is contextual neutrality, i.e. the assumption that at least some genetic changes are neutral in some environments (thus escaping selection) but affect fitness in other environments (thus contributing to standing genetic variance)(Wagner 2005). Thus, a population with a greater "neutral space" (i.e. contextually neutral variation) would be expected to have greater fitness across novel environments (Wagner 2005). Although some studies have directly measured genetic diversity through quantification of the number of mutations present (Coffey and Vignuzzi 2011), it is hard to determine how much of that diversity is functionally relevant. This is because, practically speaking, it is difficult to ascertain from the sequence data, whether a particular genomic mutation is deleterious, neutral or contextually neutral. Therefore, we favored a direct measurement of the phenotypic variation in the populations, through their ability to grow on 94 different conditions on the Biolog GEN III MicroPlate^{TM} plate (Cooper

2002). This way, we quantify those variations that can cause an observable change at the phenotypic level and hence, are functionally important.

Our results suggest that selection for unpredictable fluctuations did not increase the phenotypic variation in F populations. If anything, the mean phenotypic variation was slightly larger for the S populations (Fig 3.3A), although the difference was not statistically significant. This is consistent with a previous study on viruses demonstrating that genetic diversity (as measured by genomic mutations) is larger in populations that experience a steady environment as opposed to those facing fluctuating ones (Coffey and Vignuzzi 2011). Our results are also in sync with a previous observation that constant selection environments lead to increase in the genetic variance for fitness in novel environments (Travisano, Vasi et al. 1995). In terms of the phenotypic divergence from the ancestors, we found no consistent differences or reversal of phenotypes that were specific to the F or S populations (Fig 3.3B).

There might be several reasons for which phenotypic variation did not increase in the F populations. The duration of selection (~170 generations) might have been too less to lead to a significant divergence in terms of phenotypic variation. Moreover, the fact that the environment (and hence the selection pressure) changed every ~6 generations, might have caused a much stronger selection pressure that prevented maintenance of phenotypic variation. One way by which standing variation can be increased even in the face of changing environments, is through increased mutation rates (Ishii, Matsuda et al. 1989). However, since the mutation rates of the F populations did not evolve to be significantly larger than the S populations (Fig 3.1), this route was closed to the selected populations. It is important to note here that we only scored the presence or absence of phenotypes, a process that is biased towards catching large phenotypic differences. In principle, one can also think of variations

which affect the rate at which the substrates are metabolized or the intensity of the effect of stress substrates on the bacterial cells. However, quantifying such effects would require replicate measurements at the level of single clones and increased number of replicate clones due to the inherent variation in the metabolic rates of the cells, and hence was beyond the scope of this work.

4.4 Evolution of increased efflux activity

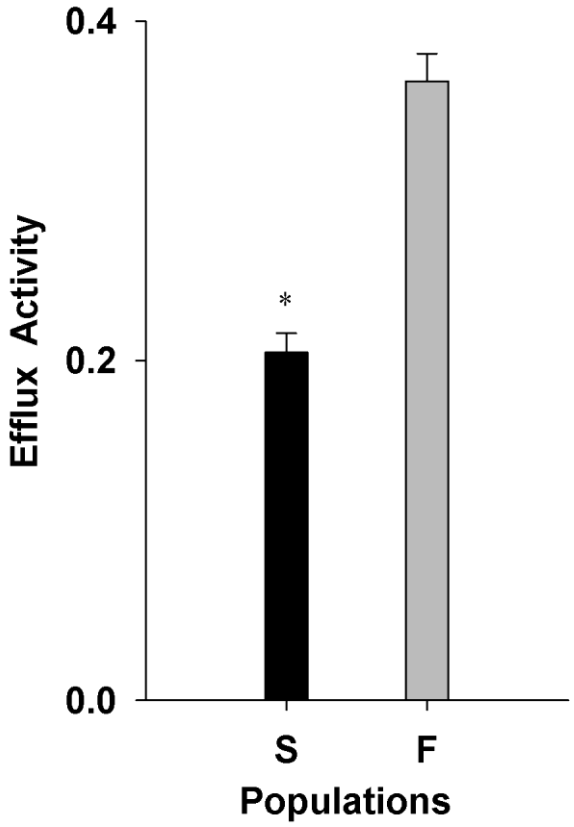

Figure 3.4: Mean (±SE) energy dependent efflux in S and F populations. F populations had significantly greater efflux activity than the S populations, which might have led to their greater fitness in novel environments.

Over the last two decades, bacteria have become resistant to a large number of antibiotics due to, *inter alia*, the action of a variety of multidrug efflux pumps (MEPs) (Li and Nikaido 2009, Nikaido and Pagès 2012). As the name suggests, MEPs are protein systems that throw out a wide range of antibiotics and biocides from the cells and can also play a role in combating environmental stresses like bile salts (Thanassi, Cheng et al. 1997) or organic solvents (Fernandes, Ferreira et al. 2003). Our results indicate that the F populations have evolved significantly higher efflux activity as compared to the controls (Fig 3.4). Increased activity of MEPs can lead to resistance to norfloxacin (Morita, Kodama et al. 1998, Nishino, Nikaido et al. 2009) and ethidium bromide (Ma, Cook et al. 1993, Nishino, Nikaido et al. 2009) in *E. coli*, both of which were observed here (Fig 2.2 from chapter 2). Multiple drug resistance is known to be associated with higher MEP activity in different kinds of bacteria (Li and Nikaido 2009, Nishino, Nikaido et al. 2009, Nikaido and Pagès 2012 and references therein) including *E coli* (Pena-Miller, Laehnemann et al. 2013). However, to the best of our knowledge, this is the first laboratory experimental evolution study in *E. coli* that reports the evolution of increased energy-dependent MEP activity and the concomitant change in growth rate in the presence of an antibiotic (norfloxacin) and a mutagen/biocide (ethidium bromide), after an exposure to fluctuating complex environments.

An increased efflux activity could have evolved in our F populations in several ways. Firstly, it could have been accidentally fixed in the population due to genetic drift. However, the chances of such an event are low due to the relatively large population sizes involved in bacterial systems and the observation that the increase in efflux happened in all three replicate F populations. Secondly, increased efflux might have also evolved as a result of a direct or a correlated response to selection. Prima facie, this appears counter-intuitive because multi-drug efflux pumps are often studied in the context of drugs or biocides, although of late they have been shown to confer resistance to components of the *E. coli* environment like

bile salts (Thanassi, Cheng et al. 1997) or steroid hormones (Elkins and Mullis 2006). Since none of these stressors were present during the selection process, it is hard to see how increased efflux activity could have been directly selected for. This inconsistency gets resolved if we take into account the suggestion made by some authors that MEPs have more functions than removing drugs, including clearing out different kinds of secondary metabolites (Poole 2005) and virulence (Piddock 2006). Recently it has been shown that a MEP called *norM* can reduce the intracellular levels of reactive oxygen species and increase the ability of *E. coli* cells to survive H_2O_2 (Guelfo, Rodríguez-Rojas et al. 2010). Since H_2O_2 was a constituent of our fluctuating environment, all else being equal, any change leading to increased levels of *norM* should be favored. Incidentally, among all the component environments, the effect size of the difference between S and H was the maximum for H_2O_2 (Table 2.2 from chapter 2). This is again consistent with the expectation that resistance to H_2O_2 has experienced positive selection. Moreover, the resistance to low pH in *E. coli* is mediated by a regulator called GadX (Ma, Richard et al. 2002) which has been shown to elevate the levels of another MEP called mdtEF (Nishino, Senda et al. 2008). Finally, the levels of acrAB (a well-studied MEP), are increased by higher concentrations of NaCl (Ma, Cook et al. 1995). Thus it was possible that somehow, the efflux activity had evolved due to a direct response to the component environments, without any role of the unpredictable fluctuations.

When exposed to constant stress (acidic pH or basic pH or salt or H_2O_2), replicate bacterial populations failed to evolve significantly larger efflux activity, compared to populations evolving in constant benign environment (see Appendix 3.2 for details). Although 40 generations seems a relatively short duration, it should be noted here that the F populations experienced a different environment every ~5.64 generations whereas the populations selected for the constant stresses did not. Thus, if indeed the component environments were

selecting for increased efflux activity, the latter set of populations were under much stronger directional selection for the same and hence were expected to show appreciable response to selection. Although the H_2O_2 selected populations had somewhat greater mean efflux activity compared to the controls (d = 1.91), the lack of statistical significance in the pooled or the individual ANOVA prevents us from concluding that exposure to H_2O_2 alone led to appreciable change in efflux activity during this span of time. It is possible that further exposure to H_2O_2 might have led to greater changes in efflux activity in the constant stress selected populations. However, given that the F populations never experienced 7 or more continuous runs of exposure to H_2O_2, the evolution of efflux activity under such a scenario has no bearing on the results of the present study.

It should be noted here that there is also a possibility of an interaction between unpredictable fluctuations and the component environments in terms of evolution of efflux activity. In other words, some of the component environments (and not others) when fluctuated unpredictably, might have led to the evolution of increased efflux activities. It is also possible that the evolution of increased efflux activity is due to the complex environments (i.e. the combinations of stresses) or an interaction of the complex environments with unpredictable fluctuations. Given that our F populations experienced the complex environments randomly and not every complex environment is likely to select for increased efflux, it is not intuitively obvious that complex environments, by themselves, would lead to the evolution of efflux activity. However, the possibility for the same cannot be completely ruled out, thus forming a fruitful avenue for future research.

4.5 No difference in fitness in cold environment

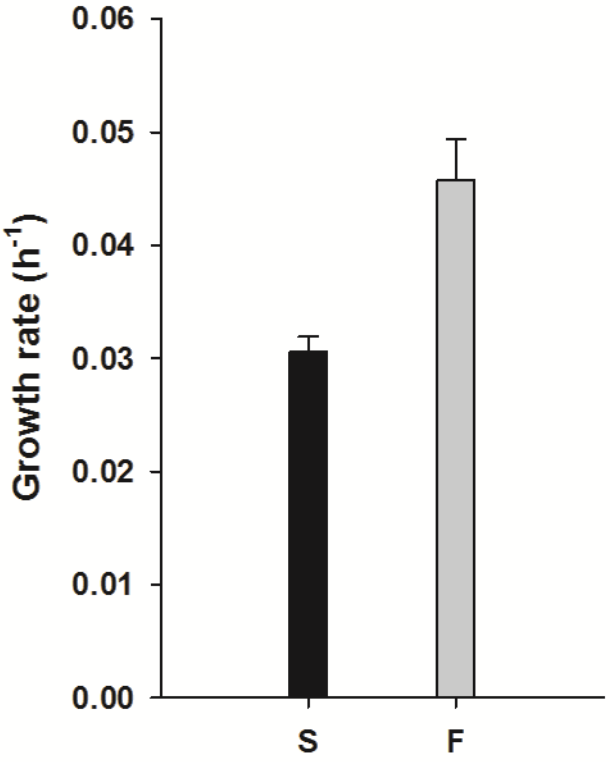

Figure 3.5: Mean (±SE) fitness in cold environment. No significant difference between the fitness of F and S populations.

Is elevated efflux activity the sole mechanism responsible for the better performance of the F populations under novel environments? To answer this question, we assayed the growth rates of F and S populations at 22^0C, since elevated efflux cannot assist growth in case of cold temperature. *E. coli* populations can grow in the range of 21^0C to 49^0C with optimum at 37^0C. Exposure to temperatures less than 37^0C induces cold shock in *E. coli* populations which is followed by an acclimation phase and adaptation (reviewed in Bárria, Malecki et al. 2013). GroEL and GroES are the rate limiting cellular determinants at lower growth temperatures (Ferrer, Chernikova et al. 2003, Strocchi, Ferrer et al. 2006). Both the proteins function as chaperonins and are involved in folding and/or assembly of approximately 30% of cellular proteins (Ferrer, Chernikova et al. 2003). We find that F populations do not show significantly better fitness in the face of cold environment. This indirectly strengthens the role of energy dependent efflux in previously observed fitness advantage in antibiotics and heavy metals.

An alternate explanation could be that fluctuating environments do not result in fitness improvement on exposure to broad stresses like cold temperature, salt, hydrogen peroxide and pH (see section 4.2 from chapter 2 for details). However, higher fitness of F populations in ethidium bromide, an intercalating agent with system wide effects, does not support this possibility (Table 2.2, Fig 2.2 A from chapter 2).

4.6 Response to novel environments does not decay with the longer duration of relaxation in F populations

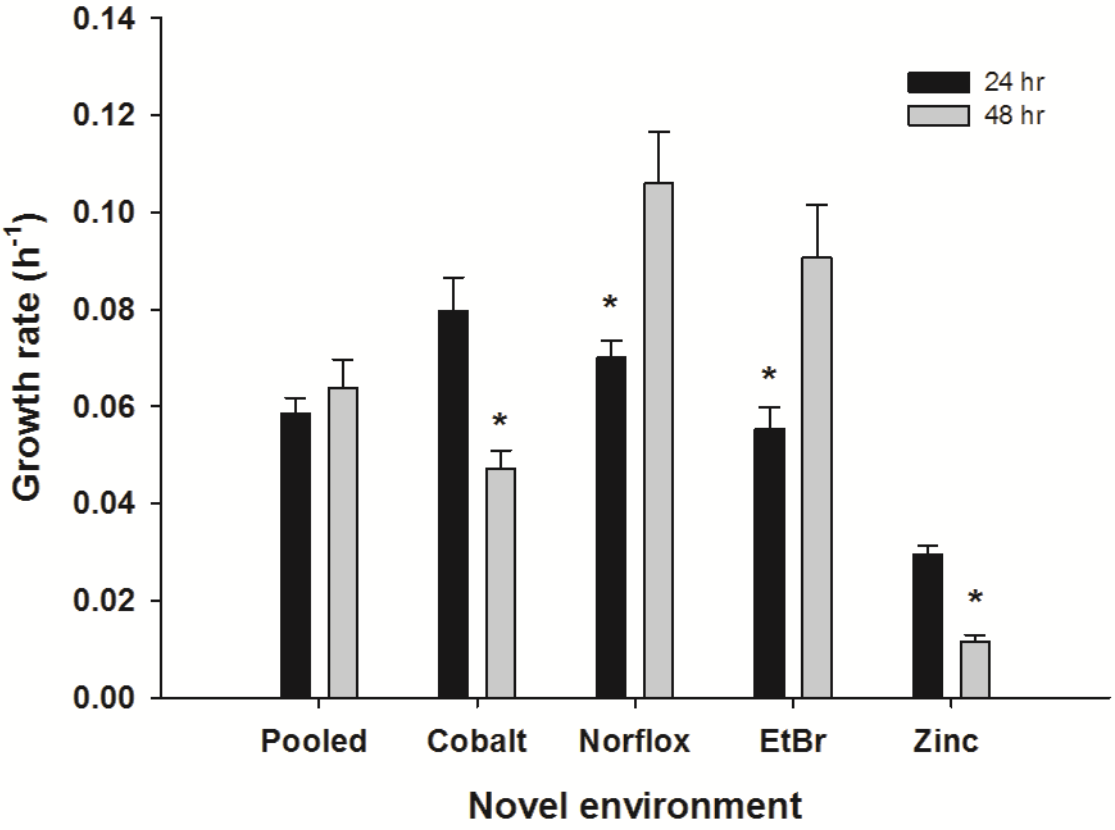

Figure 3.6: Mean (±SE) fitness in novel environments for F populations after two different durations of relaxation (24 hr and 48 hr). First pair of bars denote no significant difference in growth rate when pooled over all the four novel environments. Following four comparisons in individual environment show significance difference in the growth rate after two different durations of relaxation. 24 hr relaxation leads to higher growth rate in cobalt and zinc while 48 hr relaxed populations perform better in norfloxacin and ethidium bromide.

* denotes $p < 0.05$ (after Holm-Šidàk correction in the case of comparisons under individual environments).

Exposure to stressful environments activates multiple cellular resistance responses. For example, acid resistance system 1 and 2 are activated in response to acidic pH while alternate sigma factor RpoS are activated in response to multiple stresses (Notley-McRobb, King et al. 2002, Foster 2004). Such broad stress fighting mechanisms can provide protection against multiple environmental stresses (Cheville, Arnold et al. 1996, Chung, Bang et al. 2006). We deliberately chose the novel environments to avoid such cross protection (see section 4.3 from chapter 2) and introduced a relaxation step spanning over ~ 9 generations before the growth rate estimation. .

Fitness estimates in two out of four novel environments (Norfloxacin and Ethidium Bromide) showed that fitness improved after longer duration of relaxation (Fig 3.6). In the other two environments (cobalt and zinc) F populations had reduced fitness after longer duration of relaxation (Fig 3.6). But F populations had fitness comparable to S populations in zinc (Table 2.2, Figure 2.2 A). Thus cobalt is the only novel environment where carry over responses could have possibly played any role in the observed fitness advantage of F populations. However, in order to entertain that possibility, we need to hypothesize that resistance to cobalt is due to a mechanism which is very different from the one that leads to resistance to the other stresses. While the possibility cannot be overruled, it seems somewhat less probable that multiple mechanisms have evolved to face novel environments. Coupled with the fact that the F populations were not exposed to any of the novel environments during selection, these results make it unlikely that metabolic carry over was a mechanism leading to observed fitness advantage of F populations in novel environments.

5 SUMMARY

Exposure to complex, unpredictably fluctuating environment for short duration neither leads to increased phenotypic variation, nor a significantly increased rate of acquiring such variation. Our F populations did not evolve population-based resistance either. These observations rule out three of the most widely-believed mechanisms that are expected to enable a population to face novel environments. Instead, it was the evolution of efficient energy dependent efflux which likely conferred the advantage in the face of novel environments.

Chapter 4. Extended exposure to complex, unpredictable fluctuations does not affect the evolvability of the populations

1 INTRODUCTION

The assays described in the previous two chapters suggested that selection in complex, unpredictably fluctuating environment can elevate fitness in novel environments. Effect of selection lingers even after ~50 generations of directional selection under the novel environments. This suggests that fluctuating selection can have long-lasting effects. To further investigate the evolutionary effects of fluctuating environments, we assayed the evolvability and collateral sensitivity profiles after ~560 generations of selection.

There exist multiple definitions of evolvability (Pigliucci 2008) in the evolutionary literature. Here the term evolvability is defined as rate of change of fitness (i.e. growth rate) over time (Griswold 2006). Both selected (F) and control (S) populations are expected to adapt to constant/ directionally changing environment. Population with higher evolvability will not only show higher fitness at every junction in selection but also display higher rate of increase in fitness.

Recent studies show that resistance to one antibiotic can be accompanied by sensitivity to few others (Pál, Papp et al. 2015). These trade off profiles have been shown to be robust across multiple strains of *Escherichia coli* (Imamovic and Sommer 2013). This implies that cyclic treatment with 'antagonistic' drugs can minimize the incidence of multi drug resistance, an observation that has been hailed as replacement to the standard cocktail approach (reviewed in Pál, Papp et al. 2015).

The assays described in this chapter sought to validate these two hypotheses.

2 METHODS

2.1 Selection

Glycerol stocks of 30 day selected F and S populations were revived in 50 ml NB overnight. These revived cultures were used to restart the selection in constant and fluctuating environment (see section 2.1 of chapter 2). The selection was continued for another 70 days i.e. a total of 100 days, which translates into~ 564 generations. These populations will be henceforth referred to as the 100-day selected populations.

2.2 Fitness assays and mechanisms

Fitness was estimated (see Appendix 1.1) in component and novel environments. Component environments are those which were part of the selection i.e. salt, pH and hydrogen peroxide while novel environments are those which were not part of the selection i.e. cobalt, norfloxacin, ethidium bromide and zinc (see Appendix 2.3 for concentrations). We also determined the energy dependent efflux activity (see Appendix 1.4) and mutation rate (see Appendix 1.2) of 100 day selected populations. We followed the same statistical procedures as mentioned in section 2.3 of chapter 2 and section 2.8 of chapter 3, for each of the fitness and mechanism assay.

2.3 Evolvability

100 day selected populations were used to assess the effect of fluctuating environments on the evolvability.

2.3.1 Selection for evolvability

Glycerol stocks of 100 day selected S and F populations were revived overnight in 50 ml of NB. These revived cultures were used to initiate three different selection regimes which differed in the way the concentration of cobalt changed over time. These three regimes were:

- Treatment 1 - Constant concentration of cobalt throughout the duration of selection (0.2mg/ml)
- Treatment 2 - Directional increase in concentration every 10^{th} day (from 0.2mg/ml to 0.4mg/ml with a step size of 0.04 mg/ml)
- Treatment 3 - Directional increase in the concentration every 2^{nd} day (from 0.2mg/ml to 0.4mg/ml with a step size of 0.007 mg/ml)

First day concentration was same for all the three treatments and treatments 2 and 3 reached the same concentration by the end of the selection. Each selection (S or F) × treatment (1,2,3) combination had four replicate populations The selection for evolvability was carried out in 24 welled plates with 2 ml of culture volume. 4 µl of suspension was transferred to the new well every 24 hr. The selection was continued for 60 days i.e. ~ 530 generations, except for treatment 2, which was continued for another 10 days (i.e. ~620 generations) to reach the same end concentration as that of treatment 3. Two different rates of environmental change allowed us to study the effect of step sizes on the rate of adaptation during directional selection. Glycerol stocks were stored every 10^{th} day for all the populations. Due to an unfortunate event we lost the 60^{th} day's glycerol stocks of treatment 2. To maintain parity, we restricted our fitness assays to populations selected for 50 days (i.e. ~ 440 generations) for all the three treatments.

2.3.2 Fitness measurement

Fitness was assayed at the stress concentration same as that of the last selection environment experienced by the populations, i.e. stress concentration at which glycerol stock was made. Thus, for example glycerol stocks of treatment 2, experienced 0.24 mg/ml of cobalt on day 20

of selection, and hence were assayed at the same concentration. Every selection population was assayed for fitness (see Appendix 1.1) in triplicates.

2.3.3 Statistical analysis

Fitness was averaged over the three replicate measurements and each of the treatments (I, II and III) were analyzed separately. For every treatment, fitness estimates over time were analyzed together using 3-way mixed model ANOVA. Selection (two levels: S and F) and time (five levels: 10^{th}, 20^{th}, 30^{th}, 40^{th} and 50^{th} day of selection) were the fixed factors while replicate (three levels) was a random factor nested in selection. To compare the performance of S and F populations after different durations of selections, we performed separate 2-way mixed model ANOVA with Selection (two levels: S and F) as a fixed factor and replicate (three levels) as a random factor.

The design of the evolvability-selection treatments no. 2 and 3 allowed us to test the effects of large *vs* small step size of environmental changes in the case of directional selection. Due to the different step sizes of increase, the assay and selection concentration of treatment 3 lagged that of treatment 2 by 10 days. To test the effect of different step sizes at a given concentration, we analyzed fitness estimates from four different time durations, 20^{th}, 30^{th}, 40^{th} and 50^{th} day for treatment 2 and 10^{th}, 20^{th}, 30^{th} and 40^{th} day for treatment 3. This way, fitness is assayed at a time when both populations are at the same stress concentration in the course of their selection. The pooled data were analyzed using a 4-way mixed model ANOVA. Selection (two levels: S and F), Concentration (four levels) and Treatment (two levels) were taken as fixed factors while Replicate (three levels) was a random factor nested in Selection. We conducted eight different 2-way mixed model ANOVA for every combination of Concentration × Selection to look at the effect of large *vs* small step size. Treatment (two

levels) was a fixed factor and replicate (three levels) was a random factor nested in Treatment.

In case of multiple ANOVAs family-wise error rates were controlled through sequential Holm -Šidàk correction of the P values (Abdi 2010). To judge the biological significance of the differences in mean growth rates for F and S populations at different assay environments, we computed Cohen's *d* statistic (Cohen 1988) as a measure of the effect sizes (Sullivan and Feinn 2012). Following existing guidelines (Cohen 1988), we interpreted the effect sizes as small, medium and large for $0.2<d<0.5$, $0.5<d<0.8$ and $d>0.8$, respectively. Cohen's d statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004).

2.4 Collateral sensitivity

Following schematic shows the sequence of steps involved.

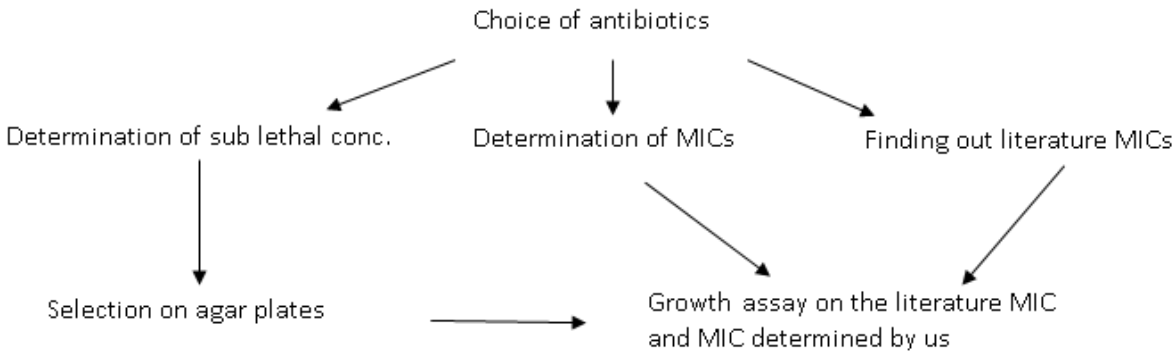

Fig 4.1 Schematic representation of the sequence of steps followed during experimentation

We used seven different antibiotics from five different antibiotic classes, to check the consistency of collateral sensitivity as reported by Immamovic *et al* (Imamovic and Sommer 2013). These antibiotics were as follows, kanamycin (aminoglycosides), chloramphenicol

(chloramphenicol), tetracycline (tetracyclines), ampicillin (penicillins), gentamycin (aminoglycosides), streptomycin (aminoglycosides) and rifampicin (antimycobacterials). Within these seven antibiotics, 9 instances of 2 fold decrease, 3 instances of 4 fold decrease and 2 instances of 8 fold decrease in MIC for one antibiotic after achieving resistance for certain other antibiotic have been reported (Imamovic and Sommer 2013).

2.4.1 Determination of MIC

We first determined the antibiotic concentration which could reduce the growth of F populations by 80 to 90% (IC90). For this purpose, glycerol stocks of 100 day selected F populations were revived in 50 ml NB overnight. These revived cultures were then inoculated into wide range of antibiotic concentrations in triplicates along with the 6 replicates in plain NB (as control). 200 µl of culture volume with 10 µl of inoculum was used in 96 welled plate with the relevant antibiotic purpose and OD_{600} was measured after 24 hr using plate reader. % inhibition was computed for every antibiotic as

$$1 - \left\{ \frac{\text{Average population density in antibiotic}}{\text{Average population density in NB}} \times 100 \right\}$$

IC90 determined by this procedure was taken as the determined MIC. We also obtained the MIC values determined for *E. coli* MG 1655 for the same seven antibiotics from literature.

2.4.2 Selection for resistance

To check whether these collateral sensitivities are retained even after selection under fluctuating environments, we first selected the three replicate F populations for resistance in all seven antibiotics. The selection was performed on agar plates using gradient plate

technique (Szybalski and Bryson 1952). The starting concentration (termed as sub lethal concentration) was chosen in such a way that it reliably produced some growth with gradient plate technique. Following table gives the sub lethal concentration and the final concentration reached after selection for the seven antibiotics used along with the MIC determined by us and the literature MIC values.

Antibiotic	Sub lethal conc on agar (µg/ml)	Final conc on agar (µg/ml)	MIC in broth (µg/ml)	Literature MIC (µg/ml)
Kanamycin	5	50	5	7
Chloramphenicol	6.8	13.6	5	8
Tetracycline	2.5	15	1	4
Ampicillin	10	150	20	8
Gentamycin	4	20	1	4
Streptomycin	25	-	10	4
Rifampicin	8.75	100	20	4

Table 4.1 : Literature MIC values along with the lab determined MIC values for the seven antibiotics chosen

The final concentration reached on agar was found to be much higher than the MIC values in broth. Well isolated colony of each population was chosen from the plates with final concentration of each antibiotic and allowed to grow overnight in NB. A glycerol stock made from this was used for assessing the sensitivity to other antibiotics.

2.4.3 Antibiotic sensitivity after selection

Glycerol stocks made from the selected clones were revived in 50 ml NB overnight. 10 µl of this revived culture was used to assess growth at the determined MIC and literature MIC of antibiotics. Every measurement had three replicates with 200 µl of culture volume. After 24 hr of growth OD_{600} was measured using a plate reader and IC90 values were computed as mentioned above. These IC90 values were then compared with the previously outlined sensitivity profiles.

3 RESULTS

3.1 No difference in fitness under component environments

After ~564 generations of selection, the maximum growth rates of the F populations did not differ, when measured under conditions that were components of the fluctuating selection regime, i.e. pH (4.5/10), high salt or H_2O_2 ($F_{1,4}$ = 0.017, p = 0.9, Table 4.2, Fig 4.2). The effect size for this difference was small (d = 0.03, Table 4.2). There was also a significant main effect of component environment ($F_{3,12}$ = 17.5, p = 0.0001) which is not surprising as the different conditions (i.e. pH 4.5, H_2O_2, etc.) are not expected to affect the growth rates similarly. However, there was no significant difference between the fitness of F and S populations when analyzed separately for each of the component environment (Table 4.3, Fig 4.2). The effect size was medium for a single component environment, pH10 and small for the remaining three component environments (Table 4.3).

3.2 No difference in fitness under novel environments

When analyzed together for the four novel environments, there was no significant difference between the fitness of F and S populations ($F_{1,4}$ = 0.87, p = 0.404, Table 4.2, Fig 4.3) and the effect size of this difference was small (d = 0.04, Table 4.2). As with component environments, there was a significant effect of the novel environment ($F_{3,12}$ = 1457.8, p < 0.0001). Fitness did not differ between S and F populations, when analyzed separately for each of the novel environment (Table 4.3, Fig 4.3). The effect sizes of the difference were small for cobalt and norfloxacin, medium for ethidium bromide and large for zinc (Table 4.3). Interestingly though, S populations showed higher fitness than F in zinc, unlike the case in the other three novel environments.

3.3 No difference in mutation rates

Although the S populations had higher mutation rates than the F populations with a medium effect size (d = 0.73, Table 4.2), this difference was not statistically significant ($F_{1,4}$ = 1.028, p = 0.368; Table 4.2, Fig 4.4). The results suggest that F populations did not evolve higher mutation rates even after ~560 generations of selection in fluctuating environments.

3.4 No difference in energy dependent efflux

F populations had higher mean energy dependent efflux abilities. But the difference was statistically insignificant with low effect size ($F_{1,4}$= 0.138, p = 0.729; d = 0.34, Table 4.2, Fig 4.5). The results suggest that the difference in energy dependent efflux, which was observed after the short duration of selection, vanishes over longer time scales.

3.5 No difference in evolvability

Treatment 1 - Fitness did not differ significantly between S and F populations when pooled over 5 measurements over 50 days of selection ($F_{1,4}$ = 6.67, p = 0.061, d = 0.77, Table 4.2). Duration of selection had marginally significant effect on the fitness ($F_{4,16}$ = 3.43, p = 0.033). When fitness was analyzed separately, there was no significant difference between the fitness of S and F populations for any of the time points (Table 4.3, Fig 4.6). Additionally, effect size for day 20 was large while all other effect sizes were either medium or small (Table 4.3).

Treatment 2 - Fitness did not differ significantly between S and F populations when pooled over 5 measurements over 50 days of selection ($F_{1,4}$ = 7.07, p = 0.056, d = 0.5, Table 4.2). Duration of selection had significant effect on the fitness ($F_{4,16}$ = 118.92, p < 0.0001). When fitness was analyzed separately, there was no significant difference between the fitness of S

and F populations for any of the time points (Table 4.3, Fig 4.6). Effect sizes for day 30 was large while all other effect sizes were either medium or small (Table 4.3). But it is important to note that F populations displayed higher fitness than S populations at every measurement point.

Treatment 3 - Fitness did not differ significantly between S and F populations when pooled over 5 measurements over 50 days of selection ($F_{1,4} = 4.88$, $p = 0.092$, $d = 0.48$, Table 4.2). Duration of selection had significant effect on the fitness ($F_{4,16} = 80.33$, $p < 0.0001$). When fitness was analyzed separately, there was no significant difference between the fitness of S and F populations for any of the time points (Table 4.3, Fig 4.6).Effect size of difference was large for three time points, day 30, 40 and 50 (Table 4.3). Not unlike treatment 2, F populations displayed higher fitness than S populations at every measurement point.

The lack of significant difference between fitness of S and F populations suggests that F populations are not more evolvable than S populations when exposed to the constant or directionally increasing concentration of cobalt.

Effect of magnitude of step size was significant with medium effect size in the pooled analysis ($F_{1, 4} = 394.05$, $p < 0.001$, $d = 0.5$, Table 4.2). Concentration affected the fitness measurement significantly ($F_{3, 12} = 60.35$, $p < 0.00010$) while selection had marginal effect ($F_{1, 4} = 7.32$, $p = 0.054$). 7 out of 8 individual ANOVAs showed a significant difference for each concentration × selection combination between small and large step size of increase and 6 out of 8 comparisons had large effect size (Table 4.4, Fig 4.7). For the first concentration (i.e. 20th day of selection for treatment 2 and 10th day of selection for treatment 3), small step size of the increase showed a higher mean fitness as compared to the large step size. But at the next concentration, the pattern reversed and stayed consistent for next two concentrations as well (Fig 4.7). The pattern was consistent for both S and F populations. This suggests that

smaller step size during directional selection is advantageous initially but large step size of change leads to the higher rate of adaptation in long term scenario.

3.6 Collateral sensitivity

For the 7 antibiotics tested here (7×7 matrix), 3 instances of conflict are noted for F populations. Selection for kanamycin resistance should collaterally increase the sensitivity for tetracycline and rifampicin and selection for resistance to ampicillin should increase the sensitivity for streptomycin (Imamovic and Sommer 2013). In all three cases sensitivity did not increase collaterally, instead decreased. These results suggest that the history of fluctuations can be one of the possible reasons leading to modification of the collateral sensitivity profiles.

Assay	Mean 1	Mean 2	ANOVA F(1,4)	ANOVA p values	Effect Size±95% CI	Inference
Fitness in Component Environments	0.104 **(S)**	0.105 **(F)**	0.017	0.902	0.03±0.33	Small
Fitness in Novel Environments	0.066 **(S)**	0.069 (**F)**	0.87	0.404	0.05±0.33	Small
Mutation rate	6.39E-09 **(S)**	3.73E-09 **(F)**	1.03	0.368	0.73±1.65	Medium
Efflux potential	0.691 **(S)**	0.74 **(F)**	0.138	0.729	0.34±0.93	Small
Evolvability Treatment 1	0.131 **(S)**	0.14 **(F)**	6.67	0.061	0.77±0.37	Medium
Evolvability Treatment 2	0.138 **(S)**	0.148 **(F)**	7.07	0.056	0.50±0.36	Medium
Evolvability Treatment 3	0.128 **(S)**	0.135 **(F)**	4.88	0.09	0.48±0.36	Small
Effect of step size in directional selection	0.146 **(T2)**	0.127 **(T3)**	394.1	3.8E-05	1.07±0.3	Large

Table 4.2: Summary of the main effects of selection in the pooled ANOVAs

Assay	Environment	Mean S	Mean F	ANOVA F(1,4)	p values (Holm-Šidàk corrected)	Effect Size ±95% CI	Inference
Fitness in component environments	pH 10	0.146	0.133	0.78	0.427	0.70±0.67	Medium
	pH 4.5	0.091	0.098	0.38	0.569	0.248±0.66	Small
	Salt	0.11	0.129	0.57	0.493	0.336±0.66	Small
	H_2O_2	0.0708	0.0634	7.33	0.0536	0.106±0.65	Small
Fitness in novel environments	Cobalt	0.033	0.04	1.32	0.314	0.4±0.66	Small
	Norfloxacin	0.134	0.135	0.22	0.665	0.03±0.65	Small
	EtBr	0.09	0.096	3.93	0.119	0.74±0.68	Medium
	Zinc	0.009	0.004	5.59	0.077	0.9±0.69	Large
Evolvability Treatment 1	Day 10	0.129	0.132	0.33	0.84	0.23±0.46	Small
	Day 20	0.136	0.148	8.8	0.19	0.97±0.49	Large
	Day 30	0.13	0.14	1.58	0.62	0.73±0.48	Medium
	Day 40	0.13	0.137	2.48	0.57	0.34±0.47	Small
	Day 50	0.129	0.142	18.68	0.07	0.73±0.48	Medium
Evolvability Treatment 2	Day 10	0.129	0.132	0.33	0.6	0.23±0.46	Small
	Day 20	0.11	0.118	11.05	0.16	0.64±0.47	Medium
	Day 30	0.147	0.163	5.55	0.22	0.9±0.48	Large
	Day 40	0.155	0.166	7.24	0.24	0.65±0.47	Medium
	Day 50	0.15	0.162	5.78	0.26	0.68±0.48	Medium
Evolvability Treatment 3	Day 10	0.135	0.136	0.054	0.83	0.09±0.46	Small
	Day 20	0.116	0.126	3.55	0.43	1.11±0.5	Large
	Day 30	0.11	0.119	4.75	0.39	1.06±0.49	Large
	Day 40	0.133	0.143	7.52	0.27	1.07±0.49	Large
	Day 50	0.147	0.15	1.67	0.46	0.35±0.47	Small

Table 4.3: Summary of the main effect of selection in the ANOVAs under individual environments

Selection	Concentration	Mean T2	Mean T3	ANOVA F(1,18)	p values (Holm-Šidàk corrected)	Effect Size ±95% CI	Inference
S	One	0.11	0.147	3098.02	0.002	3.87±1.36	Large
	Two	0.155	0.15	59.37	0.048	0.55±0.82	Medium
	Three	0.135	0.116	1939.6	0.003	3.24±1.22	Large
	Four	0.11	0.133	13.07	0.069	2.76±1.12	Large
F	One	0.118	0.163	89.84	0.043	4.57±1.52	Large
	Two	0.166	0.162	418.09	0.014	0.32±0.81	Small
	Three	0.136	0.126	120.56	0.04	1.2±0.87	Large
	Four	0.119	0.143	40.97	0.046	3.99±1.38	Large

Table 4.4: Summary of the main effect of treatment in the ANOVAs under individual concentrations of cobalt. T2 denotes treatment 2 and T3 denotes treatment 3.

4 DISCUSSION

4.1 No adaptation to component environments

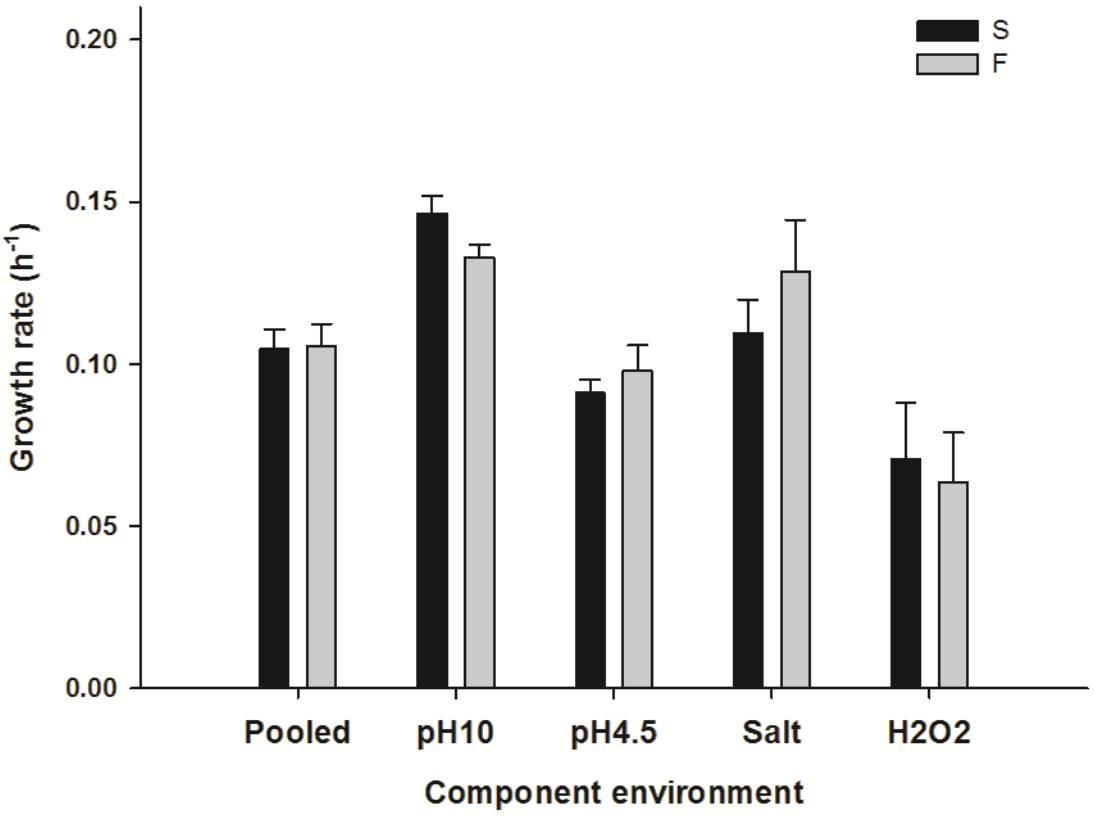

Figure 4.2: Mean (±SE) fitness in component environments. The first comparison is of the pooled means over all four component environments and the next four comparisons are for the individual component environments. For all the comparisons, no significant difference (individual ANOVAs) in the fitness of F and S populations. Fitness was measured as maximum slope of the growth trajectory over 24 hours.

Predictable changes in the environment are likely to result in adaptation for the component environments (Leroi, Lenski et al. 1994, Turner and Elena 2000, Hughes, Cullum et al. 2007, Coffey and Vignuzzi 2011, Alto, Wasik et al. 2013). But when the environment changes unpredictably, fitness in component environmnets can increase or decrease or remain unchanged (Hallsson and Björklund 2012, Alto, Wasik et al. 2013, Ketola, Mikonranta et al. 2013). Fitness assessment after ~170 generations of selection had showed no difference in fitness for the component environments between S and F populations (Fig 2.1 A). But the lack of adaptation at that stage could have been attributed to the relatively short duration of selection (see dicussion 4.2 of chapter 2). However, after ~560 generations of selection, F and S populations had similar fitness in component environments. This result indicates that even 560 generations of selection is not enough to induce difference in fitness in component environments. F populations, facing unpredicatble fluctuations, can experience the change of direction of selection every ~6 generations. Thus, a population adapting towards a certain peak for few generations can be taken in a completely different direction after every 24 hr. Such a scenario can result in little overall change in fitness, even though the F populations are continuously evolving. The results suggest that adaptation to individual stresses is diffecult to achieve even after hundreds of generations of selection when environment flutuates in complex, unpredictable manner.

4.2 No fitness difference in novel environments and no change in efflux activity

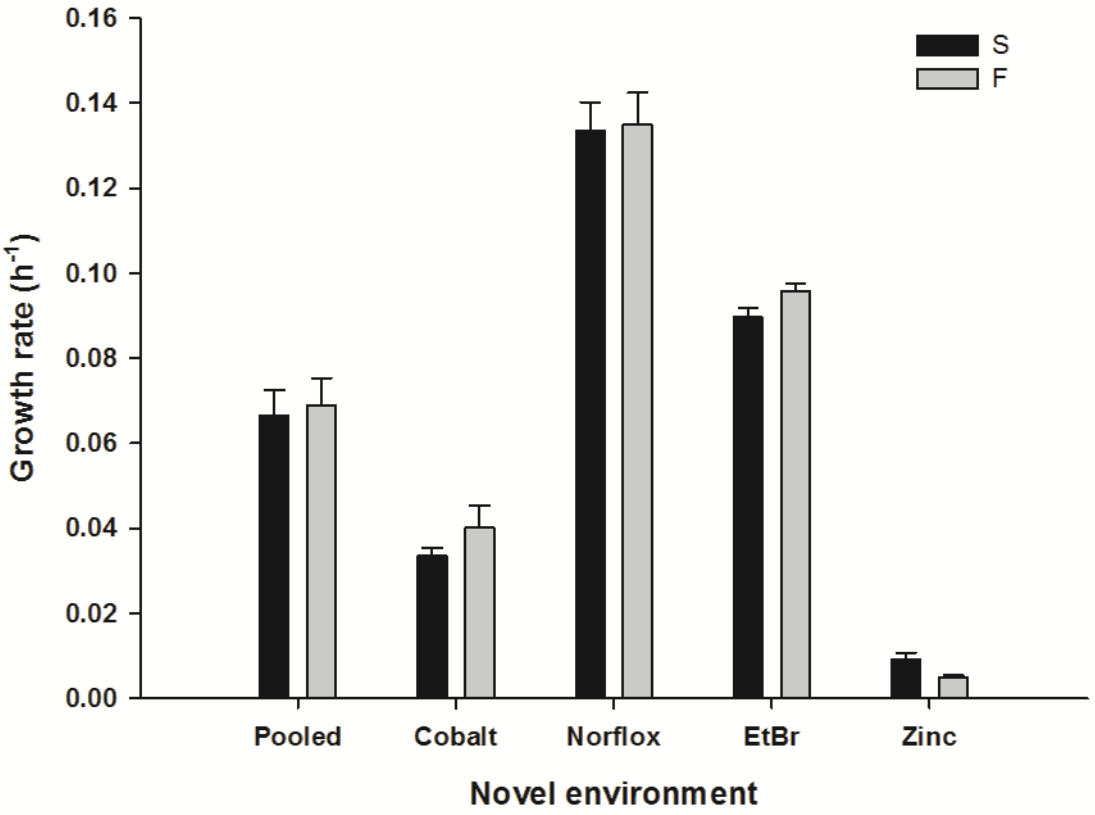

Figure 4.3: Mean (±SE) fitness in novel environments. The first comparison is of the pooled means over all four novel environments and the next four comparisons are for the individual novel environments. For all the comparisons, no significant difference (individual ANOVAs) in the fitness of F and S populations. Fitness was measured as maximum slope of the growth trajectory over 24 hours.

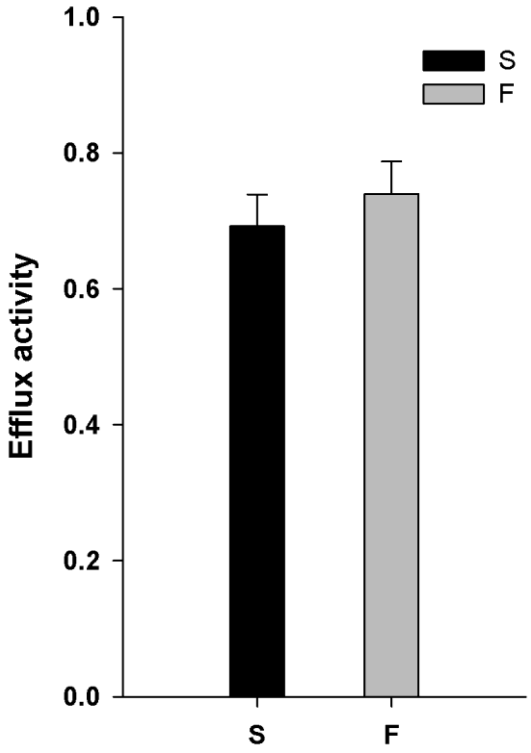

Figure 4.4: Mean (±SE) energy dependent efflux in S and F populations. S and F populations show comparable efflux activity.

In spite of lack of adaptation to the component or complex environments, F populations displayed fitness advantage in the novel environmental backgrounds after ~170 generations of selection (Fig 2.2 A). However, the fitness advantage disappeared after ~560 generations of fluctuating selection (Fig 4.3). Although in terms of a trend, F populations did have a marginally larger mean fitness in three out of four novel environments (cobalt, norfloxacin and ethidium bromide), none of the differences are statistically significant and effect sizes were low for all the comparisons. This suggests that somewhere between generations 170 and 560, the difference between the control and the selected populations in terms of resisting novel environments disappeared. This observation was corroborated by the fact that the difference in efflux activity between the S and F populations had also disappeared (Fig 4.4) .

Based on this data alone, it is not possible to infer why longer-term exposure to fluctuating environments erased the difference between the S and F populations in terms of efflux activity. One possibility might be that since efflux is an energy-intensive process, it is not possible to have a continuously elevated rate of efflux. However, comparing Fig 3.4 with Fig 4.4 suggests that the efflux rates of the F populations did not come down during this time. The difference between the F and the S populations had actually disappeared because the efflux activity of the S populations had increased about four times during the same duration, while that of the F populations merely doubled. This suggests that the S populations were actually evolving even under the NB environments, which is not a surprising result, given the ability of microbes to keep on evolving for a very long time, even under completely static environments (Satterwhite and Cooper 2015). What is more surprising is the fact that efflux activity increased in the S populations even though they were under no obvious stress. One reason for this might be the fact that the benign conditions for the S populations ensured that they reached the stationary phase in almost every generation. It is known that the stationary

phase leads to oxidative stresses and enhanced efflux activity could be one response to increased oxidative stress (Guelfo, Rodríguez-Rojas et al. 2010). One can speculate that perhaps the selection pressure imposed by these conditions is relatively less than those imposed by the fluctuating environments, which is why efflux ability takes longer to evolve in the S populations. However, at present, I am unable to comment any further on this issue and leave this as a potential avenue for future work.

Since the efflux activity of the S populations had increased, it was reasonable to assume that their ability to fight the novel stresses would also increase across the board. This prediction seemed to be borne out by the overall increase in the fitness of the S populations in the pooled data (see figure Fig 4.3 and Fig 2.2). However, when we looked at fitness under the individual stresses, the interpretations were not very intuitive. For example, increased efflux activity of the S populations in generation 560 did not seem to increase fitness under cobalt or zinc, although both are heavy metals that are expected to be cleared by the activity of the efflux pumps. This is consistent with the observation in chapter 3 that while increased efflux seems to be one mechanism that determines the fitness of the F populations in the novel environments, it is, by no means, the only one.

4.3 No significant difference in the mutation rates

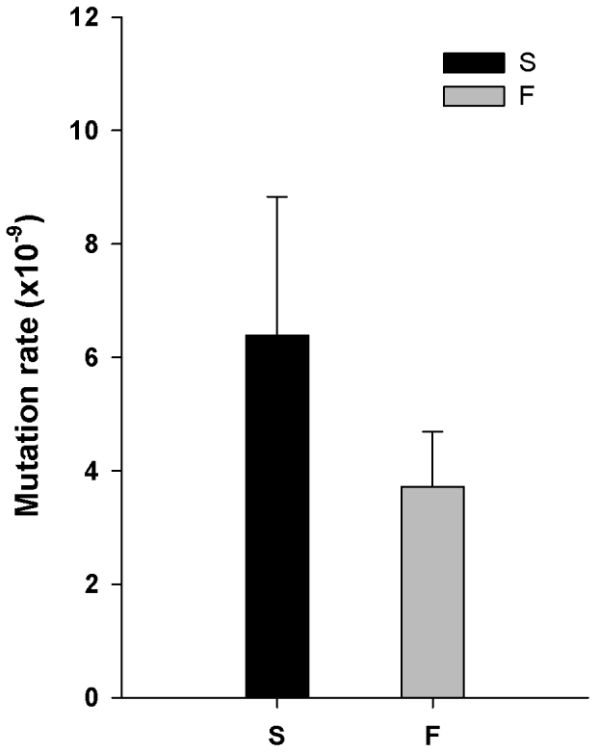

Figure 4.5: Mean (±SE) mutation rate for S and F populations estimated in the Rifampicin background. The mutation rates for S and F populations are not significantly different.

Since we observed that the S populations had also evolved over ~560 generations, we also checked the mutation rates of both these populations at this point in the evolutionary trajectory. Fluctuating environments are expected to favour the increase in the mutation rates (Leigh Jr 1970, Ishii, Matsuda et al. 1989). Short term selection in fluctuating environments did not agree with this expectation (Fig 3.1). Long duration of selection under unpredictable fluctuations supports the previous results. Mutation rates of S and F poulations are not significantly different from each other. The order of magnitude of the mutation rates (10^{-9}) shows that they did not evolve in response to the complex, unpredictable fluctuations. Constitutively-expressed mutator alleles are known to spread through population by hitch-hiking with a beneficial allele (Sniegowski, Gerrish et al. 1997, Taddei, Radman et al. 1997, Gentile, Yu et al. 2011), although see (Torres-Barceló, Cabot et al. 2013). In case of fluctuating selection though, identity of beneficial alleles keep changing frequently. This will also make it difficult for an attached mutator allele to hitch-hike to fixation with the selected mutation.

4.4 Evolvability

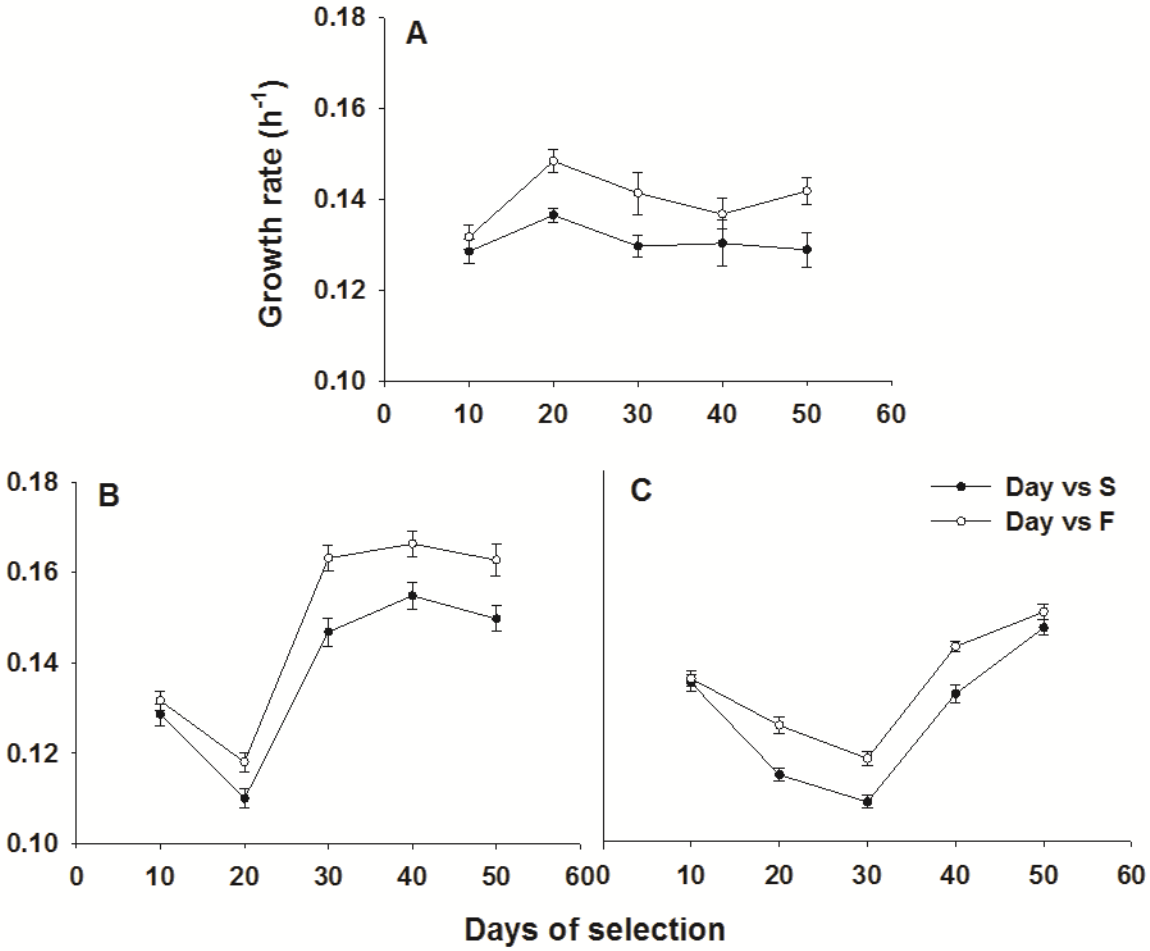

Fig 4.6 Mean (±SE) fitness for S and F populations over 50 days of selection. Filled circles denote S populations while empty circles denote F populations. **A.** Populations were selected over 50 days in constant concentration of cobalt **B.** Populations were selected over 50 days in cobalt with increase in concentration every 10th day. **C.** Populations were selected over 50 days in cobalt with every 2nd day increase in concentration.
Fitness was measured as maximum slope of the growth trajectory over 24 hours every 10th day of selection. For all the comparisons, there was no significant difference (individual ANOVAs) between the fitness of F and S populations.

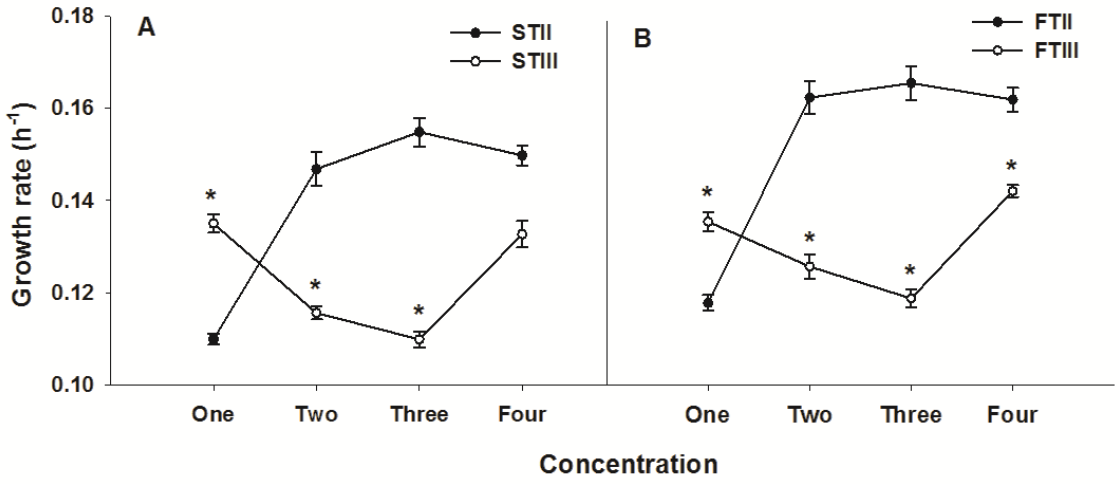

Fig 4.7 Mean (±SE) fitness for treatment 2 and 3 over four directionally increasing selection environments. A. S populations with large step size of increase (represented by filled circles) show significantly lower fitness than those selected with small step size of increase (denoted by empty circles) for the first environment. The pattern reverses for next three comparisons. **B.** F populations with large step size of increase (filled circles) show significantly lower fitness than those selected with small step size of increase (empty circles) for first environment. The pattern reverses for next three comparisons.
* denotes $p < 0.05$ (after Holm-Šidàk correction in the case of comparisons under individual environments)

Fluctuating environments are expected to promote the standing genetic variation of a population (Gillespie and Turelli 1989, Turelli and Barton 2004). This in turn can potentially increase the speed of adaptation to novel environments, i.e. evolvability (reviewed in Pigliucci 2008). To test this, previously 100 day selected F and S populations were subjected to a second round of selection, either in the constant dosage of cobalt or directionally increasing concentration of the same. It should be noted here that we had no a priori reason to believe that any one of these three conditions would lead to greater or lesser evolvability than the other. We merely sought to use these three environments as three different ways of imposing directional selection, and wished to assay for differences in evolvability between S and F populations under three different conditions. When the change in fitness over time was analyzed, F populations do not show superior adaptability compared to S populations (Fig 4.6 A, B and C). It is important to note that at every time point of every treatment, F populations showed higher mean fitness as compared to the S populations, but the difference was not statistically significant. Further investigation with different novel environment might magnify this difference.

Two different step sizes of directional selection allowed us to test the effect of magnitude of the step size during directional selection. In laboratory populations of *Chlamydomonas* smaller step size of environmental change leads to the higher fitness increase as compared to the large step size (Collins, de Meaux et al. 2007, Collins and De Meaux 2009). But this pattern was observed only for a short duration of directional selection in our case. Interestingly, we find the same to be true for the first assay concentration (20^{th} day for treatment 2 and 10^{th} day for treatment 3) but continued selection showed reversal of the pattern (Fig 4.7). Both S and F populations showed the same behaviour. This is intuitive as each time the concentration of the stress increases in the environment, the populations can potentially face selection. Populations facing the large step increase will be subjected to a

much stronger selection pressure and hence will show higher speed of adaptation (Falconer, Mackay et al. 1996). It is also important to note that at the time of the fitness assay, populations with large step size have already spents10 days in that specific concentration. Populations with smaller step size increase on the other hand, would have experienced not more than two days of the same concentration. Unlike the theoretical predictions (Collins, de Meaux et al. 2007), our results show that a large-magnitude environmental change followed by stasis leads to higher fitness over time, compared to a gradual change over time. This observation needs to be investigated in future due to its serious implications for phenomenon like antibiotic resistance. For instance, our results predict that sudden environmental changes in the clinical concentration of antibiotics might be a more potent selective force for evolution of antibiotic resistance as compared to a continuous, gradually increasing exposure to antibiotics.

4.6 Fluctuating selection can modify the collateral sensitivity profile

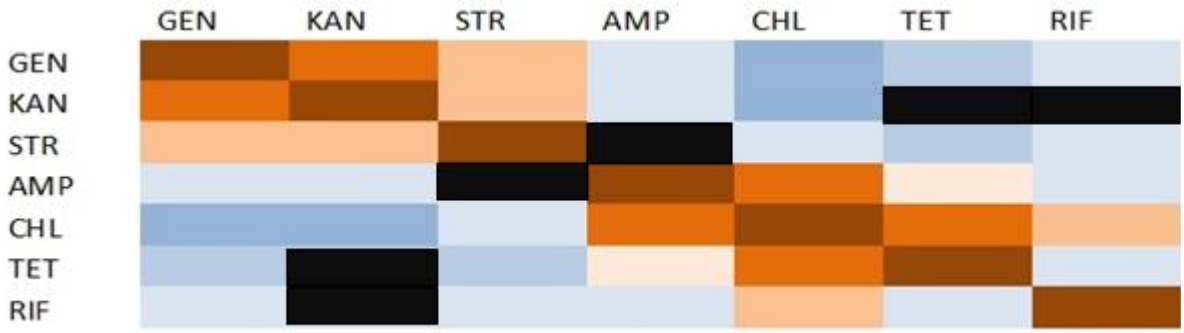

Fig 4.8: Collateral sensitivity matrix. Black coloured cells denote the instances of conflict with the proposed collateral sensitivity profiles by Imamovic *et al* 2013 (Imamovic and Sommer 2013). Abbreviations denote the names gentamycin, kanamycin, streptomycin, ampicillin, chloramphenicol, tetracycline and rifampicin (left to right and top to bottom).

Evolution of drug resistant pathogens is a global challenge to healthcare and overuse of drugs has been underlined as one of the primary causes (O'Neill 2014). A common practice of administrating antibiotic cocktails can accelerate the evolution of multi drug resistance and alternative effective approaches are being desperately sought.

Cyclic treatment of drugs which are known to have collateral sensitivity can be one of the ways to combat with the menacing problem of multi drug resistance (Pál, Papp et al. 2015). Such treatments involve cyclic dosage of paired drugs and combine effective treatment with reduced chances of evolution of multidrug resistance. The strategy heavily relies on the robust sensitivity profiles of the antibiotics involved where resistance to one antibiotic has to be accompanied by the increased sensitivity towards the other. Previous work has shown that such collateral sensitivity profiles remain robust for different populations of *Escherichia coli* (Imamovic and Sommer 2013, Lázár, Singh et al. 2013, Oz, Guvenek et al. 2014).

Our results suggest that these collateral profiles might not be robust after all. With the 7 antibiotics tested, we found 3 instances contradicting with the previously suggested collateral sensitivity profiles. The kanamycin resistance clone shows increase in MIC for rifampicin and tetracycline rather than the proposed 2 times and 4 times increase in the sensitivity respectively. Similarly, streptomycin selected clone shows increased MIC as opposed to 2 times increase in the sensitivity. Whether the modified sensitivity profiles are the outcome of fluctuating selection or is it the inherent property of the strain chosen, remains unanswered. Parallel experiment with S populations will distinguish between the two possibilities. But these results clearly warrant further investigation and put forward a note of caution. The proposed collateral sensitivity profiles are not universally robust after all and might change depending on the evolutionary history/strain under consideration. One needs to be cautious while designing treatments based on the collateral sensitivity profiles. Diverse evolutionary history of pathogens will demand case by case investigation of sensitivity profiles.

5 SUMMARY

The increase in efflux activity of the S populations over time underlines the importance of proper controls in the context of any experimental evolution study. In much of the literature on microbial evolution, all changes are measured relative to the ancestor, which does not experience any of the treatment conditions (Kassen 2014). However, in a study on evolutionary effects of fluctuating environments, it is equally valid to consider the effects of evolution under constant environments to be the appropriate control (as was done here). There is no a priori reason to believe that the two comparisons would lead to similar conclusions. As far as this study is concerned, the fact that the S populations evolved efficient efflux over long term but not over short term, underlines the importance of evolution in laboratory/control conditions. However, the crucial point to note is that there is no obvious reason to suggest that either of these controls is anyway superior / preferable to the other. Thus, an ideal (albeit logistically nightmarish) scenario would be to incorporate both kinds of controls in a study, which is what has been attempted in the next two chapters.

Chapter 5. Populations facing complex, unpredictable fluctuations for longer duration can take ‘no cost’ routes to fitness improvement

1 INTRODUCTION

Populations adapting to temporally fluctuating environments face very different challenges compared to those facing a constant environment. This has resulted in a substantial corpus of theoretical (Levins 1968, Gavrilets and Scheiner 1993, Whitlock 1996, Lande 2008) and empirical (Leroi, Lenski et al. 1994, Reboud and Bell 1997, Hallsson and Björklund 2012, Alto, Wasik et al. 2013, Ketola, Mikonranta et al. 2013, Condon, Cooper et al. 2014) studies exploring the fitness outcomes of heterogeneous environments. One major observation emerging out of such empirical studies on microbial systems is the absence of trade-offs across different values of a given environmental variable (Turner and Elena 2000, Hughes, Cullum et al. 2007, Condon, Cooper et al. 2014). One reason for this observation could be the fact that adaptation to different values of the same variable is very likely to be correlated. For instance, adaptation to pH 6 is more likely to be positively than negatively correlated to adaptation to pH 5. What remains largely unknown is whether these fitness outcomes remain same in the face of complexity i.e. environments where more than one environmental variables vary across multiple values (but see section 4.2 of chapter 2). Such environment is likely to pose more constraints to the adaptation and hence more likely to reveal trade-offs.

Trade-offs can be observed between two different life history traits in a given environment or between two different environments for a given life history trait (Agrawal, Conner et al. 2010). The latter has been thoroughly investigated in microbial systems. In such systems, trade-offs are thought to arise primarily due to two major reasons: antagonistic pleiotropy, where mutations beneficial in one environment are deleterious in another (Travisano and Lenski 1996, Cooper and Lenski 2000, MacLean, Bell et al. 2004) and mutational accumulation, where mutations neutral in one environment accumulate and prove to be deleterious in another environment (Reboud and Bell 1997) (reviewed in Kassen 2002). Antagonistic pleiotropy will always lead to trade-offs but the experimental evidence for the

same has been hard to come by (Kassen 2014 but see Travisano and Lenski 1996, Cooper and Lenski 2000, MacLean, Bell et al. 2004). On the other hand, trade-offs arising due to mutational accumulation can be easily offset by exposure to multiple environments (Barrett, MacLean et al. 2005) as selection would work against mutations which are conditionally neutral (i.e. deleterious in one environmental context but neutral in other). Experimental evidence till date favours mutational accumulation as the primary source of trade-offs (Kassen 2014). Hence, not surprisingly, when populations face multiple environments predictably during selection, they evolve to become generalists and show fitness improvements over all the environments experienced during the selection (Leroi, Lenski et al. 1994, Hughes, Cullum et al. 2007, Coffey and Vignuzzi 2011, Ketola, Mikonranta et al. 2013).

If pleiotropic interactions are nearly absent, adaptation to one selection environment can be thought of as independent to that of the other. All else being equal, the extent of adaptation will then be governed by how far the ancestor is from the different fitness optima. When pleiotropic interactions are present, the evolutionary trajectory will depend upon the sign of the interaction. Positive pleiotropic interactions will increase the speed of adaptation, since exposure to one selection environment will improve the fitness across multiple selection environments. But this will not be the case if negative genetic interactions dominate, where, in the absence of 'no cost' routes to improvement in fitness, a population will become evolutionarily stalled. On the other hand, simultaneous exposure to multiple selection environments will always disfavour the mutations which are deleterious in any one of the selection environments, leading to decreased variation for fitness and potentially stronger selection for no-cost route to fitness.

Here we test these alternate possibilities using laboratory populations of *Escherichia coli*. Replicate populations were exposed to complex, unpredictable fluctuating environment. The

selection regime consisted of four distinct selection environments, salt, pH4.5, pH9 ad hydrogen peroxide. In parallel, we select replicate bacterial populations in constant exposure to each one of the four selection environments. After ~900 generations of selection, fitness of all populations were assayed in all the selection environments. We further looked at the extent of adaptation, as compared to the ancestor. Results show that populations facing complex, unpredictable environments could adapt to the selection environments, as long as the ancestor was not already close the fitness optimum. Variation for fitness over all the selection environments was minimized due to the simultaneous exposure. Interestingly though, we observe that some selection environments act differentially across different components of the fitness.

2 METHODS

2.1 Selection experiment

Kanamycin resistant *Escherichia coli* strain K12 (see Appendix 5.1 for details) was used for this study. A single colony grown on Nutrient agar with Kanamycin (see Appendix 2.1 for composition) was inoculated in 2 ml of Nutrient broth with Kanamycin (NB^{Kan}, Appendix 2.1 for composition) and allowed to grow for 24 hr at 37^0C, 150 rpm in 24 welled plates. 4 µl of this suspension was used to initiate each of the 120 replicate populations.

These 120 replicate populations were equally divided into five treatment- and one control-regime, which led to 20 replicate populations per regime. Control populations were subcultured in NB^{Kan} for the entire duration of the selection. Four out of five selection regimes were a constant environment with either salt, basic pH, acidic pH or hydrogen peroxide in NB^{Kan}. The remaining selection environment was complex and stochastically fluctuating (henceforth termed as F) (see Appendix 5.2 for details of all selection regimes). The detailed design of the fluctuating selection regime have been mentioned elsewhere (section 2.1.2 of chapter 2).

24 welled plates with 2 ml of appropriate growth medium and 4 µl of inoculum volume for each well were used for the selection and assay experiments. The growth conditions were maintained at 37^0C, 150 rpm. All he populations were sub-cultured every 24 hr. Extinctions were identified visually and revived using 20 µl of the previous day's culture stored at 4^0C. The selection lasted for 100 days i.e. ~ 900 generations (Bennett and Lenski 1997). Every 10^{th} day the populations were stored as glycerol stocks at -80 ^{0}C for future assays.

2.2 Fitness assay in selection environments

After 100 days of selection, all the populations were assayed for fitness in every selection environment, except the fluctuating environment. Selection environments comprised of NB^{Kan} with salt or basic pH or acidic pH or hydrogen peroxide or control (see Appendix 5.2 for details).

For comparison with the ancestor, we revived the ancestral population of Kanamycin resistant *Escherichia coli* strain K12 in NB^{Kan} for 18 hrs. 20 replicate wells were inoculated with this revived culture for every selection environment. This resulted in the same number of replicates of ancestral culture for every assay environment as that of the selected populations.

Following previous studies, maximum growth rate during 24 hr of growth was used as a fitness measure (Ketola, Mikonranta et al. 2013). For the growth rate measurement, 4 µl of relevant glycerol stocks were revived in 2 ml of in NB^{Kan}. After 18 hr of growth, these revived cultures were inoculated in the appropriate assay environment. OD_{600} was measured every 2 hr on a plate reader (Synergy HT, BioTek, Winooski, VT, USA) for the duration of 24 hr. We used a QBASIC (v 4.5) script to determine the maximum growth rate of the bacterial populations. The program fits a straight line on overlapping moving windows of three points on the time series of OD_{600} values. The maximum slope obtained by this method is taken as the maximum growth rate for that population.

For estimation of trade off, we computed the difference in the fitness of every selected population from the mean fitness of ancestor (over 20 replicates) in every selection environment.

2.3 Replicates and statistical analysis

We conducted two measurements for every population in every selection environment. This resulted in a total of 1400 growth measurements (140populations × 5 assay environments × 2 measurements.

2.3.1 Overall mean fitness

1200 fitness estimates (6 selection lines × 5 assay environments × 20 replicates × 2 measurements, excluding the ancestor) were analysed using 3-way mixed model ANOVA. Selection (six levels) and assay environment (5 levels) were fixed factors crossed with each other. Replicate (twenty levels) was taken as a random factor nested in selection. To compare the overall mean fitness of all selected populations with ancestor, we performed the Dunnett post hoc test (Zar 1999) with ancestor as the reference group.

2.3.2 Variation for fitness

Since variance/ standard deviation scales with mean, we estimated coefficients of variation (CV) as a measure of variation in fitness. Two fitness estimates in a given assay environment were averaged and CV was calculated for every replicate population over five such fitness estimates. Every selection regime thus yielded 20 CV estimates. These were then analyzed using one way ANOVA with selection (six levels) as a fixed factor. To compare variation in fitness for all the selected populations with that of the ancestor, we performed Dunnett post hoc test (Zar 1999) with the ancestor as the reference group.

2.3.3 Trade off

For a given selection environment, every replicate of each selection regime had two fitness estimates. Average of these two values provided the mean fitness for every population resulting in 20 different mean fitness values for every selection regime, in every selection environment. The mean fitness of ancestor for every selection environment was computed as the average of 40 measurements (20 replicates × 2 measurements). This value was then subtracted from the mean fitness for every replicate population in the same environment. This resulted in 20 difference measurements for every selection regime, in every selection environment.

We then performed 30 different t tests (6 selection lines × 5 assay environments) for every set of difference computed from ancestor. The family-wise error due to multiple tests was controlled using Holm- Šidàk correction (Abdi 2010).

To estimate the biological significance of the difference in fitness of F populations compared to other selected populations, we computed Cohen's *d* statistics (Cohen 1988) as a measure of effect size. It was interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$, respectively.

All the ANOVAs were performed on STATISTICA v7.0 (Statsoft Inc.). Cohen's *d* statistics were estimated using freeware Effect size generator v2.3.0 (Devilly 2004).

3 RESULTS

3.1 Overall mean fitness and variation for fitness

When pooled over all the selection environments, effect of selection was highly significant ($F_{6, 700} = 12.07$, $p < 0.0001$, Fig 5.1). The effect of assay environment was highly significant ($F_{4, 700} = 247.04$, $p < 0.001$) along with the significant interaction of selection with assay environment ($F_{24, 600} = 31.73$, $p < 0.001$). The fitness of the selected populations was compared to that of the ancestor using the Dunnett's test. F populations showed highest overall mean fitness, which was significantly higher than the overall mean fitness of the ancestor with medium effect size (Table 5.1, Fig 5.1). None of the other selected populations differed significantly from the ancestor.

Selection had a significant effect on the variation in fitness in the pooled data ($F_{6, 133} = 61.65$, $p < 0.0001$, Fig 5.2). F populations showed lowest coefficient of variation compared to all other selected populations, and was significantly lower than the ancestor with large effect size (Table 5.1). Other than F, populations selected in salt also had significantly lower variation for fitness with large effect size (Table 5.1). We note however that pooled over the six environments, there is no significant relationship between standard deviation and mean (Appendix 5.4).

These results suggest that selection in fluctuating environments leads to a modest increase in overall mean fitness along with lower variation for fitness. Populations selected in salt also minimized the variation effectively but show loss of fitness as compared to the ancestor.

3.2 Fluctuating environments minimize the trade-offs

F populations did not show any significant loss in fitness compared to the ancestor, in any of the selection environments. In contrast to this, populations selected in constant environments showed a significant loss of fitness in at least one of the selection environment (Table 5.2, Fig 5.3). All the selected populations showed significant increase in fitness in hydrogen peroxide and all of them, except F populations, show loss of fitness in salt. Interestingly, even the populations selected in salt showed reduction in fitness when assayed in salt, albeit to a lesser magnitude (see section 4.3).

Taken together, these results show that fluctuating environments can minimize trade-offs across environments.

Assay	Selection regime	Mean	Dunnett's Test *p* values	Effect Size±95% CI	Interpretation
Overall mean fitness	Ancestor	0.125	-	-	-
	pH4.5	0.129	0.826	0.07±0.19	Small
	pH9	0.114	0.016	0.2±0.19	Small
	NB	0.125	1	0.002±0.19	Small
	H_2O_2	0.133	0.185	0.14±0.19	Small
	Salt	0.133	0.278	0.17±0.19	Small
	F	0.146	0.00003	0.5±0.19	Medium
Coefficient of variation for mean fitness	Ancestor	0.284	-	-	-
	pH4.5	0.367	0.007	1.13±0.67	Large
	pH9	0.491	7.0E-06	1.6±0.82	Large
	NB	0.357	0.02	0.75±0.64	Medium
	H_2O_2	0.433	7.0E-06	2.91±0.89	Large
	Salt	0.133	7.0E-06	3.13±0.92	Large
	F	0.129	7.0E-06	2.23±0.79	Large

Table 5.1 Summary of the main effect of selection in the ANOVAs for individual selection regimes. Dunnett's post hoc test was conducted with ancestor as a control group, Dunnett's test *p* values and effect size is thus in comparison with the ancestor.

Assay environment ↓	Selection environment →					
	pH4.5	**pH9**	**NB**	**H_2O_2**	**Salt**	**F**
pH4.5	0.14	1.3E-12**(-)**	0.96	0.95	0.02**(-)**	0.78
pH9	0.0004**(+)**	0.001**(+)**	0.84	0.22	0.71	0.0001**(+)**
NB	0.95	0.85	0.97	0.001**(+)**	0.02**(-)**	0.14
H_2O_2	3.1E-07**(+)**	2.4E-05**(+)**	3.1E-08**(+)**	1.1E-12**(+)**	6.4E-15**(+)**	2.2E-14**(+)**
Salt	5.4E-06 **(-)**	2.5E-05**(-)**	9.6E-05**(-)**	6.7E-15**(-)**	0.006**(-)**	0.01**(+)**

Table 5.2: Holm – Šidàk corrected *p* values of all 30 t tests are given for the differences in fitness as compared to the ancestor. The differences were computed for all selection regimes in all assay environments. In case of significant difference, sign in the bracket denotes direction of the change. '-' represents decrease while '+' represents increase in fitness from ancestor.

4 DISCUSSIONS

4.1 Complex, unpredictable fluctuations select for higher overall mean fitness

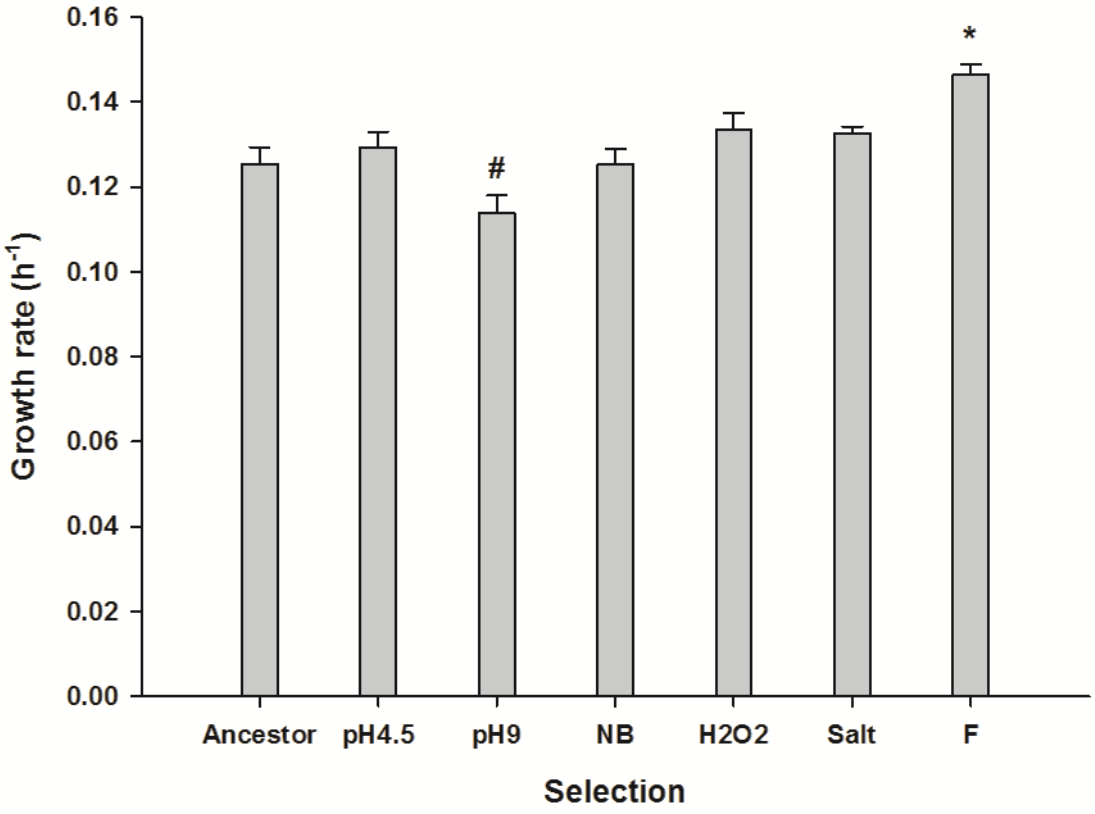

Fig 5.1 Overall mean fitness (±SE) for all the selection regimes. Overall mean fitness was computed for every selection regime over all assay environments. Fitness estimated as maximum slope of the growth trajectory over 24 hours.

* denotes significantly higher overall mean fitness ($p < 0.05$, Dunnett's post hoc statistics) than ancestor while # denotes lower overall mean fitness than ancestor.

When subjected to predictable oscillations in a single environmental variable over long time scales, microbial populations typically evolve to have higher fitness over the entire range of environments faced (Leroi, Lenski et al. 1994, Turner and Elena 2000, Hughes, Cullum et al. 2007, Coffey and Vignuzzi 2011, Puentes-Téllez, Hansen et al. 2013, Condon, Cooper et al. 2014). However, the evolutionary outcomes become much more complicated when the environment undergoes unpredictable fluctuations even for a single variable (reviewed in Collins 2011). It can result in no change (Alto, Wasik et al. 2013) or increase (Turner and Elena 2000, Ketola, Mikonranta et al. 2013) in fitness. In some cases, there is increase in fitness in with respect to a few life history traits while decrease with respect to others (Hallsson and Björklund 2012), whereas another study reported improve in fitness only in a subset of the selection environments (Hughes, Cullum et al. 2007). Moreover, natural environments typically consist of multiple variables that can change simultaneously and unpredictably (Lindow and Brandl 2003, Okafor 2011), which can potentially further constrain the evolutionary trajectories of populations. Our results show that when subjected to such complex, unpredictable fluctuations for ~ 900 generations, the bacterial populations show modest but significant overall increase fitness in the stresses under which they evolved (Fig 5.1).

Increase in overall mean fitness across different environmental variables is a challenging task as compared to the improvement in fitness for different values of the same environmental variable. Firstly, mutations advantageous in a given selection environment could have negative pleiotropic effects in other selection environments and thus get selected against when the environment changes (Travisano and Lenski 1996, Cooper and Lenski 2000). Secondly, in the absence of such trade-offs, mutations which are beneficial in one environment might be neutral in other environments. Conditionally beneficial mutations of this kind will have a lower chance of getting fixed (Whitlock 1996) and their fate will

primarily be governed by an interaction between the forces of drift and mutations rate. Due to these constraints on the fixation of beneficial mutations in unpredictably fluctuating complex environments, it can be difficult for populations to show improvement in fitness for any given component of the environment. In line with these expectations, we found little improvement in overall mean fitness when selected in complex, unpredictable fluctuations over a short duration of ~170 generations (section 4.2 in chapter 2). Our results here show that, over a longer duration of selection, populations facing complex, unpredictable fluctuations can improve fitness even for the selection environments.

Along with retarding the fixation of conditionally beneficial mutations, complex, unpredictable fluctuations will strongly select against those mutations which can reduce fitness in any of the selection environments (i.e. pH, salt or H_2O_2). This should reduce the loss of fitness across selection environments which, in turn, should lead to reduction in the variation for fitness. Our results for variation in fitness across the five selection environments support this expectation (Fig 5.2).

4.2 Fluctuating environments minimize the variation for fitness

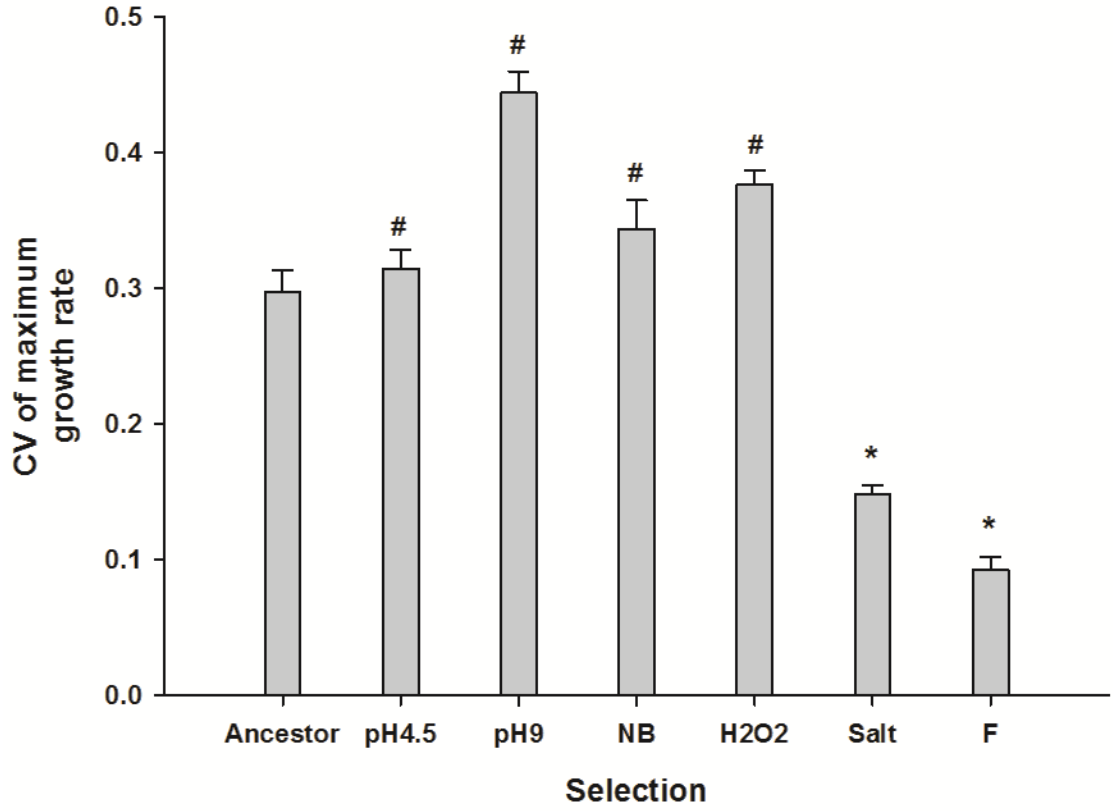

Fig 5.2 Mean coefficient of variation for fitness (±SE) for all the selection regimes. Coefficient of variation (CV) was computed for every selection regime over all the assay environments. Fitness estimated as maximum slope of the growth trajectory over 24 hours. * denotes significantly lower CV ($p < 0.05$, Dunnett's post hoc statistics) than ancestor while # denotes significantly higher CV than ancestor.

When evolved under predictable or unpredictable temporal fluctuations, microbial populations show reduced variation for fitness over the whole range of selection environments (Kassen 2014). This is because, when the environment changes temporally, it is the geometric mean (and not the arithmetic mean) of the fitness over the entire evolutionary time that plays a more important role in determining the long-term evolutionary success (Gavrilets and Scheiner 1993). Since reducing the variation in a series increases its geometric mean (Orr 2007), populations facing fluctuating environments are expected to have lower variation in fitness across the selection environments. This prediction is supported by empirical studies where temperature or host type fluctuates across time (Turner and Elena 2000, Hughes, Cullum et al. 2007). However, reducing the variation in fitness when multiple selection environments are changing unpredictably is expected to be much more challenging particularly in the presence of negative pleiotropic interactions between traits (Rainey and Travisano 1998). Our results show that mandatory negative pleiotropic interactions, where decrease in fitness in one environment is indispensable to the gain in fitness in other, were almost absent. Populations selected under complex, unpredictable fluctuations minimized the variation in the fitness across all selection environments as compared to the ancestor (Fig 5.2).

The significantly lower variation for fitness in F populations compared to the ancestor needs to be interpreted together with the observation that the F populations also showed significantly higher overall mean fitness (Fig 5.1). This indicates that F populations did not only improve the fitness in some environments but also gained fitness in at least few selection environments. To confirm this, we estimated the differences in fitness from the ancestors under different environments.

4.3 Change in fitness from ancestor is environment specific

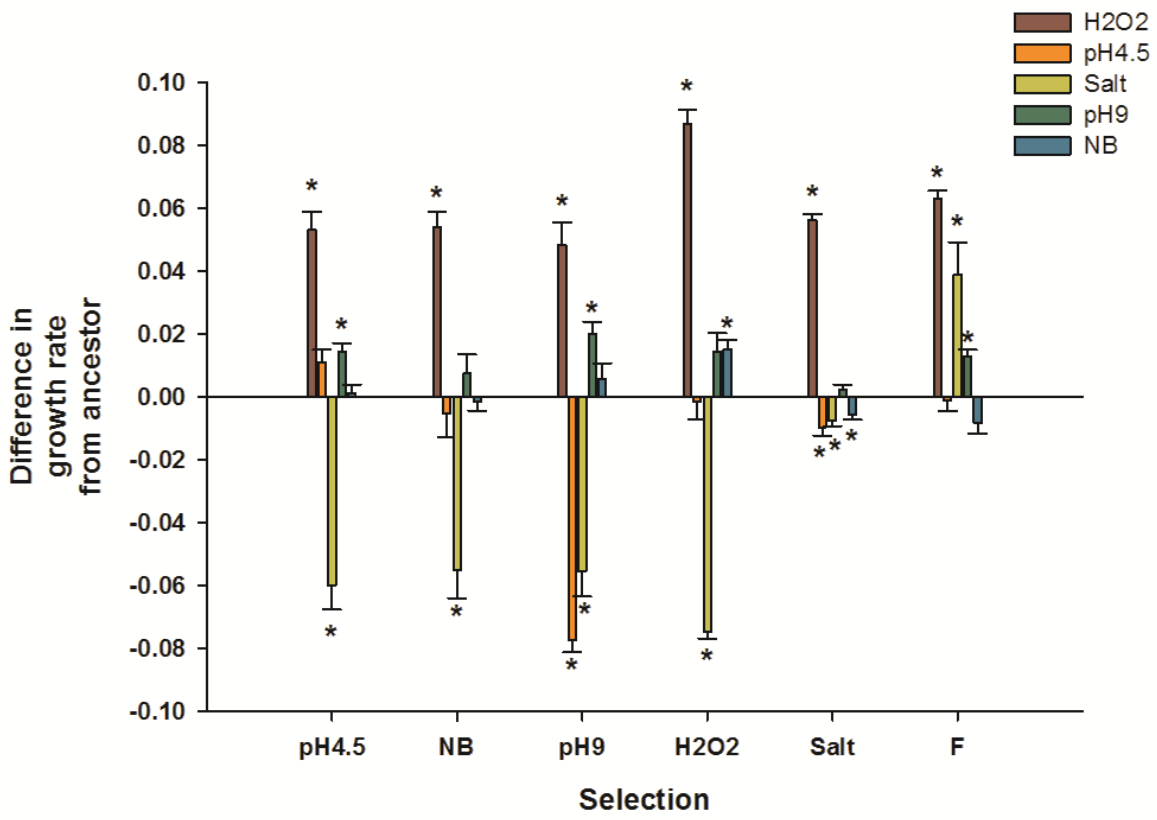

Fig 5.3 Difference in maximum growth rate from ancestor (±SE) for all the selection regimes. Difference in mean fitness (estimated as maximum growth rate) from ancestor was computed for every selection regime in every environment. Negative values indicate loss of fitness from ancestral value while positive values indicate gain of fitness, as compared to the ancestor. Every selection regime, except F, shows loss of fitness in at least one of the environments.

* denotes $p < 0.05$ after individual ANOVAs followed by Holm-Šidàk correction.

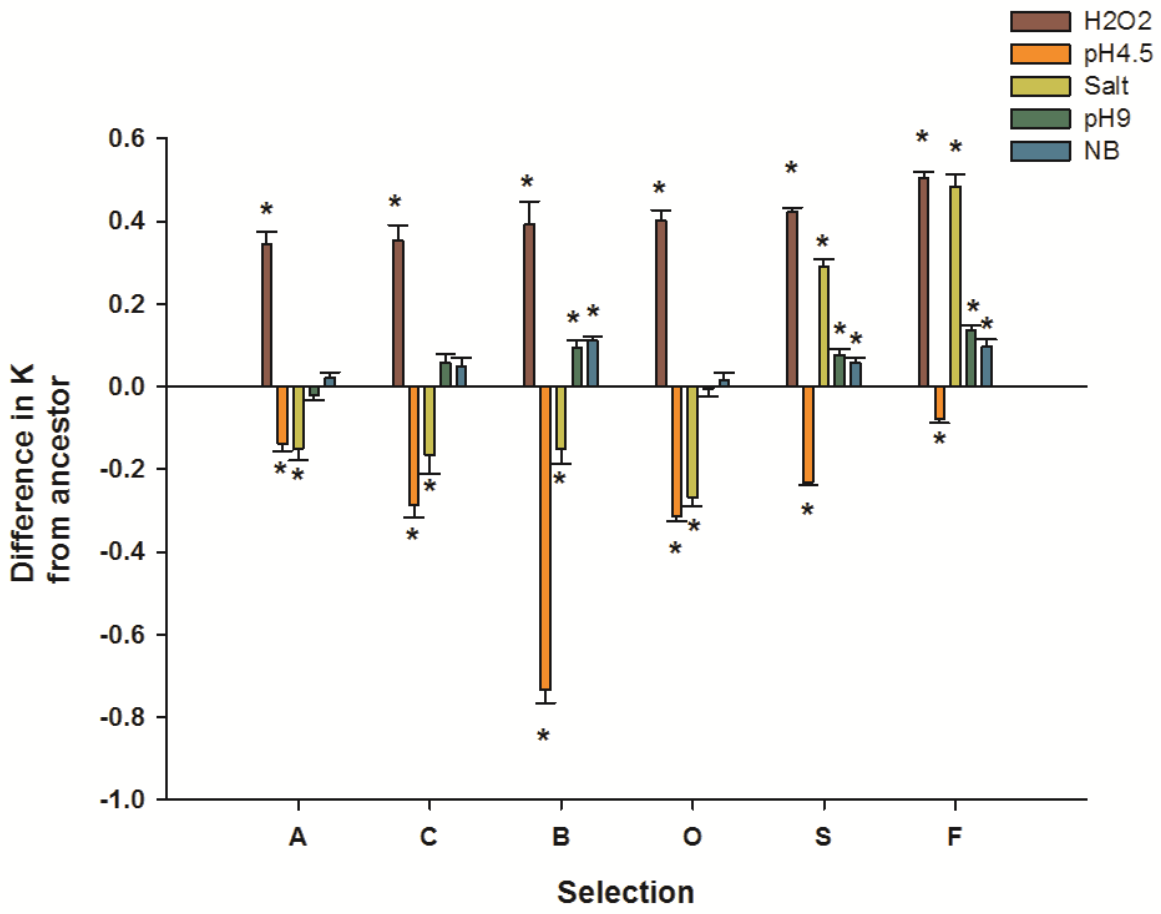

Fig 5.4 Difference in K from ancestor (±SE) for all the selection regimes. Difference in mean fitness (estimated as maximum density reached i.e. K) from ancestor was computed for every selection regime in every selection environment. Negative values indicate loss of fitness while positive values indicate gain of fitness, as compared to the ancestor. Every selection regime, except F, shows loss of fitness in at least one of the environments.
* denotes $p < 0.05$ after individual ANOVAs followed by Holm-Šidàk correction.

In a simplistic scenario, adaptation to multiple selection environments can be thought of as independent of each other. In that case, if the ancestor is already close to the fitness maxima in a given selection environment, then neither the populations with constant exposure to that environment nor the F populations will show any improvement in fitness (Kassen 2014). On the other hand, when the ancestor is far away from the fitness maxima for a given environment, populations facing only that environment, along with the F populations, will show a definite improvement in fitness. However, when the fitness improvements in different environments are correlated, the fitness of F populations can increase only via 'no cost' routes i.e. F populations can increase the fitness in one environment without loss of fitness in other.

Since trade-offs are fairly ubiquitous (Roff and Fairbairn 2007, Agrawal, Conner et al. 2010), populations facing multiple environments are expected to show lesser increase in fitness as compared to that of the specialists (Kawecki 1994, Whitlock 1996). However, results of empirical studies, involving both predictable and unpredictable fluctuations in single environmental variable or host, suggest that populations facing multiple values of a given environmental variable can improve fitness over all or some of the selection environments without a loss of fitness in other selection environments (Turner and Elena 2000, Hughes, Cullum et al. 2007, Condon, Cooper et al. 2014). Our results extend this understanding to complex environments where multiple variables fluctuate at the same time. F populations did not lose fitness in any of the selection environments as compared to the ancestor (Fig 5.3). The populations selected under constant exposure to hydrogen peroxide and pH9 showed increase in fitness in the respective selection environment. This increase in fitness was accompanied by the loss of fitness in salt for both populations and additionally in pH4.5 for populations selected in ph9. F populations on the other hand showed fitness improvements in pH9, salt and hydrogen peroxide, without any loss of fitness in any environment (Fig 5.3).

This suggests that the ancestor was not only away from the fitness maxima of some of the selection environments but also that F populations could access a 'no cost' route to increase in fitness in these environments. In case of pH4.5, neither F populations nor the populations selected under constant pH4.5 environment, improved fitness over the ancestor (Fig 5.3). Thus, the ancestor was most likely well adapted to pH4.5 which is not surprising given that *Escherichia coli* are known to be well-adapted to acidic environments (reviewed in Foster 2004). The control environment of NB^{Kan} showed similar pattern, which is intuitive given that ancestral *Escherichia coli* strain K12 is expected to be well adapted to the laboratory conditions.

Surprisingly F populations showed significant increase in fitness from ancestors in salt, while the populations selected in salt showed a loss of fitness in the selection environment (Fig 5.3). This counterintuitive observation is resolved when we look at a different proxy of fitness, namely, maximum density achieved or K (Vasi, Travisano et al. 1994, Novak, Pfeiffer et al. 2006). Populations selected in salt showed increased K relative to the ancestor (Fig 5.4). This improvement in K and lack of the same in maximum growth rate could be due to an underlying trade-off between these two aspects of fitness. Higher concentration of salt will result in strong selection for robust membrane structures, which can be negatively correlated with the growth rate of the cells (Carlquist, Fernandes et al. 2012). Consistent with the observations for maximum growth rate as a proxy of fitness, even the F populations showed corresponding improvement in K. In the light of these results, we repeated the analysis for the mean and variance for K. The patterns in overall mean and variance remained consistent with maximum growth rate, i.e. F populations showed highest mean fitness with lowest variation for fitness (Appendix 5.3). But all the selected populations, including the F populations, showed reduced K in pH4.5, (Fig 5.4). In the light of these results though, the choice of fitness proxy demands further attention.

4.4 On the measurement of fitness

In many studies on microbial experimental evolution, competitive ability relative to the ancestor is used as a proxy of fitness (Leroi, Lenski et al. 1994, Hughes, Cullum et al. 2007, Alto, Wasik et al. 2013). This measure is preferred because it integrates over all phases of a growth cycle (i.e. lag phase, log phase etc.) and is expected to show how much better the evolved population has become in terms of evolutionarily replacing the ancestor (Kassen 2014). However, we refrained from using this measure. This is because change in competitive fitness compared to the ancestor is an appropriate measure in case of constant or directionally changing selection environments, where populations are adapting towards a fixed fitness peak (Collins 2011). As opposed to this, adaptation to unpredictable environments will involve sudden changes in the underlying fitness landscape. Populations facing such environments will not show monotonic change in the fitness as compared to the ancestor, which renders the measure of competitive fitness inappropriate (Collins 2011). In addition to the unpredictability, our selection environment features complexity. Different selection environments can select different life history components which might not get detected in the composite measure of competitive fitness (Vasi, Travisano et al. 1994). The fitness measures employed in this study estimate the growth, either in terms of rate or yield, independent of the competitor. Abiotic environment is the major driver of these two parameters and hence more appropriate for the point measurement performed at the end of the fluctuating selection.

Following previous studies, we thus use maximum growth rate and maximum density achieved as two different measures of fitness (Vasi, Travisano et al. 1994, Novak, Pfeiffer et al. 2006, Ketola, Mikonranta et al. 2013). The results of maximum growth rate and K are comparable in case of overall mean fitness and variation for fitness (Fig 5.1, 5.2 and Appendix 5.3). But this is not the case when we consider the change in fitness from ancestor (Fig 5.4). Populations selected in salt show reduced maximum growth rate as compared to the

ancestor but significantly higher K. Secondly, in pH 4.5, maximum growth rate does not evolve in comparison to the ancestor in all the selected populations but K shows significant reduction (Fig 5.4). These results clearly indicate that different selection environments can select for different components of fitness. Our results suggest that widely used fitness measures like relative fitness or logistic growth rates can mask this effect and thorough investigation into all the components of fitness is more desirable.

5 SUMMARY

To our knowledge, this is the first study which shows that populations can simultaneously improve fitness and minimize the variation for fitness when exposed to complex, unpredictable fluctuating environments. Simultaneous exposure can result in fitness increase in some selection environments without any loss of fitness in other selection environments, though, selection at even longer timescale, i.e. few thousands of generations, might lead to different outcomes (Satterwhite and Cooper 2015). More importantly, our results suggest a possible explanation for the absence of trade-offs across environments in microbial populations by showing that other kind of trade-offs can exist i.e. trade-offs between different measures of fitness in the same environment. Estimating different components of fitness separately might be fruitful for future experimental studies.

Chapter 6. Predictability does not play a major role in shaping the fitness outcomes over short duration of the selection

1 INTODUCTION

Laboratory studies using bacterial populations have showed that when exposed to environments containing multiple resources, populations adapt to use all the resource components of the environment (Barrett, MacLean et al. 2005, Cooper and Lenski 2010). We found that *Escherichia coli* populations subjected to complex fluctuating environments also showed improvement in fitness with respect to some of the component environments, without the loss of fitness in the others (Fig 5.3 and 5.4 from chapter 5). Simultaneous exposure to multiple environmental variables seems to result in adaptation through 'no cost' routes where gain of fitness in some environments is not accompanied by loss of fitness in other component environments. However, the role of unpredictability of the environment by itself, in terms of affecting evolution, remained to be investigated. For instance, one could ask whether environmental complexity, in the absence of temporal unpredictability, will result in similar patterns of fitness gains or losses.

Previous experimental studies with microbial populations show that in the presence of predictable fluctuations between different values of a given stress (say pH or temperature) fitness of the selected populations improve over all the values of the stress experienced (Turner and Elena 2000, Hughes, Cullum et al. 2007, Coffey and Vignuzzi 2011, Alto, Wasik et al. 2013). On the other hand, unpredictable fluctuations can result in increase in fitness (Turner and Elena 2000, Ketola, Mikonranta et al. 2013), show no change in fitness (Alto, Wasik et al. 2013) or fitness can increase in some of the environments but not all (Hughes, Cullum et al. 2007). However, except for the systems employing host-parasite systems, fluctuating environments in these studies are almost entirely composed of different values of same environmental variable (Hughes, Cullum et al. 2007, Alto, Wasik et al. 2013). In this part of the study, we look at the evolutionary effects of predictability when the populations are exposed to qualitatively different environments (i.e. salt and pH4.5). The environmental

complexity in this study is introduced in terms of the different nature of the stresses, namely salt and pH, and the environment fluctuates predictably or unpredictably between these two different kinds of environments. In other words, the complexity is not ‘simultaneous’, like the prior selection regimes but ‘temporal’.

2 METHODS

2.1 Selection experiment

Kanamycin resistant *Escherichia coli* strain K12 (see Appendix 5.1 for details) was used for this selection experiment. A single colony grown on Nutrient agar with Kanamycin (see Appendix 2.1 for composition) was inoculated in 50 ml of Nutrient broth with Kanamycin (NB^{Kan}) (see Appendix 2.1 for composition) and allowed to grow for 24 hr at 37^0C, 150 rpm. 4 µl of this suspension was used to initiate each of the 120 replicate populations.

120 replicate populations were equally divided into five treatment and one control regime, thus resulting in 20 replicate populations per regime. Control populations (henceforth termed as Control) were sub-cultured in NB^{Kan} for the entire duration of the selection. Two out of five selection regimes constituted a constant environment with either pH 4.5 or salt in NB^{Kan}. Out of the remaining three environments, one environment alternated between pH 4.5 and salt 5g% predictably (henceforth termed as PBin, for **P**redictable **Bin**ary) and the other faced these two environments unpredictably (henceforth termed as UpBin, for **U**n**p**redictable **Bin**ary). The remaining environment fluctuated unpredictably over a range of values of salt and acidic pH (henceforth termed as UpRange, see Appendix 6.1 for details of all selection regimes). The following table summarizes the selection regimes –

Codes	Environment	Fluctuations	Predictability
Control	NB	Constant	Predictable
Acid	pH4.5	Constant	Predictable
Salt	Salt 5	Constant	Predictable
PBin	pH 4.5 <=> Salt 5	Binary	Predictable
UpBin	pH 4.5 <=> Salt 5	Binary	Unpredictable
UpRange	pH4.5/pH5/S2/S3/S/3.5/S4/S4.5	Range	Unpredictable

Summary of selection regimes

24 welled plates with 2 ml of appropriate growth medium and 4 µl of inoculum volume for each well were used throughout the selection and assay experiments. The growth conditions were maintained at 37^0C, 150 rpm. All the populations were sub-cultured every 24 hr. Extinctions were identified visually and revived using 20 µl of the previous day's culture stored at 4 ^{0}C. The selection lasted for 30 days i.e. ~ 260 generations. Every 10^{th} day, the populations were stored as glycerol stocks at -80 ^{0}C for future assays.

2.2 Fitness measurement

Growth rate was assayed (see Appendix 1.1 for details) for all the selected populations and ancestor in pH 4.5, salt 5g% and NB^{Kan}.

2.3 Statistical analysis

Pooled data were analyzed using 3 way mixed model ANOVA. Selection (seven levels: control, salt, pH 4.5, pbin, upbin, uprange, ancestor) and assay environment (two levels: pH 4.5 and salt 5g %) were fixed factors while replication (twenty levels) was a random factor nested in selection. The data were also analyzed separately for each of the assay environment to see whether predictability produces different effects across the assay environments. 2 way mixed model ANOVA was used with selection (seven levels: control, salt, pH 4.5, pbin, upbin, uprange, ancestor) was a fixed factor and replication (twenty levels) was a random factor nested in selection.

In all cases where there was a significant main effect of selection, post hoc analysis was performed using Tukey's HSD to determine which selected populations differed significantly between each other.

3 RESULTS

When analyzed together, both selection ($F_{6, 280} = 10.23$, $p < 0.0001$) and assay environment had a significant effect on fitness ($F_{1, 280} = 38.29$, $p < 0.0001$). Additionally, there was a significant interaction between assay environment and selection ($F_{6, 280} = 16.57$, $p < 0.0001$). Variation for fitness between replicates of a given selection regime was marginally significant ($F_{133, 280} = 1.35$, $p = 0.041$). When analyzed separately for pH 4.5 and salt, selection had significant effect in both assay environments ($F_{6, 140} = 7.51$, $p < 0.0001$, for pH 4.5 and $F_{6, 140} = 13.58$, $p < 0.0001$ for salt, Fig 6.1). Populations selected under constant pH 4.5 environment showed significant improvement in fitness in pH 4.5, compared to all other populations except upbin (Fig Table 6.1). Populations evolved in constant salt environment showed significant improvement in fitness from the ancestor but did not differ significantly from any of the other selected populations (Table 6.2). But surprisingly none of the three fluctuating treatments (pbin, upbin, uprange) differed significantly from each other in either salt or pH 4.5 environments (Table 6.1 and 6.2). Overall, the results suggested that while environmental fluctuations might play a role in affecting the evolutionary outcomes, the nature of predictability does not play a consistent role in the same, at least in the given environmental contexts.

	NB	Salt	uprange	upbin	pbin	pH4.5	Ancestor
NB		0.020197	0.843100	0.811992	0.996018	0.022912	0.088557
Salt	0.020197		0.463815	0.000097	0.120268	0.000026	0.998810
uprange	0.843100	0.463815		0.097953	0.992966	0.000143	0.790924
upbin	0.811992	0.000097	0.097953		0.410202	0.531997	0.000757
pbin	0.996018	0.120268	0.992966	0.410202		0.002459	0.343842
pH4.5	0.022912	0.000026	0.000143	0.531997	0.002459		0.000026
Ancestor	0.088557	0.998810	0.790924	0.000757	0.343842	0.000026	

Table 6.1 Tukey's HSD *p* values for all the pairwise comparisons of mean fitness for all the selection regimes, when assayed in pH 4.5.

	NB	Salt	uprange	upbin	pbin	pH4.5	Ancestor
NB		0.000026	0.000026	0.000026	0.000026	0.000698	0.000263
Salt	0.000026		0.675101	0.927518	0.906883	0.199029	0.314658
uprange	0.000026	0.675101		0.998749	0.999415	0.001199	0.003032
upbin	0.000026	0.927518	0.998749		1.000000	0.008427	0.018685
pbin	0.000026	0.906883	0.999415	1.000000		0.006719	0.015149
pH4.5	0.000698	0.199029	0.001199	0.008427	0.006719		0.999987
Ancestor	0.000263	0.314658	0.003032	0.018685	0.015149	0.999987	

Table 6.2 Tukey HSD *p* values for all the pairwise comparisons of mean fitness for all the selection regimes when assayed in salt.

4 DISCUSSION

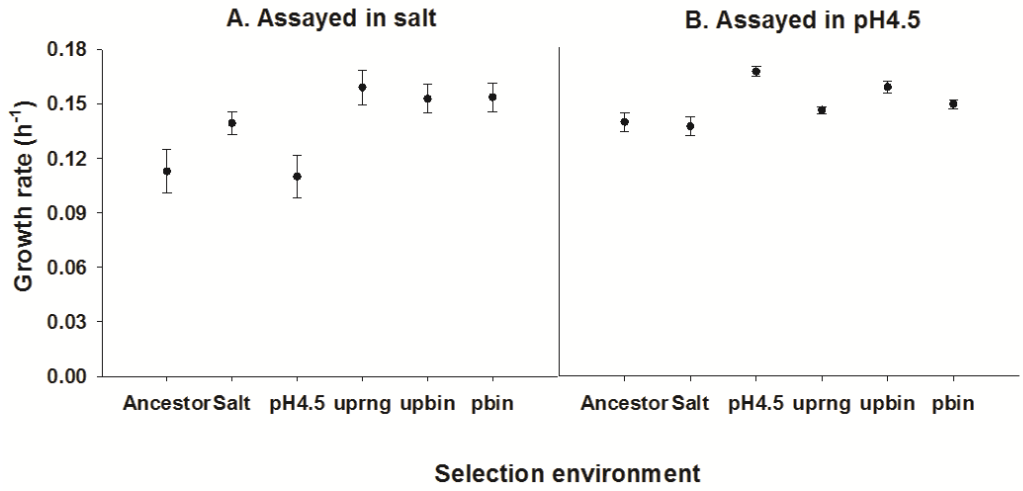

Fig 6.1 Mean (±SE) fitness in salt and pH 4.5 A. Mean fitness in salt for each selection regime and ancestor is plotted. **B.** Mean fitness in pH 4.5 for each selection regime and ancestor is plotted. None of the three fluctuating selection regimes i.e. uprng, upbin and pbin, are significantly different from each other in either salt or pH 4.5. For other significant differences, see tables 6.1 and 6.2. Fitness was measured as maximum slope of the growth trajectory over 24 hours.

Little is known about the effect of predictability on evolution in the presence of complexity. We previously showed that complex, unpredictable fluctuations do not increase fitness in the selection environments over short duration of ~ 170 generations (Fig 2.1 in chapter 2). Long term selection of ~ 900 generations results in improved fitness in selection environments (Table 5.2, Fig 5.1 in chapter 5). But we could not comment on the specific role of unpredictability in this case, due to the absence of corresponding complex predictable control. Here we compared the fitness outcomes of predictable and unpredictable fluctuations across two qualitatively different environments, salt and pH4.5.

Results show that predictability does not affect the fitness outcome in this case (Fig 6.1). Both predictable and unpredictable fluctuations show higher mean fitness as compared to the ancestor but the difference is not significant (Table 6.1 and 6.2, Fig 6.1). On the other hand, populations selected in the constant concentration of salt or pH 4.5 did show significant improvement in fitness, suggesting the scope for increase in fitness in both these environments. This lack of significant fitness increase in pbin and upbin populations cannot be attributed to underlying trade-off since previous results have shown that fitness (estimated as maximum growth rate) can improve in salt without any significant loss in pH 4.5 (Fig 5.3 in chapter 5). Though the ancestral population and the environmental variables are identical in this case, it is important to note that bacterial populations were simultaneously exposed to salt and pH 4.5 in the previous selection while here the exposure was sequential.

It has been shown previously that unpredictable binary fluctuations can result in higher fitness as compared to fluctuations over a range of values for the same environmental variable (Alto, Wasik et al. 2013). One possible reason for this observation is the greater exposure received by the populations to the assay environment, i.e. the extreme value of the chosen variable, in case of binary fluctuations. For instance, a population facing binary unpredictable fluctuations between two temperatures will face the assay temperature (one of

the two values) more often than a population selected over a range of temperatures, with one of the values being the assay temperature. We tested the possible effects of number of exposures during selection on fitness by including a selection regime with an unpredictable sequence of multiple salt and acidic pH values. The fitness of range- selected (i.e. uprange) populations was comparable to that of the predictable (pbin) or unpredictable (upbin) binary fluctuations in both the assay environments (Fig 6.1). This suggests that the number of times a population faces an environment identical to the assay environment during the process of selection, does not affect its fitness significantly.

5 SUMMARY

Our results thus suggest that predictability of the environment does not play a major role in shaping the fitness outcomes of populations in case of salt and pH 4.5. Moreover, differences in fitness of populations facing binary *vs* range of values unpredictably, cannot be attributed to the number of times the populations have been exposed to that particular environment. Longer duration of selection might yield a different picture but ~260 generations of selection in salt and pH 4.5 indicate that predictability plays little role in shaping the fitness outcomes of the evolving populations.

Chapter 7. Conclusions

In my thesis, I have used laboratory populations of *Escherichia coli*, to study short term and long term fitness effects of unpredictable, complex fluctuating environments on microbial populations. I show that selection under complex, unpredictable fluctuations over short duration (~170 generations) leads to fitness advantage in novel environments like antibiotics and heavy metals through the evolution of elevated energy dependent efflux. This observation can have serious practical implications.

Wide-spread antibiotic resistance in pathogenic bacteria is a challenge for health systems and economies worldwide. Infections by these resistant pathogens claim ~50,000 lives per year, in Europe and US alone. Though no reliable data exists for other parts of the world, the suspected numbers are no less than hundreds of thousands. By year 2050, ~10 million people are predicted to lose their lives every year, due to antimicrobial resistant pathogens (O'Neill 2014).

Increased clinical and commercial use of antibiotics is considered to be the primary driver for evolution of antibiotic resistance (O'Neill 2014). However, over the last few years, number of studies has shown that antibiotic resistance can evolve even in the absence of exposure to antibiotics (Clemente, Pehrsson et al. 2015, McArthur, Fletcher et al. 2015) This puzzling observation is typically explained in terms of horizontal gene transfer (Frost, Leplae et al. 2005, Perry and Wright 2013) or evolution of efflux activity due to exposure to heavy metals (Stepanauskas, Glenn et al. 2006, Wright, Peltier et al. 2006). My results show that even in the absence of all these causes, fluctuations in environmental variables can lead to the evolution of higher efflux abilities, which can in turn lead to multi-drug resistance. My results can potentially explain how recently discovered Amazonian tribes, who have never experienced antibiotics in their evolutionary history, can harbour microbes showing MDR (Clemente, Pehrsson et al. 2015). More critically, these results can have potential public health implications, particularly given that environmental variability has increased in many

parts of the world (Bhutiyani, Kale et al. 2010, Medvigy and Beaulieu 2012). It is already known that habitats that have a history of disturbance often harbour species that turn out to have a high invasive potential, when introduced to other areas (Lee and Gelembiuk 2008). My results sound a similar cautionary note for potential pathogens. However, the environmental complexity in nature is evidently much greater than those experienced by the *E. coli* populations in my study. Therefore, much more work needs to be done to ascertain the effects of environmental fluctuations on natural microbial and non-microbial populations.

One fruitful direction for further study in this context is an investigation of the effects of duration of exposure to fluctuating environments. I show that evolved superior efflux ability, and the associated fitness advantage under novel environments, vanishes over time (~560 generations). This suggests that over longer periods of time, elevated efflux might carry a fitness cost and hence face negative selection. Change in efflux abilities with time thus needs to be studied along with the antibiotic resistance properties. Simultaneous loss of antibiotic resistance and efflux abilities, in the absence of antibiotics in the selection environment, will confirm that antibiotic resistance evolved as a correlated response with efficient efflux. If it turns out that even under natural conditions, elevated efflux is just a transient mechanism and not a long-term one, then the potential effects of environmental fluctuations on evolved MDR is going to be less serious.

Fitness outcomes of fluctuating environments revealed intriguing patterns, not only in novel environments but also in the selection environments. There was no fitness improvement for selection environments, even after ~560 generations of selection. Approximately 900 generations of selection on the other hand, resulted in fitness improvement for environmental stresses that were part of the selection regime. This observation is very unusual given that large population sizes and substantial mutation rates in bacteria typically result in rapid adaptation in many laboratory selection experiments (Reboud and Bell 1997, Collins and De

Meaux 2009, Ketola, Mikonranta et al. 2013). However, majority of these selection regimes depict 'standard ecological scenario' (SEE) where environments remain constant throughout the selection or change monotonically (Collins 2011). Natural environments on the other hand fluctuate over time and my results show that our understanding of adaptation rates based on results coming from SEE, do not necessarily extrapolate to these fluctuating environments.

It is not only the slow rate of adaptation but also the large extent of adaptation that is surprising when bacterial populations evolve in fluctuating environments for extended duration. After ~900 generations, extent of adaptation to individual environmental stress is comparable between populations getting constant exposure to that environment and populations facing the complex, unpredictable environments (see chapter 5). But this improvement in fitness seems to be achieved through a different adaptive route by the populations facing fluctuations. This is evident when change in fitness from ancestor is characterized in all the selection environments for all the selected populations. Populations facing a single constant environment throughout the selection lose fitness in at least one of the other environments while the populations facing complex environments do not show loss of fitness in any environment. This observation suggests that trade-offs across environments (i.e. fitness advantage in one environment being accompanied by fitness loss in the other is perhaps rare in microbes which agrees in general with several studies (Turner and Elena 2000, Hughes, Cullum et al. 2007, Condon, Cooper et al. 2014 but see Reboud and Bell 1997). This is somewhat unexpected given the ubiquity of trade-offs in most organisms in the literature. I provide empirical evidence for a probable explanation for this conundrum. I show that trade-offs might exist across two different components of fitness in the same environment, for instance maximum growth rate and maximum density reached, rather than across different environments. The two different proxies of fitness can evolve to different extents and sometimes even in different directions where increase in one can be accompanied

by decrease in other, in a given environment (see chapter 5 for details). However, these results in turn raise the question as to what is the best proxy of the fitness for bacteria.

Following previous authors (Collins 2011), I argue that the most commonly employed measure of fitness i.e. relative fitness or competitive ability vis-a-vis the ancestor, is of little relevance when populations are not adapting to constant or monotonically changing environments. The measure of relative fitness assumes that the population is monotonically progressing towards an adaptive peak and hence comparison with ancestor provides a good measure of extent of adaptation. Populations adapting to fluctuating environments on the other hand, face multiple different environments and the adaptation trajectory is far from monotonic. Relative fitness thus needs to be computed with respect to the immediate ancestor, i.e. the starter population from which growth took place in the current environment, under such a scenario. Estimating such 'fitness flux', though rewarding, is logistically not possible in case of long term evolution studies. Instead, I used maximum growth rate of the population as the proxy of fitness. This is one of the simple and direct measures of fitness and logistically less demanding. The growth curve studies performed to estimate this parameter, also allowed me to estimate another components of fitness, namely 'K' (maximum density reached). In fact, comparison of 'r' (maximum growth rate) with 'K' reveals that different fitness proxies can evolve in different directions and to different extent in some environments (see chapter 5). This observation supports previous results which point out that trade-offs in the different life history components (or fitness proxies) are not absent in microorganisms (Vasi, Travisano et al. 1994, Novak, Pfeiffer et al. 2006). More importantly, this calls for cautious use of composite measures of fitness like logistic growth rate or relative fitness, as they will fail to reveal the interplay between these various facets of fitness.

I investigated some of the aspects of the evolutionary effects of complex, unpredictable fluctuations in my thesis. Along with several insights, my results also raised many questions which can be pursued in the future. For example, the role of complexity in shaping the fitness outcomes needs to be empirically investigated in greater detail. What if the fluctuations are biotic in nature rather than abiotic, and dependent on the growth and evolution of the populations rather than independently changing in background, as was studied here? What if we consider spatial heterogeneity along with the temporal one at hand? Most theoretical studies on evolutionary effects of fluctuating environments seek to model changes in mutation rates and standing variation (Leigh Jr 1970, Ishii, Matsuda et al. 1989, Taddei, Radman et al. 1997). My results suggest that such studies have perhaps failed to consider the critical mechanism that enables organisms to adapt to such situations in nature and a new class of theoretical modelling is needed to investigate this issue. Finally, although my thesis concentrated entirely on effects of environmental fluctuations on bacteria, such fluctuations are also experienced by multi-cellular organisms. Although there has been some studies on how organisms like fruit-flies (Condon, Cooper et al. 2014, Ketola, Kellermann et al. 2014), green algae (Reboud and Bell 1997) and seed beetle (Hallsson and Björklund 2012), cope with environmental heterogeneity, very little is known about the corresponding mechanisms. Thus, elucidation of the effects of fluctuating environments is likely to be a major line of work in the near future and one can safely predict interesting days ahead for this field.

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Appendix

Appendix 1.1 Estimating fitness

For all growth rate measurements mentioned, the relevant glycerol stocks were revived for 18 to 20 hours. The culture volumes for revival were same as that of the selection. 4 µl of this revived culture (20 µl in case of selection under deteriorating environment) was inoculated into 2 ml of medium in 24-well cell culture plates in triplicates and kept under 150 rpm at 37ºC. The OD_{600} of each well was measured every two hours over a period of 24 hours on a plate reader.

Following a recent study (Ketola, Mikonranta et al. 2013), we estimated fitness as the maximum growth rate over the period of 24 hours. The maximum growth rate of the bacterial population was computed using a QBASIC (v 4.5) script (available on request to the author at s.dey@iiserpune.ac.in) to fit straight lines on overlapping moving windows of three points on the time series of OD_{600} values obtained as per mentioned in the previous section. The maximum slope obtained among all the fitted lines for a given time series, was taken as an estimate of the maximum growth rate for the corresponding population.

Appendix 1.2 Mutation rate measurement

We used the method suggested by Crane et al (Crane, Thomas et al. 1996) for estimating mutation rates. One control population and one selected population were processed with a given batch of media. Glycerol stocks of control S and F populations were revived in NB for 18 hours. The revived culture was then diluted 10 fold. Eleven replicate flasks, each containing 50 ml of nutrient broth, were inoculated with 10 µl each of this diluted suspension. These replicate cultures were then allowed to grow for another 18 hours and then plated on nutrient agar with and without 100 micrograms/ml Rifampicin (Torres-Barceló, Cabot et al. 2013) for estimating mutant counts and total numbers respectively. Colonies were counted manually, after 24 hours of incubation at 37ºC. Median estimator (λ_{med}) was estimated using following formula (Jones 1994):

$$\lambda_{\text{med}} = \frac{r_m/s - 0.693}{\ln\left(r_m/s\right) + 0.367}$$

where r_m = Number of mutants found in the median culture

s = Proportion of culture plated

Mutation rate,

$\mu = \lambda_{med} / N$

where, N = Total number of living bacteria in 50 ml culture (as counted on Nutrient Agar).

Appendix 1.3 Sequencing mutS and mutL genes

Along with the mutation rate measurement, we sequenced candidate genes involved in mismatch repair pathway to address the possibility of evolution of hypermutators.

100 µl of glycerol stock from each replicate population of S and F was streaked on separate NA plates and incubated at 37^0C. After 24 hours, 3 to 4 well isolated colonies from each plate were mixed in sterile miliQ water separately. 20 minutes of heating on thermocycler (Eppendorf) was followed by 15 minutes of centrifugation. The supernatant obtained was used as a sample for the PCR reaction.

Both the genes of interest were amplified using Advantage 2 polymerase mix (Clontech Laboratories, Inc., CA, USA, Catalogue number 639137) to obtain high fidelity amplification.

mutS

Primers – Forward 5' GAGTGCAATAGAAAATTTCGA 3'

Reverse 5' TCTTCTGGTACTGACAGCAAA 3'

mutS gene is 2562 bp long. To eliminate the errors associated with long sequence reads we designed 4 additional primers with expected coverage of less than 800 bp.

Sequencing primers – mutS_619 5'CCGCTGTGGGAGTTTGAAAT 3'

mutS_1342 5'GACGGCGCGACCGATTATCTG 3'

mutS_1702 5'CGGGCCTATACCCTGAACTA 3'

mutS_2227 5'CCGGAGAAAATGGAAGGGGT 3'

mutL

Primers – Forward 5' GGCGAGCGACGATTACCAACC 3'

Reverse 5' GCGACAACCCTTCCAGCAAT 3'

mutL gene is 1850 bp long. To eliminate the errors associated with long sequence reads we designed 2 additional primers with expected coverage of less than 800 bp.

Sequencing primers – mutL_673 5'GCGCTGGCGAATGAATGGCA 3'

mutL_1200 5'ACAGCAAGGTGAAGTGTATCG 3'

Reaction mix for both mutS and mutL – For 1 reaction of 25 µl

	Volume (µl)	**Comment**
Buffer	2.5	
dNTP	1	2.5 mM concentration, $MgCl_2$ not needed
Forward + Reverse Primer	1 + 1	Final concentration needed 10 µM
Water	14	
Template	5	
Enzyme	0.5	Added separately for every reaction

PCR program for both mutS and mutL –

Lid = 105^0C

Wait

T = 94^0C for 5 min

T = 94^0C for 30 sec

T = 60^0C for 30 sec

T = 68^0C for 2 min

Go to 2 rep 35

T = 68^0C for 10 min

Hold 4^0C

The overlapping sequences obtained were aligned using MEGA (Version 5, Arizona State University, USA) and stitched together to form a whole sequence. These sequences were then compared with the library sequence in NCBI using BLAST. Any base pair substitutions or frame shift mutations were noted for each of the S and F population.

Appendix 1.4 Energy dependent efflux estimate

For characterization of efflux properties of our populations, we modified an existing protocol (Webber and Coldham 2010) for fluorescent-based estimation of active efflux in Gram negative bacteria. Glycerol stocks of S and F populations were revived for 18 to 20 hours in NB. OD_{600} was adjusted, by diluting with NB, in the range of 0.03 to 0.06 OD units on Nanodrop (Thermo scientific 2000c). 2 ml of the OD_{600} adjusted cultures were then centrifuged, supernatant was discarded and the pellet was re-suspended in the PBS buffer (pH 7.4). Part of each suspension was then boiled at 60^0C for 10 minutes to be used as positive controls. The live cells and corresponding positive controls were then loaded in 96 welled plates in triplicates (168 µl and 180 µl respectively). 20 µl of Bis-benzimide (Excitation λ 355 nm and Emission λ 465 nm) was then added to all the wells. Bis benzimide is a small molecule which can easily enter the cell and fluoresce after intercalating with DNA. Live cells were also supplied with glucose since we wanted to look at the ATP dependent active efflux (8 µl of 1% glucose solution). The total volume of each well with live cell was 196 µl and with dead cells was 200 µl.

Fluorescence was measured on a plate reader (Tecan Infinite M200 Pro) at 37^0C for forty minutes. By 35 minutes the fluorescence levels in all wells reached their steady state. The level of fluorescence at 35 minutes was taken as the total fluorescence. The reader was then paused and 4 µl of Carbonyl Cyanide m-Chlorophenylhydrazone (C2759 Sigma) was added to all the wells with live cells. Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP) is a non-specific inhibitor of active efflux in Gram negative bacteria (Webber and Coldham 2010). Fluorescence measurement was continued for another 30 minutes for steady state to reach and the reading at 70^{th} minute was taken as fluorescence without efflux.

The contribution of efflux was measured as –

$$\frac{Fluorescence\ of\ live\ cells\ at\ 70\ minutes - Fluorescence\ of\ live\ cells\ at\ 35\ minutes}{Fluorescence\ of\ live\ cells\ at\ 35\ minutes}$$

Appendix 2.1 Composition of Nutrient broth -

Ingredients	Gms/litre
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50

Final pH (at 25^0C) 7.4 ± 0.2

For slide based technique in section 2.2.6, we added 12 g/L of agar to the above mixture to make Nutrient Agar.

For making NB^{Kan}, 0.05 mg/ml of Kanamycin was added to above mixture after autoclaving.

Component space for fluctuating selection regime described in section 2.1.2 –

Salt (g%) - 1, 2, 3, 4, 4.5, 5

pH - 4.5, 5, 6, 7, 8, 9, 9.5, 10

H_2O_2 (M) – 0, 0.01, 0.012, 0.014

Appendix 2.2 Details of the selection regime faced by F populations for first 30 days

Transfer #	Combination #	pH	Salt (g%)	H_2O_2 (M)
1	46	9.5	2	0
2	54	9.5	4	0
3	31	7	5	0.014
4	64	4.5	5	0
5	31	7	5	0.014
6	30	7	4.5	0.014
7	26	7	1	0.014
8	13	7	2	0.012
9	10	9.5	0.5	0.01
10	68	8	5	0
11	51	10	3	0
12	20	6	0.5	0.012
13	22	8	0.5	0.012
14	15	7	4	0.012
15	55	10	4	0
16	1	7	5	0
17	16	7	4.5	0.012
18	53	5	4	0
19	71	10	5	0
20	54	9.5	4	0
21	8	4.5	0.5	0.01
22	29	7	4	0.014
23	38	9.5	0.5	0.014
24	37	9	0.5	0.014
25	32	4.5	0.5	0.014
26	68	8	5	0

27	15	7	4	0.012
28	49	5	3	0
29	49	5	3	0
30	51	10	3	0

Appendix 2.3 Novel, component, complex, acclimation environments used for fitness assays after fluctuating selection

	Assay Environment	**Concentration**
Novel environments	Cobalt chloride	14.2 mg%
	Zinc sulfate	18.4 mg%
	Ethidium bromide	2.5 mg%
	Norfloxacin	0.004 mg%
Component environments	Salt	5 g%
	Acidic pH	4.5
	Basic pH	10
	Hydrogen peroxide	0.058 M
Complex environments	#51	pH 10 + salt 3g% + 0 H_2O_2
	#54	pH 9.5 + salt 4g% + 0 H_2O_2
	#68	pH 8 + salt 5g% + 0 H_2O_2
	#49	pH 5 + salt 3g% + 0 H_2O_2
	#22	pH 8 + salt 0.5g% + 0.01M H_2O_2
	#13	pH 7 + salt 2g% + 0.01M H_2O_2
Acclimation environments	Cobalt chloride	17.2 mg% **
	Zinc sulfate	18.4 mg% **
	Ethidium bromide	2.5 mg%
	Norfloxacin	0.004 mg%
Fitness at individual level	Cobalt chloride	28.5 mg%
	Zinc sulfate	120 mg%
	Streptomycin	0.0065mg%
	Norfloxacin	0.0032 mg%

****** These are the concentrations used for the growth curve assay for cobalt and zinc. The concentrations for acclimation were slightly lower (14.4 mg% and 16 mg%).

2.4 Selection under deteriorating environment

Environment	Concentrations		
	Day I	**DayVIII**	**Step size**
Cobalt	6 mg%	13 mg%	1 mg%
Zinc	5 mg%	12 mg%	1 mg%
Norfloxacin	0.4 µg%	1.1 µg%	0.1 µg%

Appendix 2.5 – Novel Environments used for estimating fitness at the individual level

Assay Environment	Concentration
Cobalt chloride	28.5 mg%
Zinc sulfate	120 mg%
Streptomycin	0.0065mg%
Norfloxacin	0.0032 mg%

Appendix 2.6 – Effect size computed for the growth rates and yield measured in the three temperature environments from Figure 1 of Ketola, Mikonranta et al. 2013. Means and ANOVA p-values are as reported in that paper.

	Assay Environment	Mean C	Mean F	ANOVA p values	Effect Size±95% CI	Inference
Growth rate	24^0C	0.31	0.34	<0.001	0.43±0.25	Small
	31^0C	0.46	0.51	<0.001	0.53±0.25	Medium
	38^0C	0.37	0.41	<0.001	0.61±0.25	Medium
Yield	24^0C	0.086	0.089	<0.001	0.21±0.25	Small
	31^0C	0.1	0.11	0.478	0.12±0.25	Small
	38^0C	0.075	0.079	<0.001	0.45±0.25	Small

3.1 Protocol for using the Biolog plates

We used GEN III MicroPlate^{TM} along with inoculating fluid A (IF-A) for estimating the phenotypic variation. Both plates and inoculating fluid were stored at 4^0C and thawed at room temperature before use.

A part of glycerol stock was streaked on nutrient agar plate for every replicate population of S and F. The plates were incubated at 37^0C overnight. 8 isolated clones of comparable sizes were selected for every population and inoculated into the separate inoculation fluid tube. The transmittance was in the range of 95% to 98% for the selected clones. 100 µl of this well mixed inoculation fluid was used to inoculate the GEN III plate. The plates were incubated at 37^0C for 24 hours after which they were stored at 4^0C for another day, during which time we measured optical density for all the 48 plates at 590 nm (Cooper and Lenski 2000) using a microplate reader (SynergyHT Biotek, Winooski, VT, USA).

Appendix 3.2 Efflux activity under selection for adaptation to constant environment

Median values for each of the stress variable (Salt 4g%, pH5, pH9.5 and 0.012M H_2O_2) were chosen for the selection in constant environment. *Escherichia coli* (strain NCIM 5547) was revived overnight in Nutrient Broth. This revived culture was used to initiate three replicate populations in each of the selection environments and Nutrient Broth, a total of 15 populations. Culturing conditions and transfer volume was as mentioned in section *2.1.1.* The selection lasted for seven days (i.e ~40 generations), without any extinction events, after which the populations were stored as glycerol stocks. These stocks were then used for efflux measurement as per Appendix 1.4.

Statistical Analysis – The average of the three efflux measurements for each population was used for analyzing all the environments together. The pooled data was analyzed using 1-way ANOVA where Selection (5 levels: Salt, pH5, pH9.5, H_2O_2 and NB) was a fixed factor. For analyzing each stress separately, we performed four separate 2-way mixed model ANOVAs where selection (2 levels: selected and control) and replicates (3 levels, nested in selection) were treated as fixed and random factors respectively.

Results – Efflux did not differ across environments ($F_{4,10}$= 0.577, p = 0.686). Results of individual ANOVA, summarized in the following table, show no difference in the energy dependent efflux of control and any of the selected populations when analyzed separately.

Selection environment	Mean Control	Mean Selected	ANOVA F(1,4)	ANOVA *p* values	Effect Size±95% CI	Inference
Salt	0.648	0.49	1.52	0.285	1.13±0.99	Large
pH 5	0.551	0.507	0.1	0.765	0.29±0.93	Small
pH 9.5	0.59	0.622	0.04	0.851	0.19±0.93	Small

H_2O_2	0.458	0.626	4.74	0.095	1.91±1.11	Large

Note that no Holm-Šidàk correction was done since even the lowest *p*-value was not significant at the 0.05 level.

Appendix 5.1 Details of the ancestral *Escherichia coli* population used for the selection

lacY gene from lac chromosome was deleted in *Escherichia coli* K12 MG1655. Kanamycin resistance gene was inserted in this place creating a non-revertible Kanamycin resistant bacteria. Colonies of this bacterium are white colored on MacConkey's agar as opposed to the red colored colonies as opposed to the red colored colonies produced by other *Escherichia coli*.

Appendix 5.2 Details of all the selection regimes

1. Salt – 5g% of sodium chloride
2. Acidic pH – pH 4.5
3. Basic pH – pH 9
4. Hydrogen peroxide –
5. Component space for fluctuating selection regime –
    a. Salt – 0.5 g%, 2 g%, 3 g%, 3.5 g%, 4 g%, 4.5 g%, 5 g%
    b. pH – 4.5, 5, 7, 8.5, 9
    c. Hydrogen peroxide – 2.4 mM, 2.9 mM, 3.4 mM, 3.9 mM

Selection regime faced by F populations for 100 days

Transfer #	**Combination #**	**Salt g%**	**pH**	**H_2O_2 mM**
1	39	5	7	2.9
2	59	5	7	3.9
3	2	3.5	5	0
4	46	3.5	7	3.4
5	23	5	10	0
6	10	4.5	4.5	0
7	62	0.5	8.5	3.9
8	1	3	5	0
9	19	3	10	0
10	9	4	4.5	0
11	9	4	4.5	0
12	30	0.5	5	2.4
13	7	3	4.5	0
14	39	5	7	2.9
15	45	3	7	3.4
16	32	0.5	8.5	2.4

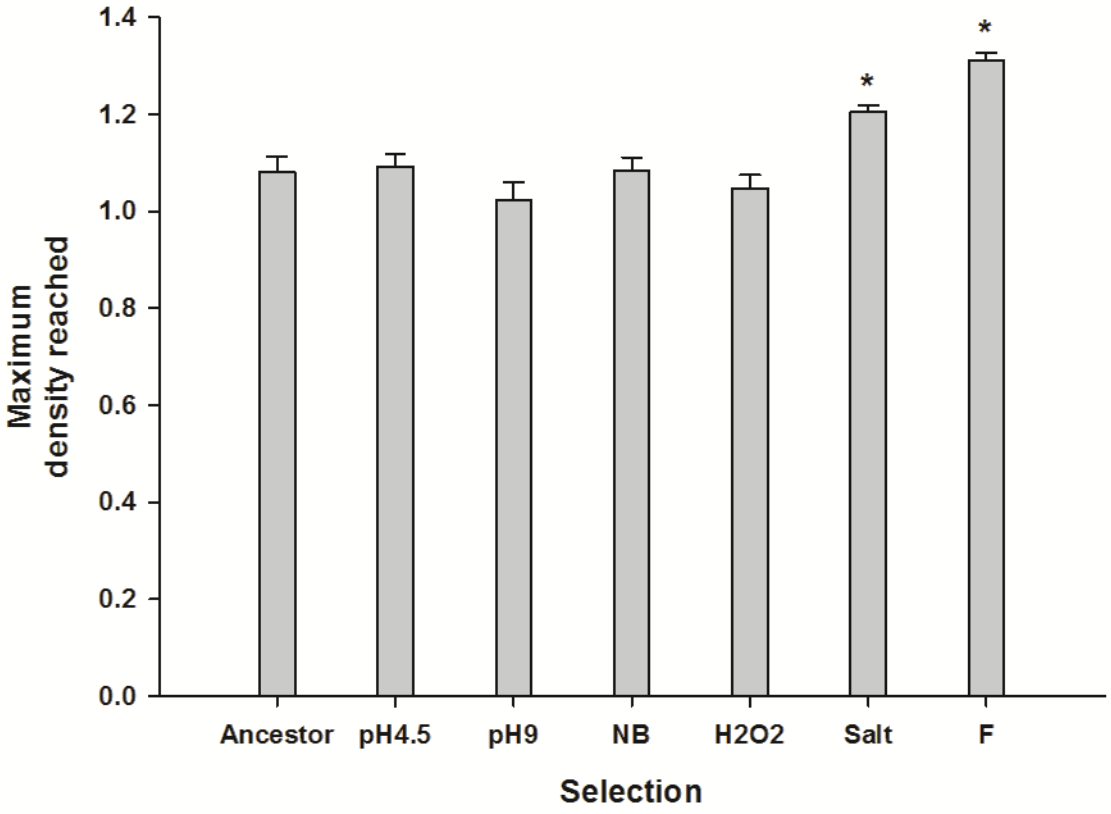
17	44	2	7	3.4
18	58	4.5	7	3.9
19	60	0.5	5	3.9
20	36	3.5	7	2.9
21	9	4	4.5	0
22	45	3	7	3.4
23	25	3	7	2.4
24	12	2	8.5	0
25	8	3.5	4.5	0
26	11	5	4.5	0
27	25	3	7	2.4
28	34	2	7	2.9
29	26	3.5	7	2.4
30	26	3.5	7	2.4
31	53	0.5	9	3.4
32	18	2	9	0
33	55	3	7	3.9
34	43	0.5	9	2.9
35	27	4	7	2.4
36	24	2	7	2.4
37	48	4.5	7	3.4
38	1	3	5	0
39	21	4	9	0
40	14	3.5	8.5	0
41	48	4.5	7	3.4
42	63	0.5	9	3.9
43	30	0.5	5	2.4
44	49	5	7	3.4
45	30	0.5	5	2.4
46	21	4	9	0
47	46	3.5	7	3.4
48	62	0.5	8.5	3.9
49	17	5	8.5	0

50	49	5	7	3.4
51	41	0.5	4.5	2.9
52	63	0.5	9	3.9
53	25	3	7	2.4
54	25	3	7	2.4
55	28	4.5	7	2.4
56	16	4.5	8.5	0
57	46	3.5	7	3.4
58	55	3	7	3.9
59	31	0.5	4.5	2.4
60	3	4	5	0
61	30	0.5	5	2.4
62	5	5	5	0
63	42	0.5	8.5	2.9
64	28	4.5	7	2.4
65	2	3.5	5	0
66	47	4	7	3.4
67	1	3	5	0
68	24	2	7	2.4
69	22	4.5	9	0
70	38	4.5	7	2.9
71	7	3	4.5	0
72	18	2	9	0
73	46	3.5	7	3.4
74	2	3.5	5	0
75	0	2	5	0
76	11	5	4.5	0
77	40	0.5	5	2.9
78	24	2	7	2.4
79	25	3	7	2.4
80	37	4	7	2.9
81	56	3.5	7	3.9
82	21	4	9	0

83	43	0.5	9	2.9
84	62	0.5	8.5	3.9
85	53	0.5	9	3.4
86	4	4.5	5	0
87	63	0.5	9	3.9
88	42	0.5	8.5	2.9
89	62	0.5	8.5	3.9
90	33	0.5	9	2.4
91	48	4.5	7	3.4
92	43	0.5	9	2.9
93	50	0.5	5	3.4
94	11	5	4.5	0
95	8	3.5	4.5	0
96	35	3	7	2.9
97	17	5	8.5	0
98	59	5	7	3.9
99	11	5	4.5	0
100	15	4	8.5	0

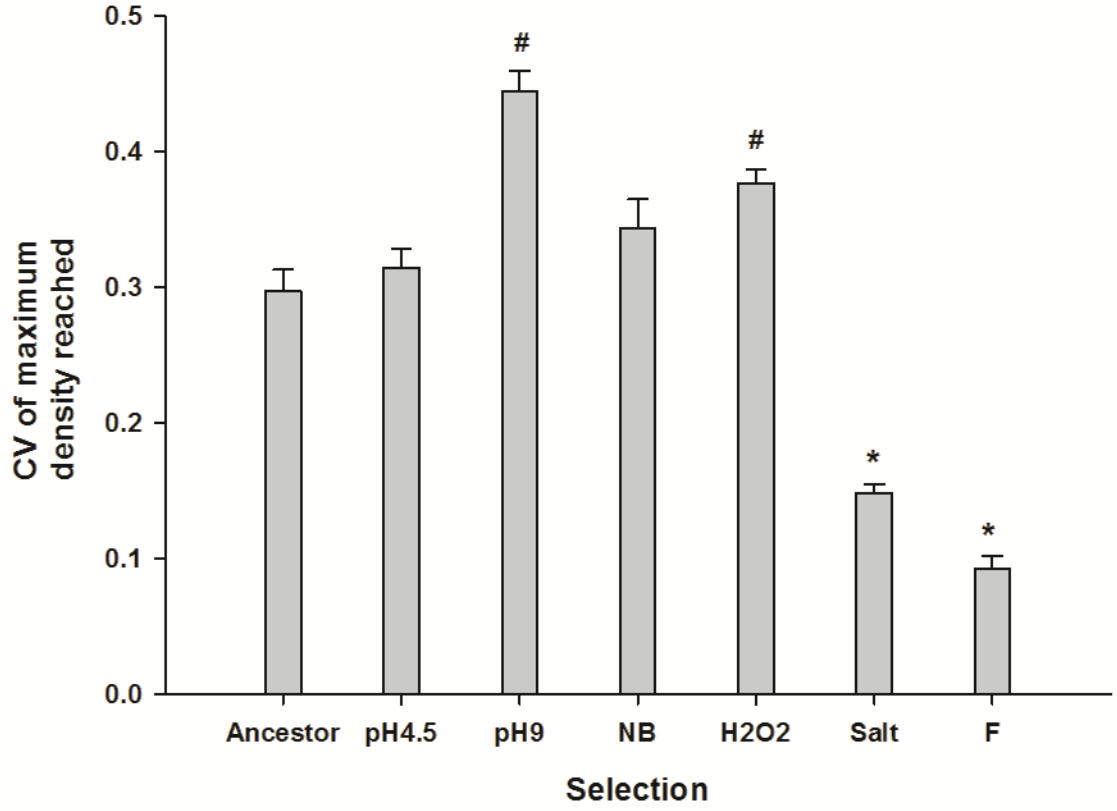
Transfer #5 and #9 comprised of pH 10 which was initially part of the fluctuating component space. Both the environments lead to extinction and hence the component space was modified to the present state.

Appendix 5.3 Overall mean and coefficient of variation for fitness, measured as maximum density reached



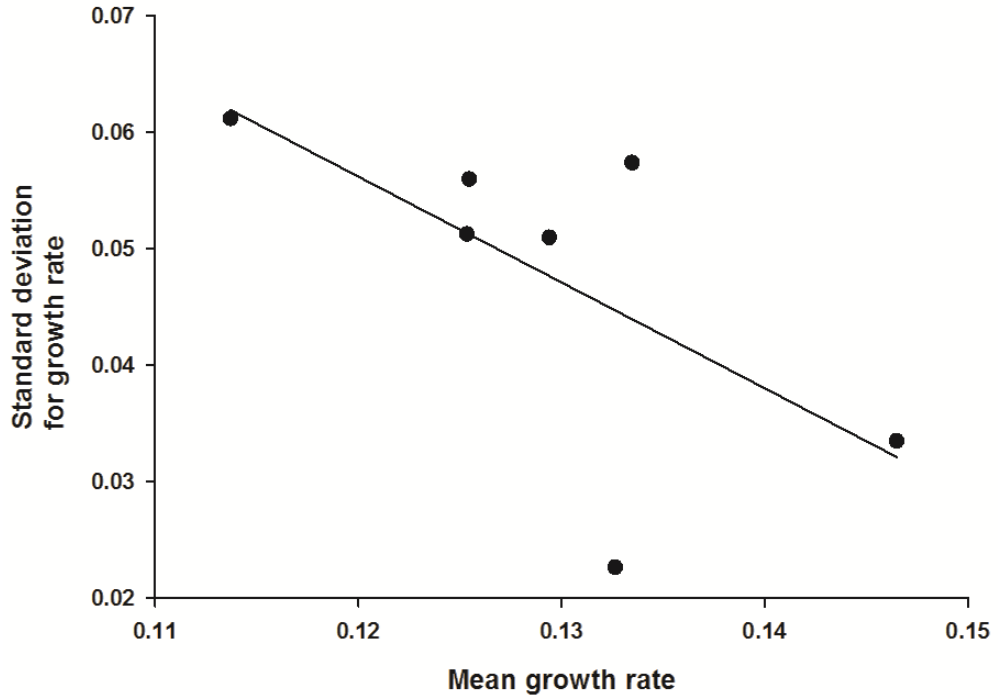
Overall mean fitness (±SE) for all the selection regimes. Overall mean fitness was computed for every selection regime over all assay environments. Fitness estimated as maximum density reached (K) during 24 hours growth period.

* denotes significantly higher overall mean fitness ($p < 0.05$, Dunnett post hoc statistics) than ancestor.



Mean coefficient of variation for fitness (±SE) for all the selection regimes. Coefficient of variation (CV) was computed for every selection regime over all the assay environments. Fitness estimated as maximum density reached in 24 hours growth period. * denotes significantly lower CV ($p < 0.05$, Dunnett post hoc statistics) than ancestor while # denotes significantly higher CV than ancestor.

Appendix 5.4 Relationship between overall mean and standard deviation for fitness, measured as maximum density reached



Relationship between overall means of all the selection regime and corresponding standard deviations. Standard deviation and means were computed for every selection regime over all the assay environments. The relationship between these two estimates is not significant with $R^2 = 0.41$.

Appendix 6.1 Details of all the selection regimes

1. Continuous growth in NB
2. Continuous growth in pH 4.5
3. Continuous growth in 5 g% salt
4. pbin – predictable fluctuations every 24 hr, between pH 4.5 and 5 g% salt
5. upbin - predictable fluctuations every 24 hr, between pH 4.5 and 5 g% salt
6. uprange –

   Component space for selection –

   pH: 4.5, 5

   salt: 2 g%, 3g%, 3.5 g%, 4 g%, 4.5 g%, 5 g%

   Following table shows 30 environments faced in 30 days of selection

Transfer #	Environment
1	pH 4.5
2	Salt 5
3	Salt 3.5
4	pH 4.5
5	pH 4.5
6	pH 4.5
7	pH 4.5
8	pH 4.5
9	Salt 5
10	pH 4.5
11	Salt 4
12	Salt 4
13	Salt 3.5
14	Salt 5
15	pH 4.5
16	Salt 4.5
17	Salt 4
18	Salt 3.5
19	Salt 4
20	pH 4.5
21	Salt 3
22	Salt 3
23	Salt 4
24	Salt 2
25	Salt 3.5
26	Salt 2
27	Salt 4
28	Salt 2

29	Salt 3.5
30	Salt 4.5

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