The Effects of Population Size on Adaptation and Trade-offs: Insights from Experimental Evolution with *Escherichia coli* and Individual-based Models

A thesis

submitted in partial fulfillment of the requirements

of the degree of

Doctor of Philosophy

by

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Dedicated to my family

CERTIFICATE

Certified that the work incorporated in the thesis titled "The Effects of Population Size on Adaptation and Trade-offs: Insights from Experimental Evolution with *Escherichia coli* and Individual-based Models," submitted by Yashraj Chavhan, 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.

Prof. Sutirth Dey

Date: 30/08/2019

(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 the 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.

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Contents

SYNOPSIS

Thesis title: The Effects of Population Size on Adaptation and Trade-offs: Insights from Experimental Evolution with *Escherichia coli* and Individual-based Models

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Chapter 1. Introduction

The size of a biological population determines the relative importance of selection and drift during its evolution (Charlesworth, 2009; Lanfear et al., 2014; Lang et al., 2011; McShea and Brandon, 2010; Rice, 2004). This thesis aims to investigate how population size influences adaptation and maladaptation in asexual systems by modulating the interplay of selection and drift. I begin this introductory chapter with a brief discussion of how population size plays different roles in shaping the adaptation of asexual and sexual populations. Restricting the scope of this thesis to asexual systems, I discuss the importance of population size in influencing a variety of evolutionary aspects including the rate of adaptation, efficacy of natural selection, mutational meltdown, valley crossing, biological complexity, success of mutators, etc. This chapter focuses on the evolutionarily relevant measures of population size, with a particular emphasis on how fluctuating population size shapes the process of adaptation. I also introduce how population size can be relevant in influencing the extent of fitness trade-offs across environments. Highlighting the gaps in the current understanding of these topics, I end this chapter with an outline of the specific questions addressed in the rest of the thesis.

In this thesis, I have used a combination of experimental evolution and individual based simulations to understand how population size affects the adaptive and maladaptive dynamics of asexual systems.

Chapter 2. Larger numbers can impede adaptation in asexual populations despite entailing greater genetic variation

Periodic bottlenecks play a major role in shaping the adaptive dynamics of natural and laboratory populations of asexual microbes (Abel et al., 2015; Kawecki et al., 2012; Lenski et al., 1991; Wahl and Gerrish, 2001). Here I study how they affect the 'Extent of Adaptation' (*EoA*), in such populations. *EoA*, the average fitness gain relative to the ancestor, is the quantity of interest in a large number of microbial experimental-evolution studies which assume that for any given bottleneck size (N_0) and number of generations between bottlenecks (g), the harmonic mean size ($HM=N_0g$) will predict the ensuing evolutionary dynamics (Desai and Fisher, 2007; Lachapelle et al., 2015; Lang et al., 2011; Lenski et al., 1991; Rozen et al., 2008; Samani and Bell, 2010; Vogwill et al., 2016). However, there are no theoretical or empirical validations for *HM* being a good predictor of *EoA*. Using experimental-evolution with *Escherichia coli* and individual-based simulations, I show that *HM* fails to predict *EoA* (i.e., higher N_0g does not lead to higher *EoA*). This is because although higher g allows populations to arrive at superior benefits by entailing increased variation, it also reduces the efficacy of selection, which lowers *EoA*. I show that *EoA* can be maximized in evolution experiments by either maximizing N_0 and/or minimizing g. I also conjecture that N_0/g is a better predictor of *EoA* than N_0g. Our results call for a re-evaluation of the role of population size in predicting fitness trajectories. They also aid in predicting adaptation in asexual populations, which has important evolutionary, epidemiological and economic implications.

This chapter has been published as the following research article:

Chavhan, Y.D., Ali, S.I., and Dey, S. (2019). Larger Numbers Can Impede Adaptation in Asexual Populations despite Entailing Greater Genetic Variation. Evol. Biol. *46*, 1–13.

Chapter 3. Adapting in larger numbers can increase the vulnerability of *Escherichia coli* populations to environmental changes

Larger populations generally adapt faster to their existing environment (Desai and Fisher, 2007; Lanfear et al., 2014; Sniegowski and Gerrish, 2010). However, it is unknown if the population size experienced during evolution influences the ability to face sudden environmental changes. To investigate this issue, I subjected replicate *Escherichia coli* populations of different sizes to experimental evolution in an environment containing a cocktail of three antibiotics. In this

environment, the ability to actively efflux molecules outside the cell is expected to be a major fitness-affecting trait (Morita et al., 1998; Nikaido and Pagès, 2012; Nishino et al., 2009). I found that all the populations eventually reached similar fitness in the antibiotic cocktail despite adapting at different speeds, with the larger populations adapting faster. Surprisingly, whereas efflux activity enhanced in the smaller populations, it decayed in the larger ones. The evolution of efflux activity was largely shaped by pleiotropic responses to selection and not by drift. This demonstrates that quantitative differences in population size can lead to qualitative differences (decay/enhancement) in the fate of a character during adaptation to identical environments. Furthermore, the larger populations showed inferior fitness upon sudden exposure to several alternative stressful environments. These observations provide a novel link between population size and vulnerability to environmental changes. Counter-intuitively, adapting in larger numbers can render bacterial populations more vulnerable to abrupt environmental changes.

This chapter has been published as the following research article:

Chavhan, Y., Karve, S., and Dey, S. (2019). Adapting in larger numbers can increase the vulnerability of *Escherichia coli* populations to environmental changes. Evolution *73*, 836–846.

Chapter 4. Minimal requirements for divergent character fates in populations adapting to the same environment at different sizes

Sign epistasis is expected to be a common feature of natural fitness landscapes (Szendro et al., 2013). Moreover, most genomes are known to have a substantial variation in the lengths of individual genes (Eyre-Walker, 1996; Moriyama and Powell, 1998; Wright, 1990), which entails that some biological characters can have more mutational supply than others. Here I use Wright-Fisher simulations to study the interactive effects of these two phenomena (sign epistasis and differential mutational supply) on the dynamics of evolution in asexual populations of different sizes. Specifically, I investigate the minimal set of conditions that can translate differences in the sizes of asexual populations adapting to the same environment into antagonistic fates of an important fitness-affecting character. Such character divergence was observed in terms of efflux activity in Chapter 3 (Chavhan et al., 2019). I find that the simultaneous presence of sign epistasis and differential mutational supply are essential to obtain divergent evolution of a fitness-affecting character during adaptation to the same environment. Importantly, the removal of any one of these two conditions results in convergent

(and not divergent) character evolution. These results have important implications for understanding how selection makes large and small asexual population take different adaptive paths, particularly in the presence of sign epistasis.

This chapter is being written up as the following research article: '**Chavhan, Y.**, Shah, S., and Dey, S. (2019). Minimal requirements for divergent character fates in populations adapting to the same environment at different sizes (Manuscript under preparation).'

Chapter 5. Larger *Escherichia coli* populations suffer greater fitness trade-offs and undergo more ecological specialization

Evolutionary studies over the last several decades have invoked fitness trade-offs to explain why species prefer some environments to others (Agrawal et al., 2010; Futuyma and Moreno, 1988; Levins, 1962, 1968). However, the effects of population size on trade-offs and ecological specialization remain largely unknown. To complicate matters, trade-offs themselves have been visualized in multiple ways in the literature (Andersson and Hughes, 2010; Bell and Reboud, 1997; Bono et al., 2017; Jessup and Bohannan, 2008; Kassen, 2014; Lee et al., 2009; Schick et al., 2015). Thus, it is not clear how population size can affect the various aspects of trade-offs. To address these issues, I conducted experimental evolution with *Escherichia coli* populations of two different sizes in two nutritionally limited environments and studied fitness trade-offs from three different perspectives. I found that larger populations evolved greater fitness trade-offs, regardless of how trade-offs are conceptualized. Moreover, although larger populations adapted more to their selection conditions, they also became more maladapted to other environments, ultimately paying heavier costs of adaptation. To enhance the generalizability of this study, I further investigated the evolution of ecological specialization across six different environmental pairs and found that larger populations specialized more frequently and evolved consistently steeper reaction norms of fitness. This is the first study to demonstrate a relationship between population size and fitness trade-offs and the results are important in understanding the population genetics of ecological specialization and vulnerability to environmental changes.

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'**Chavhan, Y.**, Malusare, S., and Dey, S. (2019). Larger *Escherichia coli* populations suffer greater fitness trade-offs and undergo more ecological specialization (Under review).'

Chapter 6. An interaction of environmental heterogeneity and population size explains the rarity of detectable fitness costs

Although fitness costs are a fundamental assumption of a large number of models of evolution (Futuyma and Moreno, 1988; Levins, 1962, 1968; Lynch and Gabriel, 1987; Stearns, 1989), several evolutionary studies spanning diverse taxa have failed to detect them (Coustau et al., 2000; Friman and Buckling, 2013; Nidelet and Kaltz, 2007; Rausher, 1984; Vasilakis et al., 2009; Via, 1984). Explaining such rarity of detectable fitness costs has been a major challenge for evolutionary studies over the last two decades (Agrawal et al., 2010; Fry, 1996; Joshi and Thompson, 1995). Chapter 5 showed that when evolution occurs in a homogenous environment, population size plays a key role in shaping fitness trade-offs. Moreover, a recent meta-analysis of microbial experimental evolution studies suggested that environments imposing a single (homogenous) selection pressure frequently lead to fitness costs that can be avoided in heterogeneous environments (which fluctuate across multiple individual selection pressures) (Bono et al., 2017). However, it is unknown if and how population size and environmental heterogeneity interact with each other to shape fitness costs. To investigate this issue, I conducted experimental evolution with *Escherichia coli* populations of two different sizes in heterogenous and homogenous environments and studied the evolution of fitness costs. I demonstrate a previously unreported interplay of population size and environmental heterogeneity that determines the evolutionary emergence (or avoidance) of fitness costs. I show that population size has opposite relationships with fitness costs in homogenous versus heterogenous environments. Interestingly, large population size and environmental heterogeneity led to fitness cost avoidance when present together but not on their own. Moreover, based on these observations, I discuss how fitness costs can be avoided even when most mutations show antagonistic pleiotropy. Finally, I show that heterogenous environment can make larger populations avoid fitness costs despite giving rise to steeper reaction norms of fitness. Taken together, these observations provide a novel explanation for the rarity of fitness detectable costs in evolutionary and ecological studies.

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Chapter 7. Conclusions, implications, and future avenues

Here I discuss the major implications of the observations made in this thesis. I also introduce some possible future extensions of the work presented here.

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Chapter 1

Introduction

Since the origin of life on planet Earth more than 3 billion years ago, the process of organismal evolution has been giving rise to a nearly limitless diversity of life forms. The ultimate source of such diversity resides in the imperfect fidelity in the process of genetic material replication (Futuyma, 2005; Gillespie, 2002; Haldane, 1932), which leads to spontaneous errors (mutations) that are essentially random with respect to their effects on the organisms' ability to survive and reproduce in their immediate environments (McShea and Brandon, 2010). The biological variation thus generated is, in turn, acted upon by random genetic drift on the one hand, and the natural selection on the other. While genetic drift is blind to fitness and thus changes the compositions of biological population randomly (Futuyma, 2005), natural selection causes non-random changes in the population-wide distributions of individuals based on the latter's fitness in the environmental context in question (Rice, 2004). Therefore, the interplay of these two fundamental forces of evolution (drift and selection) is key to understanding how evolution unfolds to give rise to the diversity of life as we know it. One of the parameters known to determine the relative importance of selection and drift is the size of the population (Charlesworth, 2009; Lanfear et al., 2014; Lang et al., 2011; McShea and Brandon, 2010; Rice, 2004). This thesis aims to investigate how population size influences adaptation and maladaptation in asexual systems by modulating the interplay of selection and drift.

In general, whereas drift is more powerful in smaller populations, selection is more effective in larger populations (Desai and Fisher, 2007; Futuyma, 2005; Gillespie, 2002; Ohta, 1992). Consider a population (sexual or asexual) where N individuals can successfully pass on their genetic material to the next generation. In such a population, a mutation of fitness effect s can be categorized into three different classes depending on the whether its evolution is shaped largely by the deterministic force of selection or by random drift (Lanfear et al., 2014): If $0 \approx |s| << 1/N$, the mutation's fate is governed largely by random drift and selection has negligible effect. If $|s| \approx 1/N$, both selection and drift play important roles in shaping the evolutionary fate of the mutation. If $|s| >> 1/N$, the mutation's fate is determined primarily by selection and drift has a relatively minor role.

Interestingly, a *de novo* mutation needs to rise to large enough frequencies in order to escape random genetic drift before selection can start influencing its fate. In haploid populations, the probability that a beneficial mutation of effect s would survive drift is equal to s (Desai et al., 2007; Haldane, 1927). However, once the mutation in question has survived the stochastic effects of drift, sexual and asexual populations pose fundamentally different conditions and

challenges to the mutation's enrichment via natural selection (Rice, 2004). In sexual populations, the eventual fixation (rise to frequency = 1) of a beneficial mutation that has survived drift is not only certain but also independent of the population size (Haldane, 1927; Sniegowski and Gerrish, 2010). On the other hand, in asexual populations, there is no guarantee that a mutation that has survived drift would eventually fix. Importantly, in asexual populations, the fixation probability of a beneficial mutation that has survived drift depends, among other parameters, on the population size (Desai et al., 2007; Sniegowski and Gerrish, 2010). Thus, theory suggests that population size plays different roles in shaping the adaptation of asexual and sexual populations. In this thesis, I study the effects of population size on the microevolutionary processes of adaptation and maladaptation in asexual populations. Here I present a brief review of the extant literature on these topics. I first highlight the gaps in the current understanding in these areas and then present an outline of the specific questions addressed in the subsequent chapters.

In asexual systems, for a given rate of spontaneous mutation, larger populations have access to greater amounts of variation (Cvijović et al., 2018; Neher, 2013; Sniegowski and Gerrish, 2010). Unsurprisingly, therefore, the size of an asexual population is thought to influence a large number of evolutionary processes (Fig. 1.1). For example, larger asexual populations are generally expected to adapt faster (Cvijović et al., 2018; Lanfear et al., 2014; Sniegowski and Gerrish, 2010). This expectation is primarily based on two notions. First, larger populations can not only access more variation, they can also arrive at relatively fitter mutations that may remain inaccessible to smaller populations (Desai et al., 2007; Neher, 2013). This is because only a small fraction of all possible mutations are beneficial in any given environment and among beneficial mutations, those with greater effect sizes are generally rarer (Eyre-Walker and Keightley, 2007; Fisher, 1930; Kassen and Bataillon, 2006). Second, natural selection enriches beneficial mutations and eliminates deleterious ones more effectively in larger populations (Charlesworth, 2009; Rice, 2004). Moreover, since weakly deleterious mutations can become fixed via drift, smaller asexual populations (in which drift is relatively more powerful) are more likely to irreversibly accumulate deleterious mutations (Felsenstein, 1974; Metzger and Eule, 2013; Muller, 1964). Such irreversible accumulation (conventionally known as Muller's ratchet) can potentially drive small asexual populations to extinction (Chipkin et al., 2018; Lynch et al., 1993, 1995). However, larger asexual populations are not always expected to have an evolutionary advantage over smaller ones, particularly if the fitness landscape (the regression of fitness on the space of genotypes) is rugged and not smooth

(Handel and Rozen, 2009; Szendro et al., 2013a; Vahdati and Wagner, 2017). On rugged fitness landscapes, populations often need to cross fitness valleys in order to reach higher fitness peaks (Rozen et al., 2008; Szendro et al., 2013a). Theory predicts that the probability of valley crossing is a non-monotonic function of population size, with both very small and very large populations being likely to cross fitness valleys but intermediate-sized populations likely to be trapped on local fitness peaks (Ochs and Desai, 2015). Moreover, since population size plays a key role in deciding the importance of selection over stochastic drift, it is also a key determinant of the repeatability of evolution (Lachapelle et al., 2015; Szendro et al., 2013b; de Visser and Krug, 2014). The existing theory presents a clear expectation regarding the relationship between population size and the repeatability of evolution: the latter should be maximum at a range of population sizes where $N\mu^2 << 1 << N\mu$ and much lower when $N\mu < 1$ or $N\mu^2 > 1$ (where μ is the rate of spontaneous mutation) (Szendro et al., 2013b; de Visser and Krug, 2014). Interestingly, population size has also been shown to exhibit a similar non-monotonic relationship (albeit within different range of N) with biological complexity (LaBar and Adami, 2016). Finally, the evolutionary success of mutators (genotypes that elevate the rates of spontaneous mutation) is also expected to be non-monotonically dependent on population size. Specifically, when $NU_b ln(Ns) < 1$, (where U_b is the rate of beneficial mutations and s is the selection coefficient) mutator fixation is likelier in larger populations. However, when $NU_b ln(Ns) > 1$, mutators are more likely to spread in smaller populations.

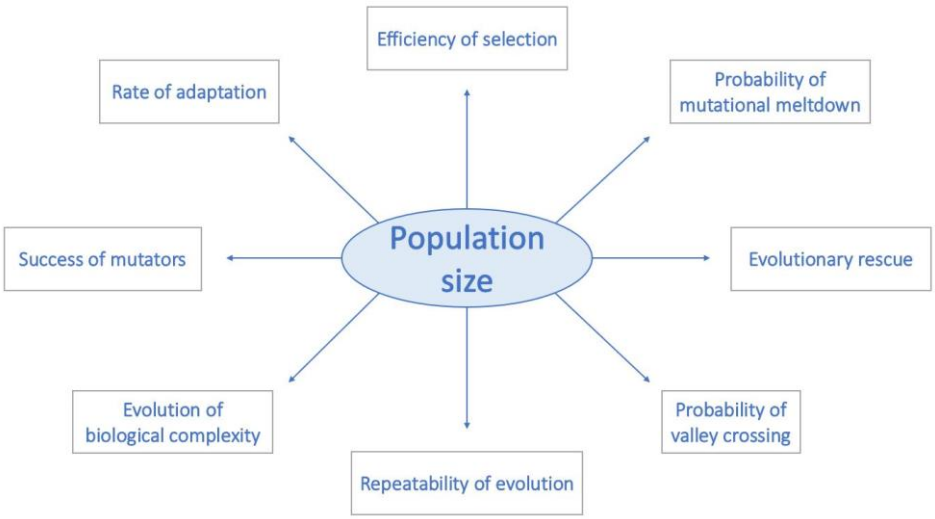

Fig. 1.1. Population size is an important parameter that influences a diverse set of evolutionary processes in asexual systems.

All the discussion regarding population sizes up to this point has not touched upon the following questions: What exactly does the phrase 'population size' refer to? Which measure of population size is appropriate while making statements such as 'larger populations generally adapt faster'? Can there be a common measure of population size that can be relevant in all population-genetic contexts? The next section addresses these issues.

1.1. Evolutionarily relevant measures of population size

The concept of effective population size, which offers a measure of the power of random genetic drift in biological populations (Wright, 1931), aids in answering the above questions. Most models in population genetics and evolution deal with idealized populations that do not fluctuate in size over time and involve the transmission of alleles from one generation to the next via random sampling (Charlesworth, 2009). There are several other ways a population can be idealized, depending upon the assumptions of the model in question (Kimura and Crow, 1963). However, real biological populations mostly depart from idealized populations in several important ways, which is the primary reason behind conceptualizing the idealization. The effective size of a real biological population is the size of an idealized population that would show the same population genetic behaviour as the real population in question (Kimura and Crow, 1963; Rice, 2004). This also means that the effective size of a given biological population is dependent on the population genetic quantity of interest. Indeed, one can define several different effective population sizes for the same biological population, depending upon the population genetic context (Ewens, 1979; Rice, 2004; Whitlock and Barton, 1997). Moreover, these various effective sizes of the same real population represent different values, each conveying a different information about the real population in question (Rice, 2004). In general, lower the effective size of a population, the more strongly it is affected by random genetic drift (Charlesworth, 2009; Lanfear et al., 2014; Rice, 2004).

Most of the theoretical studies discussed up to this point deal with asexual populations whose size (= N) remains constant across generations (Desai and Fisher, 2007; Gerrish and Lenski, 1998; Jain et al., 2011; Ochs and Desai, 2015; Szendro et al., 2013b; Weissman et al., 2009; Wilke, 2004). These theoretical populations are thus themselves idealized in their own way. However, the experiments that test the predictions of such theoretical studies involve asexual populations which experience substantial changes in their sizes over time (Cvijović et al., 2018; Kawecki et al., 2012). When the population in question undergoes changes in its size across

generations while retaining other aspects of an idealized population, its effective size (in terms of the variance in allele frequencies) is equal to the harmonic mean (or $N_e = HM$) of its population sizes over the course of evolution (Charlesworth, 2009; Kimura and Crow, 1963; Rice, 2004). In other words, the amount of drift experienced by the population in question would be the same as that experienced by a constant population of size N_e (= HM).

Interestingly, most experimental evolution studies conducted with asexual microbes employ populations which face a sudden reduction in their sizes (bottlenecks) at regular intervals during their propagation (Garland and Rose, 2010; Kassen, 2014; Kawecki et al., 2012). Specifically, in such populations, periods of growth are punctuated by sudden reductions (bottlenecks) which occur when a small sample (inoculum) from fully-grown populations is introduced into fresh medium in batch culture (or, equivalently, when chemostat tubes are changed) (Wahl and Gerrish, 2001). Furthermore, such periodic bottlenecks are also common in many natural asexual populations, where dissociation from hosts (e.g., during the spread of an infection) cause abrupt reductions in population size, which eventually grows back to larger numbers (reviewed in (Abel et al., 2015)). Thus, to understand how population size affects the extent and dynamics of adaptation in such asexual populations, it is crucial to understand the role played by periodic bottlenecks.

Although the harmonic mean population size (*HM*) has been conventionally considered an appropriate measure of population size in terms of the fate of neutral mutations (Charlesworth, 2009; Kimura, 1983), some theoretical studies posit that *HM* can also predict the probabilities of fixation of individual beneficial mutations of a given size in periodically bottlenecked asexual systems (Patwa and Wahl, 2008; Wahl and Gerrish, 2001). Periodic bottlenecks have two opposing effects on genetic variation in asexual populations. First, harsher (more severe) bottlenecks increase the chances of random loss of variation due to sampling errors. Second, harsher bottlenecks also increase the scope for periodic growth, ultimately entailing increased variation, the very substrate for evolution (Wahl et al., 2002). Theoretical studies have predicted that, when it comes to affecting the probabilities of fixation of beneficial mutations, the second effect (entailing increased variation) should overwhelm the first one (increasing loss of variation to sampling) (Heffernan and Wahl, 2002). More recent and nuanced theoretical studies have also predicted that increasing the harmonic mean size should lead to enhanced adaptation rates in terms of fixation probabilities (Campos and Wahl, 2009, 2010).

Although fixation probabilities are the focal quantity in most theoretical studies, it is not feasible to track them in most evolution experiments, even in relatively simple model systems like asexual microbes (Cvijović et al., 2018; Patwa and Wahl, 2008). As stated in Patwa and Wahl (2008), a decade ago, there was no evolution experiment that had determined fixation probabilities. Instead of fixation probabilities, most evolution experiments are interested in the dynamics of average population-wide fitness, which is an easily tractable quantity (Cvijović et al., 2018; de Visser and Rozen, 2005; Desai et al., 2007; Kassen, 2014; Kawecki et al., 2012; Lachapelle et al., 2015; Lenski et al., 1991; Rozen et al., 2008; Samani and Bell, 2010). Although a recent study determined fixation probabilities in populations of different sizes by conducting whole-genome-whole-population sequencing at several time points during experimental evolution, it also determined the dynamics of average fitness increase (Lang et al., 2013). Curiously, most studies concerned with quantities like average fitness increase in a given time (or the rate of increase in average fitness) also use *HM* as the measure of population size despite the absence of any theoretical or empirical justification to do so (de Visser and Rozen, 2005; Desai et al., 2007; Lachapelle et al., 2015; Lang et al., 2013; Lenski et al., 1991; Rozen et al., 2008; Samani and Bell, 2010). Most asexual microbial populations that are employed in evolution experiments are large enough to undergo clonal interference (a phenomenon where multiple independently arising beneficial mutations compete with each other within an asexual population) (Bataillon et al., 2013; Cvijović et al., 2018; Sniegowski and Gerrish, 2010). In such populations, fixation probabilities decrease with increasing population sizes but the rate of average fitness change increases with increase in population size (Sniegowski and Gerrish, 2010). Therefore, it is not obvious that the measure of population size relevant for predicting fixation probabilities would also predict the rate of average fitness gain. Clearly, direct experimental and/or theoretical tests are required to verify if *HM* can indeed be an appropriate predictor of the speed of average fitness increase. If *HM* cannot predict the trajectories of average fitness, we need to come up with measures of populations size that can do so. This is the topic of Chapter 2 of this thesis.

Up to this point, this chapter has dealt with phenomena and processes that are known to be influenced by population size (Fig. 1.1). In the next section, I introduce two interrelated aspects of evolution that can have potential relationships with population size, namely fitness trade-offs and vulnerability to environmental change. The relationships of these two aspects with population size have received scant attention in the existing literature and are the topics of Chapters 3 to 6.

1.2. The effects of population size on fitness trade-offs and vulnerability to environmental change

When a biological population adapts to a given environment for a certain number of generations, there are no reasons to assume that it would become similarly adapted to other environments that have not been encountered during this period (Anderson et al., 2013; Bono et al., 2017; Cooper, 2014; Kassen, 2002, 2014). As a matter of fact, such a population may even become maladapted in certain environments (Andersson and Hughes, 2010; Bono et al., 2017; Lee et al., 2009). Evolutionary studies since the early 1960s have invoked the concept of fitness trade-offs to explain such incongruities in fitness changes across environments (Agrawal et al., 2010; Futuyma and Moreno, 1988; Levins, 1962, 1968), where it is assumed the jack-of-all-trades is a master-of-none (MacArthur, 1984). Indeed, such trade-offs are a basic assumption in a majority of theoretical studies that deal with multiple environments (Rodríguez-Verdugo et al., 2014). Moreover, fitness trade-offs are the basis of ecological specialization, which explain why species prefer some environments over others (Bono et al., 2017; Fry, 1996; Kassen, 2002). The large number of evolutionary studies dedicated to understanding fitness trade-offs notwithstanding, it still remains largely unknown how such trade-offs are shaped by a key population-genetic parameter like population size. Specifically, no experimental evolution studies of fitness trade-offs have been carried out with population size as a variable treatment, an observation that has been made many times in the literature (Bataillon et al., 2013; Cooper, 2014; Kawecki et al., 2012; Kraemer and Boynton, 2017). The absence of such studies precludes any direct tests of a potential relationship between population size and fitness trade-offs. Furthermore, as described in Chapter 5, fitness trade-offs have themselves been studied using several different perspectives that are not always consistent with each other. This has contributed to the lack of understanding of the population genetics of fitness trade-offs and the resulting ecological specialization.

There are several reasons to expect a significant effect of population size on fitness trade-offs. As described earlier, adaptation in large asexual populations is likely to be driven by rare large-effect beneficial mutations that are not explored by small populations. Interestingly, theoretical studies that link the size of a beneficial mutation to its deleterious pleiotropic effects have a rich history. Fisher was the first to suggest a relationship between the size of a mutation's effect on fitness and its pleiotropic effects (Fisher, 1930). In his geometrical model of adaptation, he

imagined a multidimensional phenotypic space, with each dimension representing an orthogonal aspect of the organism's total phenotype. His model predicted that the chance that a given mutation is beneficial would fall steeply with the extent of its pleiotropy (the number of dimensions it affects). Along somewhat similar lines, Lande constructed a model to investigate the behaviour of beneficial mutations of variable fitness sizes, with the key assumption that large-effect beneficial mutations carry considerable pleiotropic disadvantages, but small effect beneficial mutations carry none (Lande, 1983). Orr and Coyne (1992) described a model where the magnitude of pleiotropic disadvantages carried by beneficial mutations was proportional to the magnitude of their direct (beneficial) effects. Overall, these studies assume that larger beneficial mutations also lead to heavier pleiotropic disadvantages to fitness in environments that are not encountered by populations (Fisher, 1930; Lande, 1983; Orr and Coyne, 1992; Otto, 2004). Combining this assumption with the notion that larger populations adapt primarily via large beneficial mutations, one can expect that larger populations should suffer heavier pleiotropic disadvantages and should evolve bigger fitness trade-offs. However, the existing literature offers no formal theoretical or empirical test of this hypothesis. Moreover, this line of reasoning also predicts that evolving in larger numbers in an unchanging environment for several hundred generations should lead to greater fitness trade-offs and render asexual populations more vulnerable to sudden changes in the environment.

Interestingly, evolution in heterogenous environments can lead to very different fitness trade-offs as compared to evolution in homogenous environments (Kassen, 2002; Bono et al., 2017 and references therein). On the one hand, homogenous environments challenge populations with a uniform (unchanging) selection pressure, making natural selection blind to mutational fitness-effects in other (unexplored) environments, ultimately leading to substantial fitness trade-offs (Duffy et al., 2007; Kassen, 2014). On the other hand, when populations evolve in a heterogeneous (fluctuating) environment, selection is not blind to mutational fitness-effects in the several states faced during the environmental fluctuations (Bono et al., 2013, 2015). Thus, the magnitudes of fitness trade-offs are expected to be lower in heterogenous environments as compared to homogenous ones (Bono et al., 2017). Although it is possible that population size has qualitatively different effects on fitness trade-offs in homogenous and heterogeneous environments, the existing literature does not offer any experimental tests of this idea (Bataillon et al., 2013; Kraemer and Boynton, 2017).

This thesis attempts to fill the aforementioned gaps in the existing understanding of the effects of population size on adaptation and fitness trade-offs in asexual populations. Fig. 1.2 presents an outline of the thesis, highlighting the links between different chapters.

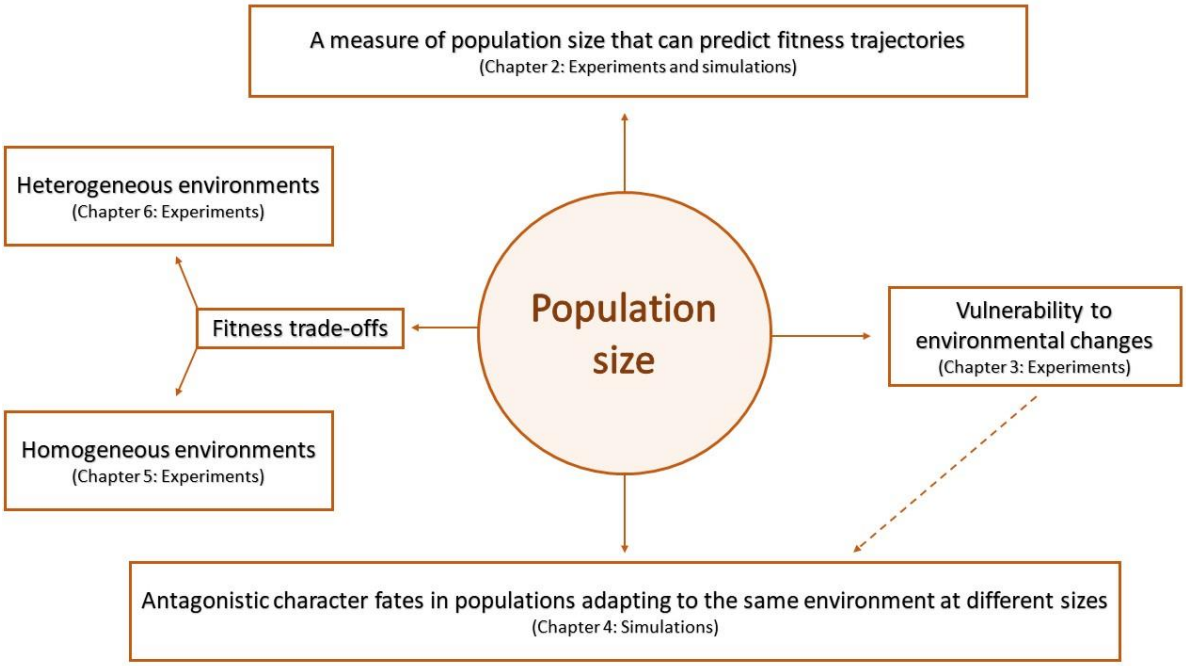

Fig. 1.2. The topics of the different chapters in this thesis and the links between them.

Chapter 2 deals with periodic bottlenecks, which play a major role in shaping the adaptive dynamics of natural and laboratory populations of asexual microbes. It tests how periodic bottlenecks influence the 'Extent of Adaptation' (*EoA*) in such populations. *EoA*, the average fitness gain relative to the ancestor, is the quantity of interest in a large number of microbial experimental evolution studies that assume that for any given bottleneck size (N_0) and number of generations between bottlenecks (g), the harmonic mean size ($HM = N_0 g$) will predict the ensuing evolutionary dynamics. However, there are no theoretical or empirical validations for *HM* being a good predictor of *EoA*. Using experimental-evolution with *Escherichia coli* and individual-based simulations, this chapter shows that *HM* fails to predict *EoA* (i.e., higher $N_0 g$ does not lead to higher *EoA*). This is because although higher g allows populations to arrive at superior benefits by entailing increased variation, it also reduces the efficacy of selection, which lowers *EoA*. The simulations also demonstrate that *EoA* can be maximized in evolution experiments by either maximizing N_0 and/or minimizing g. Finally, this chapter proposes and

demonstrates that N_0/g is a better predictor of *EoA* than $N_0 g$. These results call for a re-evaluation of the role of played by periodic bottlenecks in shaping fitness trajectories of asexual populations. They also aid in predicting adaptation in such populations, which has important evolutionary, epidemiological and economic implications.

Chapter 3 investigates how the population size experienced during evolution influences the ability of asexual systems to face sudden environmental changes. To this end, it follows up on the evolution experiment of Chapter 2, in which replicate *Escherichia coli* populations of different sizes had been subjected to experimental evolution in an environment containing a cocktail of three antibiotics. In this environment, the ability to actively efflux molecules outside the cell is expected to be a major fitness-affecting trait. Surprisingly, we found that whereas efflux activity enhanced in the smaller populations, it decayed in the larger ones. This evolution of efflux activity was largely shaped by pleiotropic responses to selection and not by drift. This demonstrates that quantitative differences in population size can lead to qualitative differences (decay/enhancement) in the fate of a character during adaptation to identical environments. Furthermore, the larger populations showed inferior fitness upon sudden exposure to several alternative stressful environments. These observations provide a novel link between population size and vulnerability to environmental changes. Counter-intuitively, adapting in larger numbers can render bacterial populations more vulnerable to abrupt environmental changes.

Chapter 4 presents generalizable individual-based simulations that aim to replicate an interesting observation made in Chapter 3 (that differences in the sizes of asexual populations adapting to the same environment can translate into antagonistic fates of an important fitness-affecting character). To this end, we used Wright-Fisher simulations to evolve asexual populations on a two-locus three-allele fitness landscape with two key conditions: (1) unidirectional sign epistasis (where the first locus shows sign epistasis on the second locus' background but the fitness-effects of the second locus are independent of the first one) and (2) differential mutational supply across the two loci. Then we relaxed the two conditions, both singly and in combination to arrive at the minimal set of requirements under which a fitness-affecting focal character can evolve divergently in populations of different sizes adapting to the same environment. We find that the simultaneous presence of both conditions (sign epistasis and unidirectional mutational supply) are essential to obtain antagonistic evolution of a fitness-affecting character. Specifically, removal of any one of these two conditions results in convergent (and not antagonistic) character evolution. We also show that it is important to

add another hypostatic locus to the model to match all the aspects of the antagonistic efflux evolution of Chapter 3.

Chapter 5 deals with the effects of population size on trade-offs and ecological specialization in homogeneous environments. Fitness trade-offs have been visualized in multiple ways in the existing literature, and it is not clear how population size affects the various aspects of trade-offs, even in relatively simple organisms like asexual microbes. To address these issues, this chapter presents the results of an evolution experiment conducted with *Escherichia coli* populations of two different sizes in two nutritionally limited environments for ~480 generations. We studied fitness trade-offs from three different perspectives, and found that larger populations evolved greater fitness trade-offs, regardless of how trade-offs are conceptualized. Moreover, although larger populations adapted more to their selection conditions, they also became more maladapted to other environments, ultimately paying heavier costs of adaptation. To enhance the generalizability of our results, we further investigated the evolution of ecological specialization across six different environmental pairs and found that larger populations specialized more frequently and evolved consistently steeper reaction norms of fitness. This is the first study to demonstrate a relationship between population size and fitness trade-offs and the results are important in understanding the population genetics of ecological specialization and vulnerability to environmental changes.

Chapter 6 provides a novel explanation for the rarity of detectable fitness costs in evolutionary and ecological studies. It describes an evolution experiment conducted with *Escherichia coli* populations of two different sizes in both heterogenous and homogenous nutritionally limited environments for several hundred generations. To the best of our knowledge, this chapter provides the first demonstration that population size has opposite relationships with fitness costs in homogenous versus heterogenous environments. On the one hand, in homogenous environments, larger populations evolved greater fitness costs than the smaller ones. On the other hand, in heterogenous environments, smaller populations suffered greater fitness costs than the larger ones, with the latter avoiding fitness costs across all the environmental pairs under consideration. The phenomenon of cost avoidance in our experiments could not be accounted for by any of the conventional explanations for the rarity of costs in the existing literature. Instead, we found that large population size and environmental heterogeneity led to cost avoidance when present together but not on their own. This chapter also shows that,

counterintuitively, evolving in a heterogenous environments in large numbers can lead to cost avoidance even if most mutations show antagonistic pleiotropy in fitness effects across environments. Based on an interplay of the multiplicity of selection pressures and the supply of variation, our results explain why costs are rarely detected in evolutionary studies, particularly when the latter deal with organisms that have a history of evolution in unstable environments in large numbers.

Chapter 7 concludes the thesis with a discussion of the major implications of its observations, while also introducing some possible future extensions of the work presented here.

Chapter 2

Larger numbers can impede adaptation in asexual populations despite entailing greater genetic variation

Highlights

- We studied how periodic population bottlenecks influence the extent of adaptation (*EoA*) in asexual populations.
- Experimental evolution with *Escherichia coli* and individual-based simulations revealed that the conventional measure of population size (harmonic mean (*HM*)) cannot predict fitness trajectories.
- Simulations showed that the *EoA* varies positively with bottleneck size (N_0) but negatively with number of generations (g). This explains why *HM* (= $N_0 g$) fails to predict *EoA*.
- Harsher periodic bottlenecks are double-edged swords—they entail greater variation but reduce the efficacy of selection, ultimately impeding adaptation.
- Our simulations and experiments show that N_0/g predicts fitness trajectories much better than $N_0 g$ and should be used as the measure of population size in evolution experiments that deal with average fitness increase.

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2.1. Introduction

Population size is a key demographic parameter that affects several ecological and evolutionary processes including the rate of adaptation (Desai and Fisher, 2007; Desai et al., 2007; Gerrish and Lenski, 1998; Lanfear et al., 2014; Samani and Bell, 2010; Wilke, 2004), efficacy of selection (Petit and Barbadilla, 2009), organismal complexity (LaBar and Adami, 2016), fitness decline (Katju et al., 2015), repeatability of evolution (Lachapelle et al., 2015; Szendro et al., 2013b; Vogwill et al., 2016), etc. Interestingly though, what constitutes a useful measure of population size for predicting evolutionary outcomes often depends on the ecological/evolutionary question being addressed and the population-genetics quantity in question (Charlesworth, 2009). Consequently, it is crucial to use the relevant measure of population size while constructing or empirically validating any evolutionary theory.

Experimental evolution using asexual microbes has been one of the key tools in validating several tenets of evolutionary theory (Kassen, 2014, reviewed in Kawecki et al., (2012)). Most such studies deal with populations that face regular and periodic bottlenecks during their propagation (Kawecki et al., 2012). The absolute population size keeps changing regularly because of these periodic bottlenecks. Therefore, in order to make predictions and claims based on population size in such experiments, it is important to define a proper measure of population size depending upon the question of interest (Charlesworth, 2009; Kawecki et al., 2012; Lanfear et al., 2014; Wang et al., 2016).

Previous theoretical studies have shown that the harmonic mean of population size over time acts as the measure of population size that can explain and predict the fixation probabilities of beneficial mutations in such systems (Patwa and Wahl, 2008; Wahl and Gerrish, 2001). Specifically, if a population grows from size N_0 to N_f via binary fissions within a growth phase, and is diluted back periodically to N_0 by random sampling at the end of the growth phase, then the relevant population-size measure for fixation probabilities is given by $N_e \approx N_0 * log_2(N_f/N_0) = N_0 g$, where g refers to the number of generations between successive bottlenecks and $N_0 g$ is the harmonic mean size (Lenski et al., 1991). From an evolutionary perspective, periodic bottlenecks play two opposite roles in such experiments. On the one hand, harsher bottlenecks (entailed by higher g) reduce the probability that a given beneficial mutation would fix due to sampling errors during the bottleneck. On the other hand, higher values of g also imply an increase in N_f, which causes an increase in mutational opportunities (binary fissions) during exponential growth (Wahl et al., 2002). This is expected to increase the total supply of

mutations that would survive drift, which in turn should increase the raw material available for evolution. It has been predicted that exponential growth between N_0 and N_f influences fixation probabilities more than the elimination by sampling (Heffernan and Wahl, 2002). More nuanced and complex measures of population size (Campos and Wahl, 2009, 2010) also suggest that adaptation rates in terms of fixation probabilities would have a positive relationship with N_0 and g, the two population size parameters amenable to experimental manipulation.

Unfortunately, most experimental evolution studies with serially bottlenecked asexual populations do not focus on the fixation probabilities of beneficial mutants. Instead, they are interested in the average amount of fitness gained with respect to the ancestor at a given time (we call this quantity the extent of adaptation, *EoA*) during the course of evolution (de Visser and Rozen, 2005; Desai et al., 2007; Lachapelle et al., 2015; Lenski et al., 1991; Rozen et al., 2008; Samani and Bell, 2010). Several experimental studies, dealing with quantities akin to *EoA* for periodically bottlenecked asexual populations, have used the harmonic mean ($= N_0 g$) for quantifying the evolutionarily relevant (i.e., predictive of the magnitude of evolutionary response) population size (de Visser and Rozen, 2005; Desai et al., 2007; Lachapelle et al., 2015; Lenski et al., 1991; Rozen et al., 2008; Samani and Bell, 2010). However, to the best of our knowledge, there is no theoretical basis or empirical justification (Raynes et al., 2014) for this usage of the harmonic mean. Here we use a combination of agent-based simulations and long-term evolutionary experiments using *Escherichia coli* to investigate the interplay of N_0 and g in shaping the *EoA* of asexual populations. Since the harmonic mean has been widely used by experimentalists in the context of *EoA*-like quantities, we begin by testing the suitability of the harmonic mean as a predictor of *EoA*. We show that populations with similar values of $N_0 g$ can have markedly different *EoA* trajectories, and this result applies to both real (bacterial) as well as simulated populations. Secondly, we demonstrate that although increasing the value of g (making the periodic bottleneck harsher) promotes adaptation through an increased supply of variation, it also reduces the efficacy of selection which impedes adaptation by restricting the spread of large-effect beneficial mutations. When these two opposing aspects of bottlenecks are considered together, counterintuitively, *EoA* turns out to have a negative relationship with g. Thirdly, we show that populations with similar harmonic mean ($= N_0 g$) can not only have different fitness trajectories but can also differ markedly in terms of how frequency-distribution of fitness amongst individuals changes during adaptation. Finally, we show that, for a given mutation rate, N_0/g can be a better predictor of *EoA* trajectories, *i.e.*,

populations with similar N_0/g have similar fitness trajectories and populations with higher N_0/g adapt faster. Our findings thus introduce a new way of thinking about the relationship between population size and adaptive trajectories.

Our approach differs from previous studies in two important ways. First, unlike many studies (Campos and Wahl, 2009, 2010; Heffernan and Wahl, 2002; Wahl and Gerrish, 2001) we focus on how *EoA* (and not long-term fixation probabilities) is shaped by bottleneck size (N_0) and bottleneck ratios (N_0/N_f). This makes our study directly relevant to a rich body of microbial experimental evolution literature (de Visser and Rozen, 2005; Desai et al., 2007; Lachapelle et al., 2015; Lenski et al., 1991; Rozen et al., 2008; Samani and Bell, 2010), reviewed in (Kawecki et al., 2012). Second, many previous theoretical studies on periodically bottlenecked systems (where $N_f = N_0 2^g$), assume that the culture volume (and therefore N_f) is a constant, and then go on to explore what value of N_0 or g leads to the minimum loss of variation during bottlenecks and/or in the long run (Campos and Wahl, 2009, 2010; Heffernan and Wahl, 2002; Wahl and Gerrish, 2001; Wahl and Zhu, 2015; Wahl et al., 2002). In our simulations, we remove this restriction and seek to compare loss of variation in those cases where both N_0 and N_f can be different (e.g. between a population grown in 50 ml of medium versus one grown in (say) 1 ml of medium). Clearly, it is possible to have two populations with very different N_0 and N_f values that can nevertheless have similar values of $N_0 g$. One of the questions that we investigate is whether such populations have similar fitness trajectories or not. Thus, our results make it possible to compare the expected *EoA* across experimental studies that employ similar environments but different culture volumes, which is a rather common scenario in experimental evolution studies (Lachapelle et al., 2015; Raynes et al., 2012, 2014; Rozen et al., 2008; Samani and Bell, 2010).

2.2. Methods

Experimental evolution

Our primary aim was to investigate if a commonly used measure of population size in experimental evolution, namely harmonic mean (N_0g), could predict *EoA* trajectories. We also wanted to see if populations with similar values of N_f have similar *EoA*. To this end, we experimentally evolved three different population regimens (LL, SL, and SS) in Nutrient Broth containing a sub-lethal cocktail of three antibiotics (Norfloxacin, Rifampicin and Streptomycin) for ~380 generations in batch culture (see Appendix 1 for more details regarding the culture medium). The first letter in the name refers to the harmonic mean size and the second letter refers to N_f; L means 'large' and S means 'small'. Each regimen consisted of 8 independently evolving replicate populations, all of which were started from a single *Escherichia coli* MG 1655 colony. The three population regimens were propagated at different bottleneck sizes: LL faced lenient bottlenecks (1/10), whereas SS ($1/10^4$) and SL ($1/10^6$) experienced much harsher bottlenecks. LL and SL were grown at larger culture volumes (100 ml, culture in flasks) than SS (1.5 ml, culture in 24 well-plates). Thus, in terms of N_f, LL = SL >> SS but in terms of N_0g LL >> SL = SS (see Table 2.1 for the values of these parameters).

Regime type	Starter population size (N_0)	Final population size (N_f)	Dilution during bottleneck	No. of generations per dilution (g)	Harmonic mean size (HM)	Culture volume
SS	1.5x	15000x	1: 10^4	≈13.28	**~20x**	**1.5ml**
SL	x	10^6x	1:10^6	≈19.93	**~20x**	**100ml**
LL	10^5x	10^6x	1:10	≈3.32	**~3.32*10^5x**	**100ml**

Table 2.1. A summary of the experimental populations. x ≈ 10^5 in our experiments.

Fitness assays

We reconstructed the fitness trajectories of our experimental bacterial populations by measuring bacterial growth using an automated multi-well plate reader (Synergy HT, BIOTEK ® Winooski, VT, USA). We conducted these fitness assays in the same medium (containing

the antibiotic cocktail) that the populations experienced during evolution. We used optical density (OD) at 600 nm as a proxy for bacterial growth. Bacteria from the cryostocks belonging to each of the 24 populations were grown in 96 well plates. Each cryostock-derived population was assayed in three measurement-replicate wells in a 96 well plate. Each well contained 180 µl growth medium (Nutrient Broth with the antibiotic cocktail) containing $1:10^4$ diluted cryostock. The plate was incubated at 37^{O}C and shaken continuously by the plate-reader throughout the growth assay. OD readings taken every 20 minutes during this incubation resulted in sigmoidal growth curves. Fitness measurements were done using cryostocks belonging to multiple time-points in order to reconstruct evolutionary trajectories. While reconstructing fitness trajectories, it was made sure that every 96 well-plate contained populations belonging to similar time-points (in terms of number of generations) during the course of evolution. We used the carrying capacity (*K*) and maximum population-wide growth rate (*R*) as the measure of fitness. *K* of a population was defined as the maximum OD value attained over a period of twenty-four hours (the highest value in the sigmoidal growth curve) (Karve et al., 2016; Novak et al., 2006). *R* was estimated as the maximum slope of the growth curve over a running window of four OD readings (each window spanning one hour) (Karve et al., 2015, 2016; Vogwill et al., 2016). Fitness measurements were done using cryostocks belonging to multiple time-points in order to reconstruct evolutionary trajectories.

Statistical analysis

To analyze the data, we first performed separate repeated measures (RM) ANOVA for each of the two growth parameters (*K* and *R*). "Regimen-type" (SS, SL or LL) was treated as the categorical factor, and TIME (9 time-points) as the repeated measures factor. We also included the interaction of the two factors (Regimen-type and TIME) in the ANOVA model. We did not perform post-hoc tests in the RM ANOVA analysis as it involved very large number of comparisons that were irrelevant for the present question and thus reduced statistical power. We also repeated the above RM analysis with only SL and SS (the regiment-types with the same harmonic mean population size). Furthermore, we analyzed the two growth parameters (*K* and *R*) independently at each time point in the *EoA* trajectory (Fig 2.2) using nested-design ANOVAs with Regimen-type (SS, SL or LL) as the fixed factor and replicate-line (1-8, nested in population-type) as the random factor. For each of these ANOVAs, we further corrected the *p*-value of the main-effect of Regimen-type using the Holm-Šidàk correction (Abdi, 2010) to

control the family-wise error rate. The means from all those ANOVAs which showed a significant (< 0.05) *p*-value after the Holm-Šidàk correction were further subjected to Tukey's HSD to identify which pair-wise differences were significant.

Simulations of microbial evolution

Any difference between the three regimens in our experiment can, in principle, be due to some idiosyncratic properties of the experimental organism (*E. coli*) or potential differences between the selection environments in flasks and plates. In order to account for that possibility and enhance the generalizability of our results, we used an individual based model to simulate bacterial growth under resource-limited conditions (Wahl et al., 2002). Except for differences in the amount of resources, our model contained no other parameters specific to *E. coli* or related to differences in culture conditions. Thus, in terms of differences between the *EoA* of the regimens, if the model output matched the empirical observations then our results were likely to be applicable for other asexual systems. Treating our experiment as a case-study, we used our model to investigate if our results were generalizable.

Our simulations start with a nearly clonal distribution of fitness effects. In our model, an individual bacterium was characterized by three principal parameters: efficiency, threshold, and body-mass. The simulation (coded in the C programming language) began with a fixed amount of resources available in the environment, utilized by the bacteria for growth. A typical individual was represented by an array that specified three principal parameters: (1) Bodymass, (2) Efficiency, and (3) Threshold. Efficiency and Threshold were the only two evolvable parameters. Bacteria consumed resources in an iterative and density-dependent manner. The parameter $Bodymass_i$ of the i^{th} individual represented how big that individual was during a given iteration. Its efficiency (K_eff_i) specified how much food it assimilated per iteration. If $population\ size/\ K\_eff_i < 1$, $10(1 - (population\ size/\ K\_eff_i))$ units were added to $Bodymass_i$. Otherwise, $Bodymass_i$ remained unchanged. $Bodymass_i$ increased with cumulative assimilation. When $Bodymass_i$ becomes greater than or equal to $thres_i$ (its threshold parameter), the individual *i* underwent binary fission and divided into two equally sized daughter individuals. Each fission event had a fixed probability of giving rise to mutations based on a mutation rate that remained constant for all individuals in the population. K_eff_i and $thres_i$ mutate independently and were the only two parameters that could undergo mutation. The mutated value was drawn from a static normal distribution with the frequency of deleterious

mutations being much higher than that of beneficial mutations, which is in line with experimental observations (See Table A3.2 for the distribution parameters (Appendix 3); Kassen and Bataillon, 2006; Eyre-Walker and Keightley, 2007). The distribution of mutational effects remained fixed throughout the simulation (Kassen and Bataillon, 2006) due to which, *EoA* was expected to eventually approach a plateau. When the population ran out of resources (once the amount of body-mass accumulated per unit time by the population went below a pre-decided threshold so that the sigmoidal curve reached a plateau), it was sampled according to the sampling ratio being studied. The above process was repeated for 400 generations, where each generation represented two-fold growth in population size (see Appendix 2 for the algorithm used in the model).

Density-dependent growth, clonal interference, the presence of deleterious mutations, the presence of variable fitness effects of mutations, etc. are some key features that are instrumental in shaping the adaptive dynamics of periodically bottlenecked asexual populations (Patwa and Wahl, 2008; Sniegowski and Gerrish, 2010). Unfortunately, the complex interactions of so many features are difficult to capture in analytical models (Sniegowski and Gerrish, 2010). Consequently, previous theoretical studies have been forced to make simplifying assumptions like the absence of deleterious mutations (Desai and Fisher, 2007; Wahl and Gerrish, 2001), constancy of beneficial mutational effects (Desai and Fisher, 2007), constancy of N_f (Campos and Wahl, 2009, 2010; Wahl and Gerrish, 2001; Wahl and Zhu, 2015), the presence of discrete generations (Campos and Wahl, 2009, 2010; Desai and Fisher, 2007), etc. (see Table A2.1 (Appendix 2) for further details). Our model avoids these simplifying assumptions, which might explain why some of the features captured by our model have not been reported earlier. Moreover, our study is in the context of *EoA*, while most of the earlier studies have investigated fixation probabilities.

2.3. Results

Harmonic mean failed to predict and explain the EoA trajectories of experimental populations

Repeated measures ANOVA on all three regimens indicated a significant Regimen-type × TIME interaction for both *K* ($F_{16, 168}$ = 5.72; $P < 10^{-6}$) and *R* ($F_{16, 168}$ = 7.306; $P < 10^{-6}$). However, in principle, this interaction could be driven by the fact that the LL populations had a much larger increase in *K* and *R* compared to the SL and SS populations. Since our primary interest was to check whether the SL and SS populations differed in terms of these two fitness measures, we performed the repeated measures ANOVA for only these two regimens and again found a significant Regimen-type × TIME interaction for both *K* ($F_{8, 112}$ = 2.070; P = 0.0446) and *R* ($F_{8, 112}$ = 3.594; P = 0.000948). Since the interaction term was significant, we chose not to interpret the main effects of Regimen-Type or TIME.

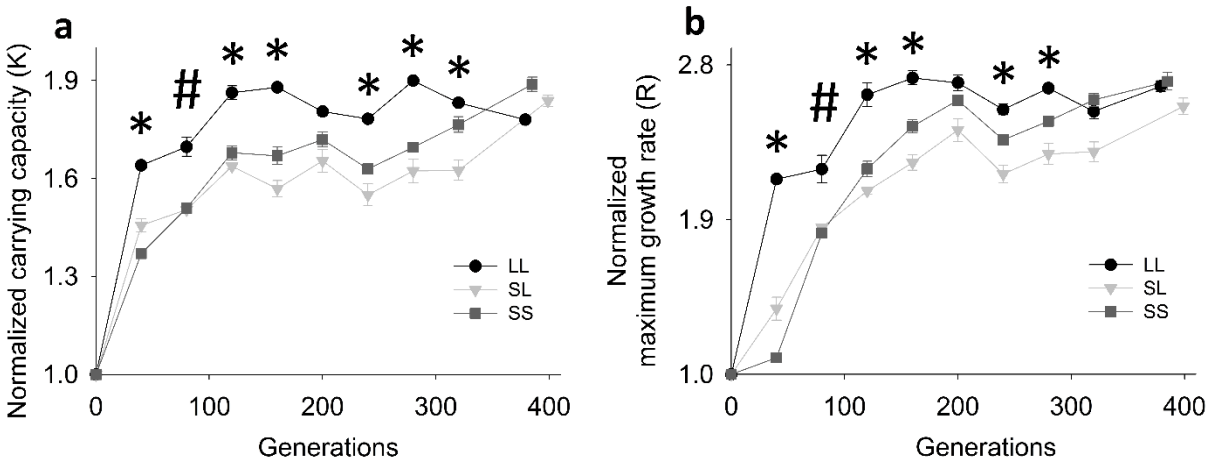

Fig. 2.1. Experimental *EoA* trajectories in terms of carrying capacity and maximum growth rate. (a) *EoA* of carrying capacity *(K)*. (b) *EoA* of maximum growth rate *(R)*. Data points show mean ± SEM for 8 replicates. * refers to cases when all three pairwise differences (LL-SL, LL-SS, and SL-SS) are significant (Tukey post hoc $P < 0.05$). # refers to significant difference across LL-SL and LL-SS, but not SL-SS (See Tables A4.1 and A4.2 (Appendix 4)). SS and SL have markedly different adaptive trajectories despite having similar harmonic mean population sizes.

Individual ANOVAs showed that the *EoA* of SS was greater than that of SL at 5/6 and 4/5 time-points which had significant difference in terms of *K* (Fig. 2.1a) and *R* (Fig. 2.1b). The *p*-values and the *F*-values (with corresponding *df*) for each time-point for *K* and *R* are presented in Tables A4.1 and A4.2 respectively (see Appendix 4). Thus, particularly during the last two thirds of the evolution experiment, the *EoA* of SS was consistently higher than that of SL. The

effect sizes (Cohen's *d* (Cohen, 1988)) of *EoA* differences between SL and SS were found to be either medium or large (with the majority being large effects; see Table 2.2) for several points on the *EoA* trajectory. Thus, similar harmonic mean can give rise to fairly different adaptive trajectories. This observation is consistent with recent empirical findings that question the validity of harmonic mean as an evolutionarily relevant population size (Raynes et al., 2014). Surprisingly, SS had a larger overall *EoA* than SL despite having lower N_f. Interestingly, despite having similar N_f, LL typically had much larger extent of adaptation than SL, which is explainable by the fact that the latter regimen suffered more severe bottlenecks. This shows that similar N_f does not lead to similar extents of adaptation if the bottleneck ratios are different.

In summary, the harmonic mean failed to predict the adaptive trajectories of our experimental populations as, in spite of having similar values of N_0g, the SL and SS regimens had markedly different adaptive trajectories for *K* (Fig. 2.1a) as well as *R* (Fig. 2.1b).

Fitness measure	**Generation**	**Cohen's *d***	**Inference about Effect Size**
K	40	1.112	Large effect
	120	0.527	Medium effect
	160	0.786	Medium effect
	240	0.606	Medium effect
	280	0.514	Medium effect
	320	1.000	Large effect
R	40	1.172	Large effect
	120	0.770	Medium effect
	160	1.024	Large effect
	240	1.037	Large effect
	280	0.836	Large effect

Table 2.2. Analysis of the differences in *EoA* of populations with similar *HM* in terms of effect sizes. Cohen's d (Cohen, 1988) was used to determine the effect sizes of the differences in the *EoA* of SS and SL. $0.2 < d < 0.5$ was interpreted as small effect, $0.5 < d < 0.8$ as medium effect, and $d > 0.8$ as large effect. The majority of differences between SL and SS were found to be of large effect size. The analysis was performed only at time points when the post-hoc (Tukey) *P* – values corresponding to SL-SS were < 0.05 (see Appendix 4).

Our simulations matched the empirical fitness trajectories in numerically similar populations

We found that the results of our experiments and simulations agree well in terms of the range and dynamics of adaptation over identical time-scales in numerically similar populations (Fig. 2.2). This was true for both the measures of population-level fitness: carrying capacity *(K)* (Fig. 2.2a) and maximum growth rate *(R)* (Fig. 2.2b).

We also checked if our simulations met other well-established theoretical expectations from the extant literature that had not been coded directly. As expected, despite following the same distribution for mutations, large populations showed smooth curves of fitness increase while adapting, whereas very small populations showed stepwise increase in fitness with long periods of stasis (Fig. 2.3). Theory expects this because very small populations (but not large ones) need to wait for beneficial mutations to arise (Sniegowski and Gerrish, 2010). Furthermore, the trajectories of fitness increase are expected to show curves of diminishing returns (Chou et al., 2011; Kassen, 2014; Lenski and Travisano, 1994). Indeed, we found such trajectories throughout our experiments and simulations.

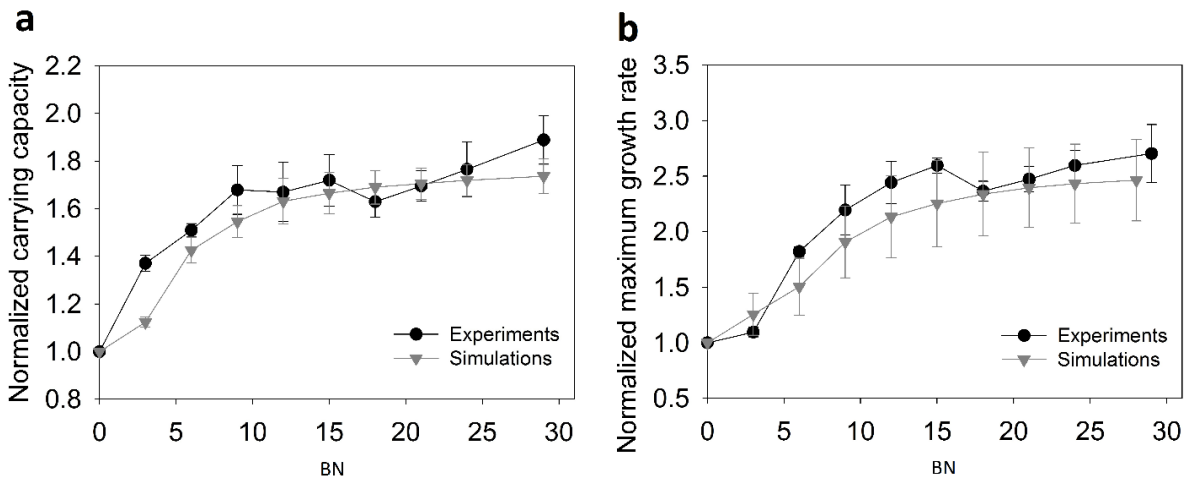

Fig. 2.2. Agreement between experiments and simulations in terms of adaptive dynamics over identical timescales in numerically similar populations. (a) Carrying capacity *(K)* versus bottleneck number *(BN)* (b) Maximum growth rate *(R)* versus bottleneck number *(BN)*. Data points represent mean ± SD over 8 replicates. Each data point corresponds to the respective measure of fitness *(K or R)* derived from the sample taken after *BN* bottlenecks. Range of population size: $N_0 \approx 10^{4.5}$; $N_f \approx 10^{8.5}$; bottleneck ratio = $1/10^4$. Each bottleneck corresponds to approximately 13.28 generations. Both carrying capacity and maximum growth rate are normalized with the ancestral values.

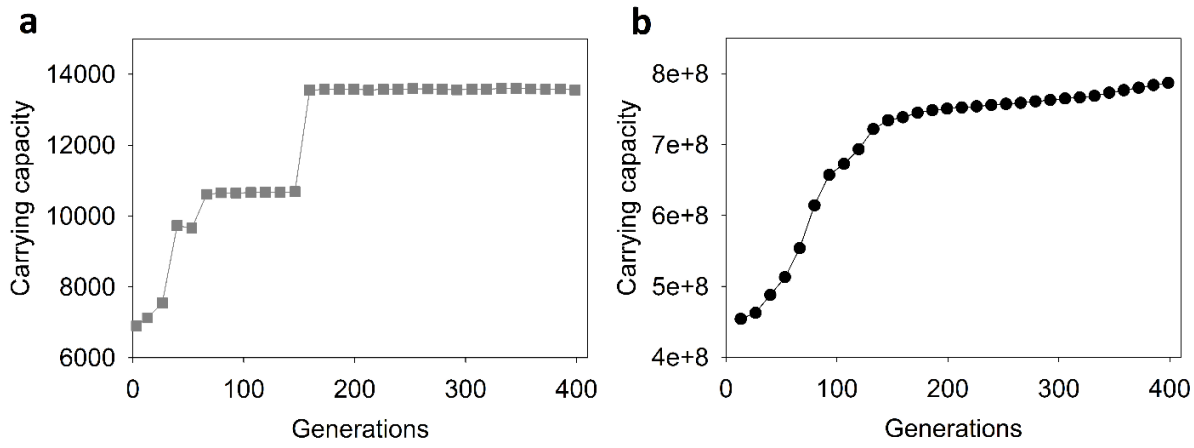

Fig. 2.3. Qualitative differences in adaptive trajectories corresponding to populations with a large different in their sizes. Stepwise increase in fitness (with long periods of stasis) occurred in typically small populations such as the one shown in (a) as compared to smooth curves of diminishing returns in typically large populations such as the one shown in (b) (See the ordinates for absolute ranges of N_f during adaptation). The population shown in (a) experienced a periodic bottleneck of 1/10 while the population shown in (b) was bottlenecked $1/10^4$ periodically.

We also found that the standard deviation of fitness parameters in our simulations did not increase when the sample size was increased from 8 to 20 (Fig. 2.4). Since most of our simulations deal with millions of individual-based parameters they take a very long time to run. Therefore, we decided to operate on a sample size of 8 replicates per population type throughout our study. This also matched the sample size of our experimental regimens.

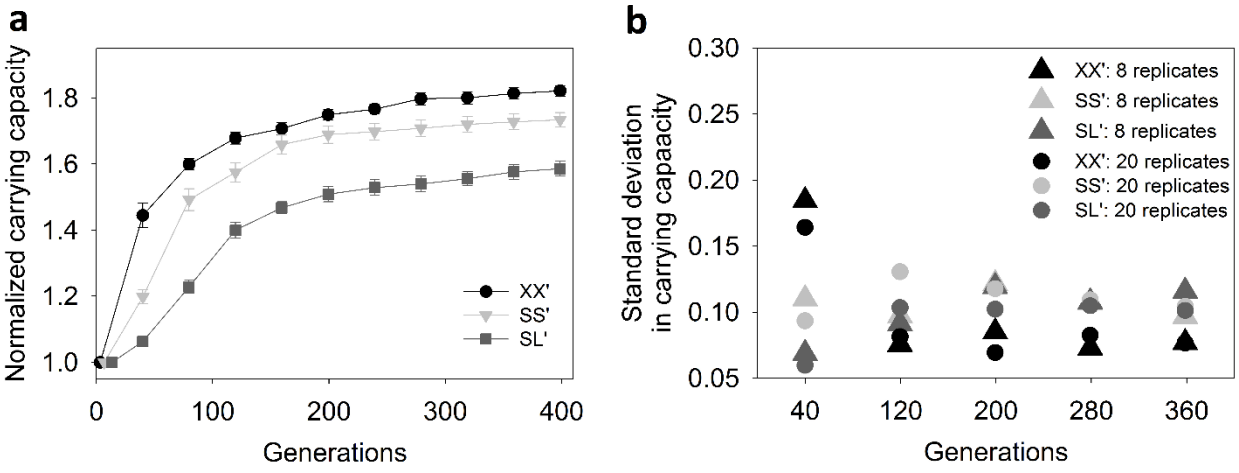

Fig. 2.4. Increasing the number of replicate simulations from 8 to 20 did not result in increase in variation across replicates. (a) Increasing the number of independent replicate simulations from 8 to 20 didn't result in qualitative changes (ranks) of three populations with similar harmonic mean size but different N_0/g (mean ± SEM; N=20) (b) This increase in replicate number also didn't result in major changes in the standard deviation in carrying capacity during the course of adaptation. XX': $N_0 \approx 3.6*10^3$, bottleneck ratio: 1/10; SS': $N_0 \approx 1.8*10^3$, bottleneck ratio: $1/10^2$; SL': $N_0 \approx 9*10^2$, bottleneck ratio: $1/10^4$.

Simulations also revealed that the harmonic mean fails to predict adaptive trajectories

We simulated evolution in populations with identical harmonic mean sizes but with different values of N_0 and g, such that the product (N_0g) remained constant. If the harmonic mean (= N_0g) were a good predictor of how much a population is expected to adapt, then these treatments were expected to show similar *EoA*. This was not found to be the case for both *K* (Fig. 2.5a) and *R* (Fig. 2.5b), which was consistent with our experimental observations of *EoA* trends in SL and SS (Fig. 2.1). The simulated populations with identical harmonic mean sizes (XX', SS', and SL') were also found to be remarkably different in terms of the adaptive increase in average efficiency of individuals (Fig. 2.6a). Thus, multiple measures of fitness in our study revealed that harmonic mean is not a good predictor of adaptive trajectories because populations with similar harmonic mean size can have markedly different adaptive trajectories.

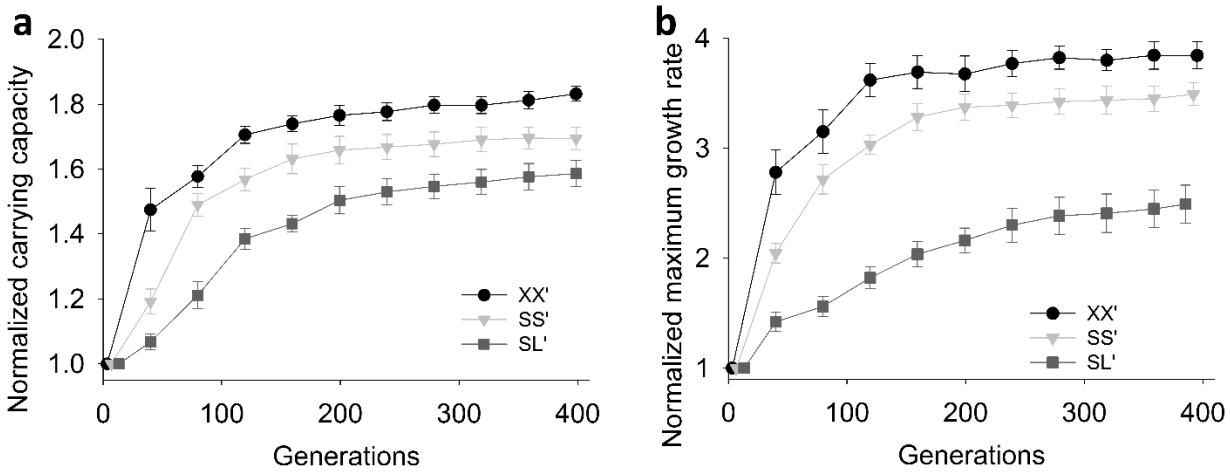

Fig. 2.5. Simulations: Adaption in three population types with similar harmonic mean size. Data points show mean *EoA* ± SEM for 8 replicates. (a) Adaptation in terms of normalized carrying capacity *(K)*. (b) Adaptation in terms of normalized maximum growth rate *(R)*. XX', SS' and SL' had similar harmonic mean sizes and represent lenient, medium and harsh bottlenecks with $N_0 \approx 3.6\times10^3$, 1.8×10^3, 9×10^2 and bottleneck ratio of 1/10; $1/10^2$,$1/10^4$ respectively. These simulations suggest that populations with similar harmonic mean size can have markedly different *EoA* trajectories.

In terms of fitness at the level of individuals, efficiency showed the same trend as *R* and *K* (Fig. 2.6a). However, the adaptive trajectories corresponding to XX', SS', and SL' were almost identical when expressed in terms of threshold (Fig. 2.6b). Threshold evolved (decreased) so quickly and to such a large extent in almost all population types we simulated in this study that most populations had similar trajectories of threshold decrease, regardless of their population size parameters (Fig. 2.7). Consequently, despite threshold being an important determinant of

fitness, adaptive differences amongst populations were best expressed and explained in terms of trajectories of increase in efficiency and not in terms of decrease in threshold. Therefore, we focussed on population-wide trait distributions only in terms of efficiency.

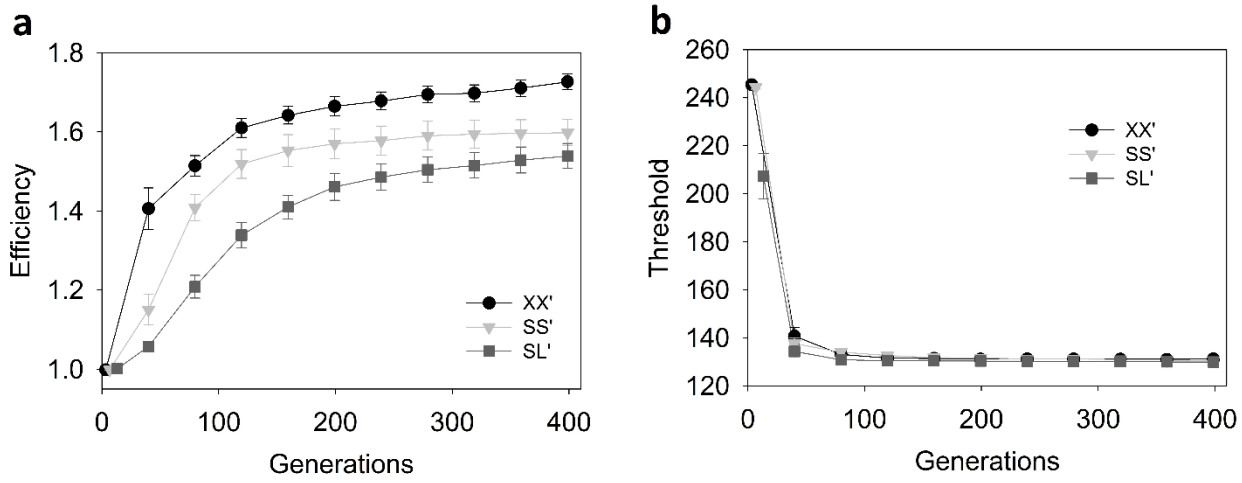

Fig. 2.6. Adaptation in three populations with similar *HM* in terms of measures of fitness at the level of individuals (a) Adaptive increase in averge individual efficiency within populations with similar harmonic mean size (mean ± SEM; 8 replicates). (b) Adaptive decrease in average individual threshold in populations with similar harmonic mean size (mean ± SEM; 8 replicates). Threshold evolved so quickly that its adaptive decrease did not refelct the differnce observed in *EoA* trjectories for *K* and *R* (Fig. 2.5). XX': $N_0 \approx 3.6*10^3$, bottleneck ratio: 1/10; SS': $N_0 \approx 1.8*10^3$, bottleneck ratio: $1/10^2$; SL': $N_0 \approx 9*10^2$, bottleneck ratio: $1/10^4$.

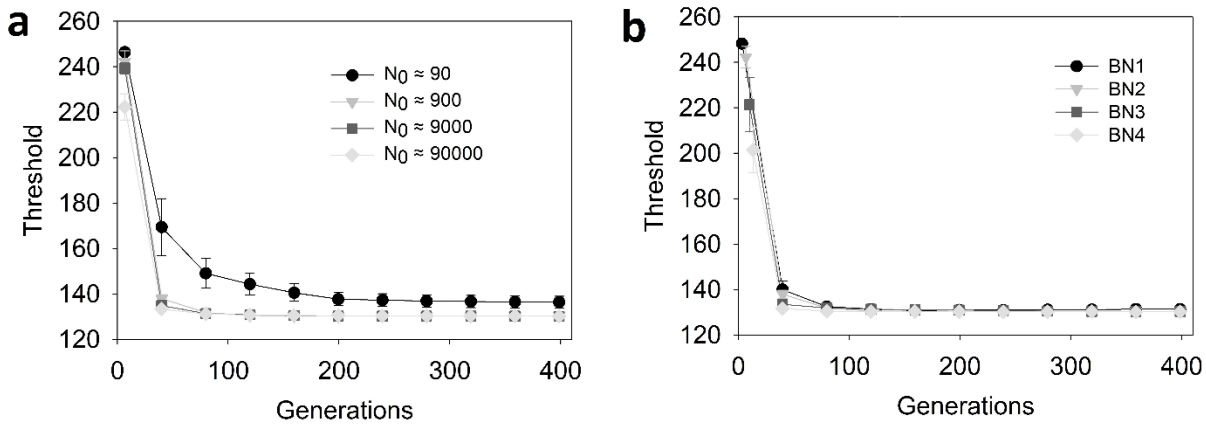

Fig. 2.7. Rapid and convergent reduction in threshold. Threshold evolved (decreased) so quickly and convergently in most populations that we simulated that the effects The data points show mean ± SEM (N=8). The populations shown in (a) were bottlenecked $1/10^2$ periodically. The populations shown in (b) had $N_0 \approx 900$. Bottleneck ratios: BN1: 1/10; BN2: $1/10^2$; BN3: $1/10^3$; BN4:$1/10^4$.

Interestingly, populations with similar harmonic mean were also found to differ in terms of the frequency distributions of the efficiency parameters amongst their constituent individuals (Fig. 2.8). To determine why $N_0 g$ could not explain *EoA* trajectories, we determined how *EoA* varied with N_0 and g, independently.

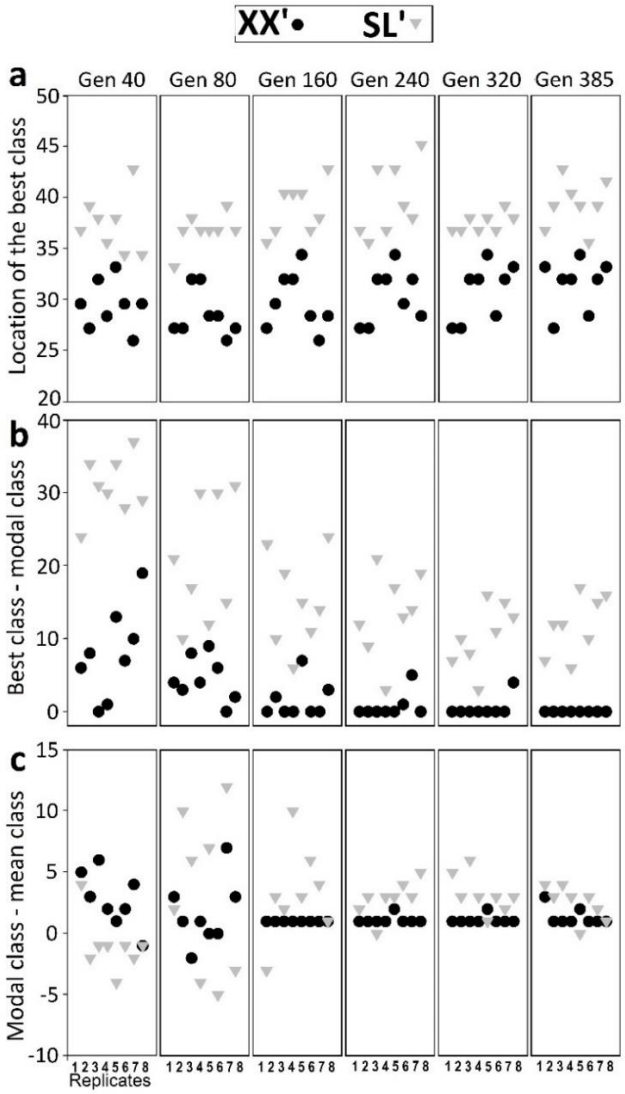

Fig. 2.8. The distributions of efficiency across constituent individuals during adaptation in populations with similar HM. The individuals of each simulated population (8 replicate populations each of XX' and SL') were classified into to a discrete frequency distribution of their efficiency values (50 static classes) just prior to the bottleneck. Higher class indices correspond to higher efficiencies. The best phenotype (in terms of fitness) explored by SL' was consistently fitter than the best phenotype explored by XX' (a). The modal phenotype quickly converged with the best available phenotype in most XX' populations but failed to do so in all SL' populations (b). The mean phenotype in XX' approached the best phenotype very closely (b and c). However, there was a consistently larger gap between the best phenotype and the modal phenotype in SL' (b) and an even larger one between its best and mean phenotype (b and c). Our simulations revealed that populations with similar harmonic mean size can differ appreciably from each other not only in terms of their adaptive trajectories but also in terms of how the distribution of fitness amongst their constituent individuals changes during adaptation.

***EoA* varied positively with N_0 but negatively with *g*.**

If $N_0 g$ were a good measure of the population size that has a positive relationship with *EoA*, then increasing either N_0 or *g* or both should lead to greater *EoA*. We tested this intuitive prediction via simulations using several combinations of N_0 and *g*, spanning four orders of magnitude for both N_0 and the sampling ratio (N_0/N_f). Although *EoA* was found to increase with greater N_0 (Fig. 2.9a; Fig. 2.10), the relationship between *EoA* and *g* turned out to be negative, which was reflected in terms of both individual-level (Fig. 2.9b (in terms of efficiency)) and population-level (Fig. 2.11 (in terms of *R*)) fitness parameters. This implied that larger values of N_f impeded adaptation in populations when the population size during the bottleneck (N_0) was held constant.

Importantly, the negative dependence of *EoA* on *g* was robust to changes in mutation rate (μ) over a 100-fold range in our simulations (Fig. 2.13). We also found that *EoA* exhibited a non-monotonous relationship with μ in both BN1 and BN4 populations, which is in line with theoretical expectations (Orr, 2000). Thus, the relationship between *EoA* and μ can be influenced by bottleneck ratio, which is in agreement with recent empirical findings (Raynes and Sniegowski, 2014; Raynes et al., 2014).

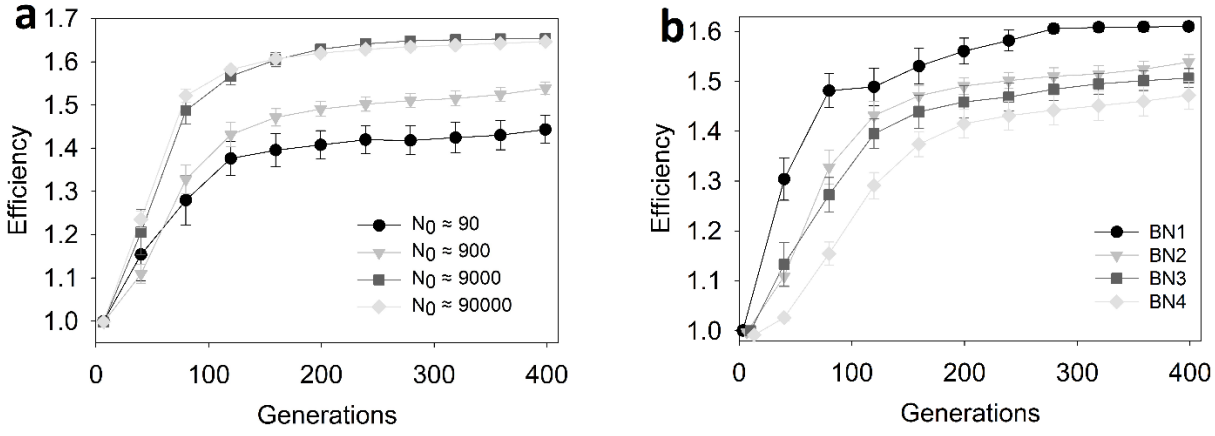

Fig. 2.9. Simulations: The relationship of *EoA* (expressed in terms of efficiency) with N_0 and *g*. Data points show mean ± SEM; 8 replicates. The populations shown in (a) had the same bottleneck ratio ($1/10^2$) but different bottleneck sizes (= N_0). *EoA* varies positively with N_0. On the other hand, the populations shown in (b) had identical bottleneck size (= N_0) but different bottleneck ratios (reflected by different values of *g*. Bottleneck ratios: BN1: 1/10 (g = 3.32); BN2: $1/10^2$ (g = 6.64); BN3: $1/10^3$ (g = 9.96); BN4:$1/10^4$ (g = 13.28). *EoA* varies negatively with *g*. Also see Fig. 2.10, 2.11 and 2.12.

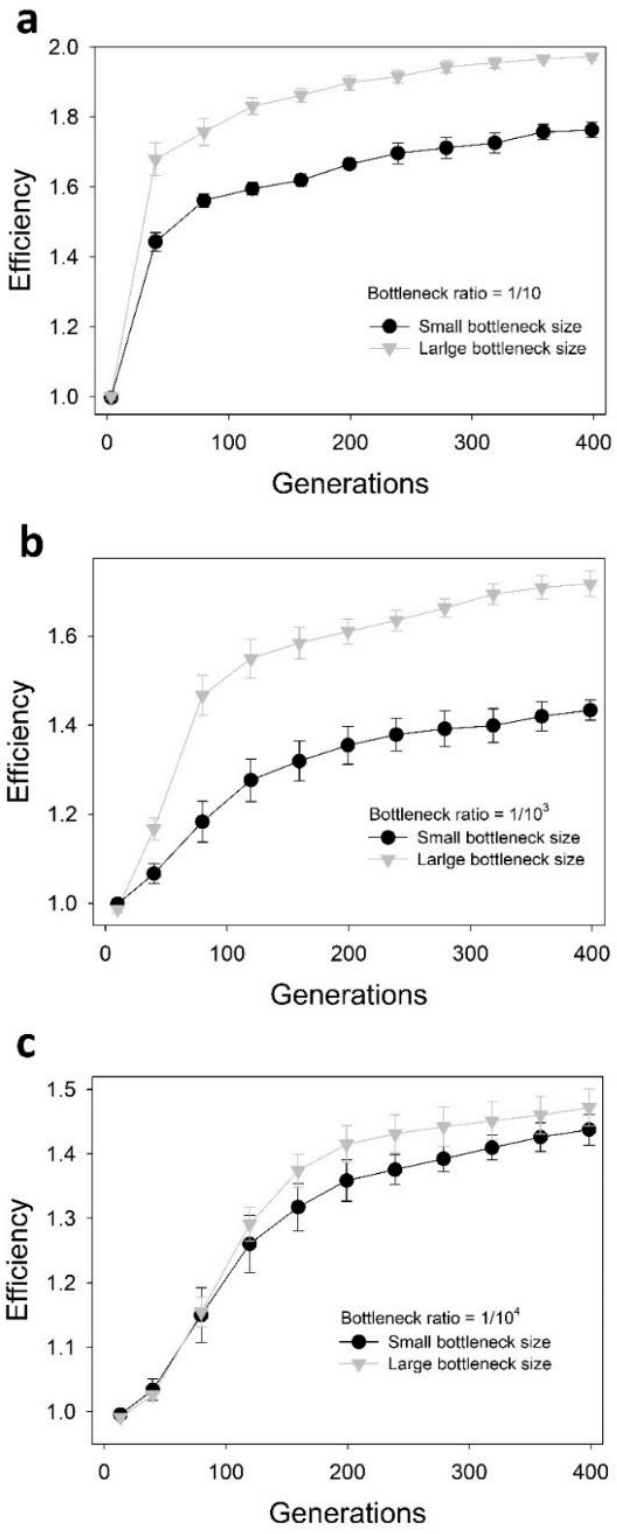

Fig. 2.10. Simulations: *EoA* trajectories of populations with similar bottleneck ratio but different bottleneck sizes (N_0). Data points show mean ± SEM; 8 replicates. (a) Bottleneck ratio = 1/10; Small bottleneck size (N_0) ≈ 9000; Large bottleneck size (N_0) ≈ 900000. (b) Bottleneck ratio = $1/10^3$; Small bottleneck size (N_0) ≈ 90; Large bottleneck size (N_0) ≈ 9000. (c) Bottleneck ratio = $1/10^4$; Small bottleneck size (N_0) ≈ 90; Large bottleneck size (N_0) ≈ 900.

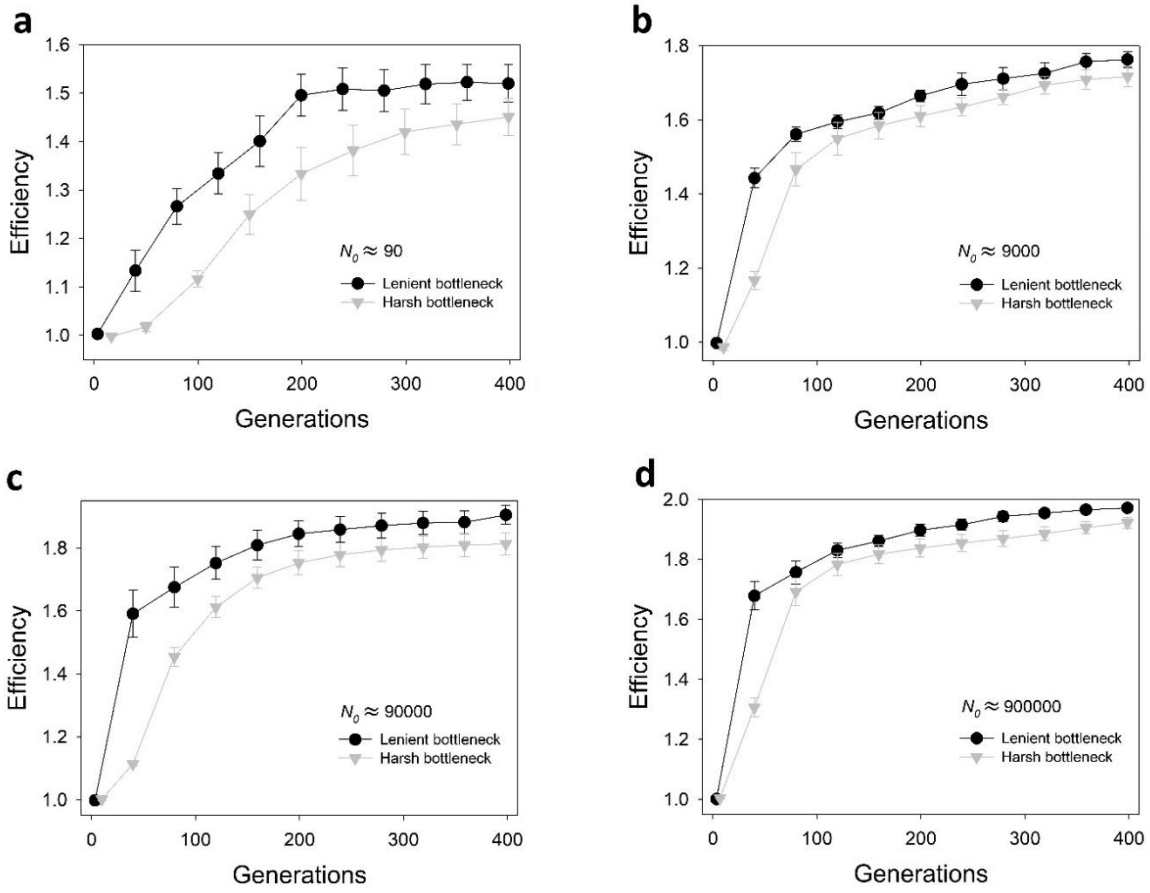

Fig. 2.11. Simulations: *EoA* trajectories of populations with similar bottleneck size (N_0) but different bottleneck ratios. Data points show mean ± SEM; 8 replicates. (a) $N_0 \approx 90$; Lenient bottleneck = 1/10 (g = 3.32); Harsh bottleneck = $1/10^5$ (g = 16.61) (b) $N_0 \approx 9000$; Lenient bottleneck = 1/10; Harsh bottleneck = $1/10^3$ (c) $N_0 \approx 90000$; Lenient bottleneck = 1/10; Harsh bottleneck = $1/10^3$ (d) $N_0 \approx 900000$; Lenient bottleneck = 1/10; Harsh bottleneck = $1/10^2$.

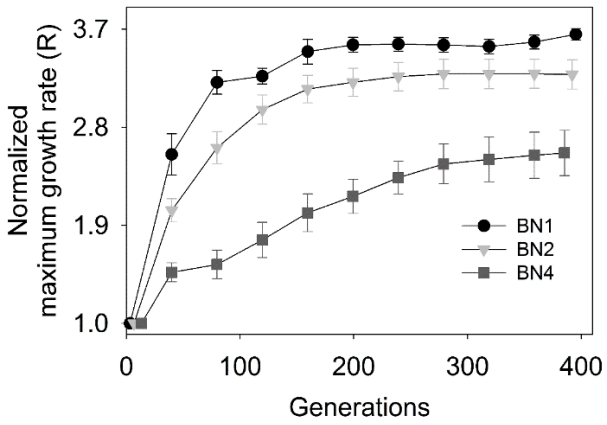

Fig. 2.12. Simulations: *EoA* trajectories (in terms of *R*) of populations with similar bottleneck size (N_0) but different bottleneck ratios. Data points show mean ± SEM; 8 replicates. All the population regimens shown here had $N_0 \approx 900$. Bottleneck ratios: BN1: 1/10; BN2: $1/10^2$; BN4:$1/10^4$. Larger values of g lead to reduced *EoA* for a given number of generations.

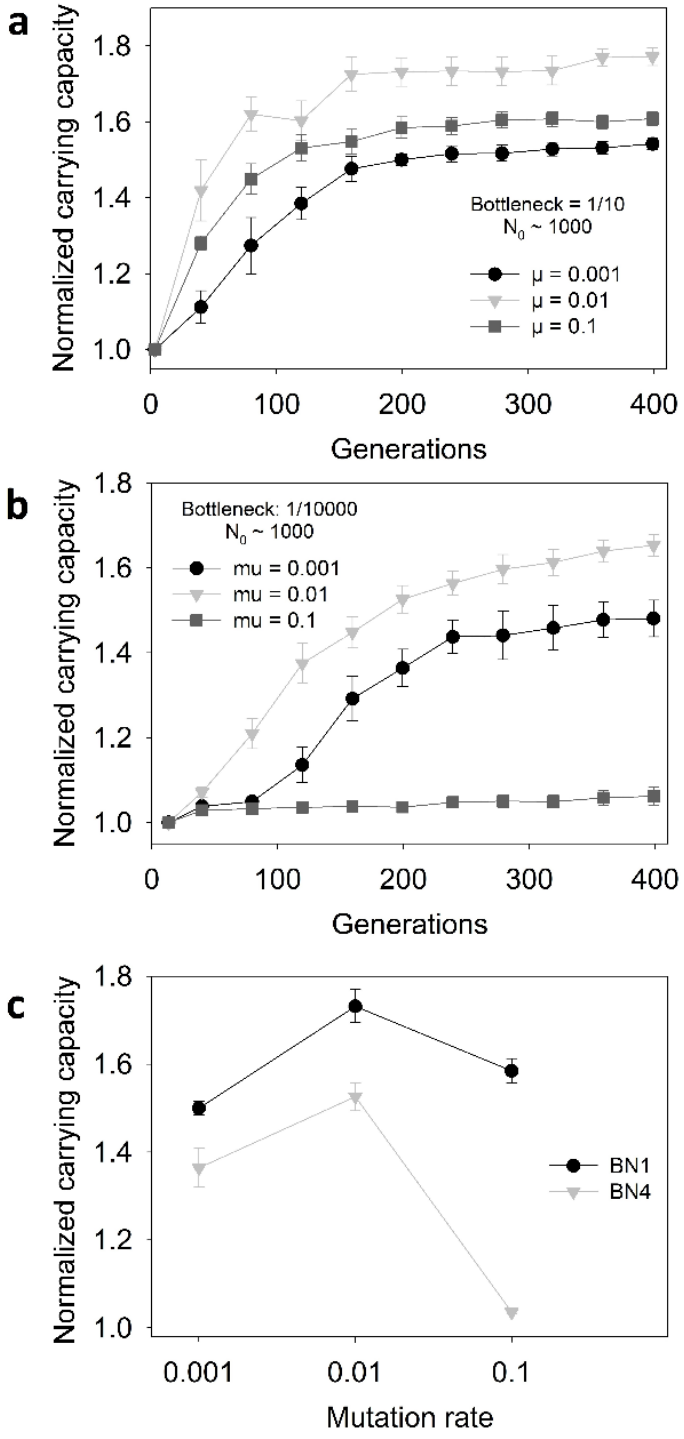

Fig. 2.13. The relationship between *EoA* and *g* at three different mutation rates. (a) Adaptive increase in normalized carrying capacity in BN1 populations at three mutation rates. (b) Adaptive increase in normalized carrying capacity in BN4 populations at three mutation rates. (c) Normalized carrying capacity in BN1 and BN4 at generation 200 at three μ values. Both BN1 and BN4 had similar bottleneck size ($N_0 \approx 900$). BN1 experienced a periodic bottleneck of 1/10 whereas BN4 experienced a periodic bottleneck of $1/10^4$.

A negative relationship between the extent of adaptation and g is particularly surprising because, in populations with similar N_0, increase in g is expected to lead to an increase in the available variation. All else being equal, this should have led to greater adaptation. Since that was not the case, we went on to check if these slowly adapting populations (with similar N_0 but higher g) were limited, qualitatively and/or quantitatively, by the availability of variation.

The quantitative availability of beneficial traits could not explain why EoA varied negatively with g

To determine why the extent of adaptation varied negatively with g, we probed population regimens that had similar starting population size (N_0) after the first bottleneck but also had g values of 3.32 and 13.28 respectively (SM1 and SM4, where SM refers to sampling ratio, expressed in terms of log(10). SM1 grew to a final size of $10N_0$ in one growth phase (*i.e.*, before bottleneck), while SM4 grew to 10^4N_0. Consequently, SM1 faced a periodic bottleneck of 1/10 whereas SM4 was sampled $1/10^4$. Since SM4 experienced ~ 278 times more fission events than SM1 per evolutionary generation, the former was expected to stumble upon more mutations and consequently show many more variation. Furthermore, SM4 was also expected to arrive at

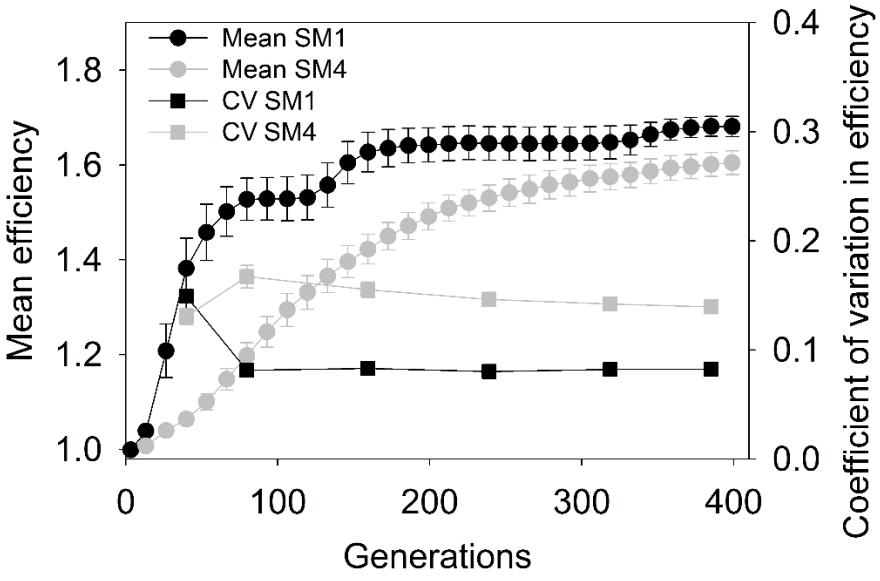

Fig. 2.14. Simulations: Trajectories of efficiency in terms of across-replicate mean and within-replicate coefficient of variation. The within-populations coefficient of variation (CV) was computed for each replicate population across its constituent individuals using discrete frequency distributions. The error bars represent SEM (8 replicates). Both SM1 and SM4 had similar bottleneck size ($N_0 \approx 900$). SM1 experienced a periodic bottleneck of 1/10 whereas SM4 experienced a periodic bottleneck of $1/10^4$. SM4 had a consistently lower *EoA* than SM1 despite having consistently more variation.

very large-effect benefits that were so rare that the probability of SM1 stumbling upon them was vanishingly low due to its lower mutational supply. As expected, compared to SM1, SM4 had a greater within-population coefficient of variation in terms of efficiency values (Fig. 2.14) and therefore was not limited by the supply of variation. To better understand the contributions of phenotypes of different magnitudes to the extent of adaptation, we classified the phenotypes into 50 discrete static classes. We found that SM4 could continually access to rare beneficial

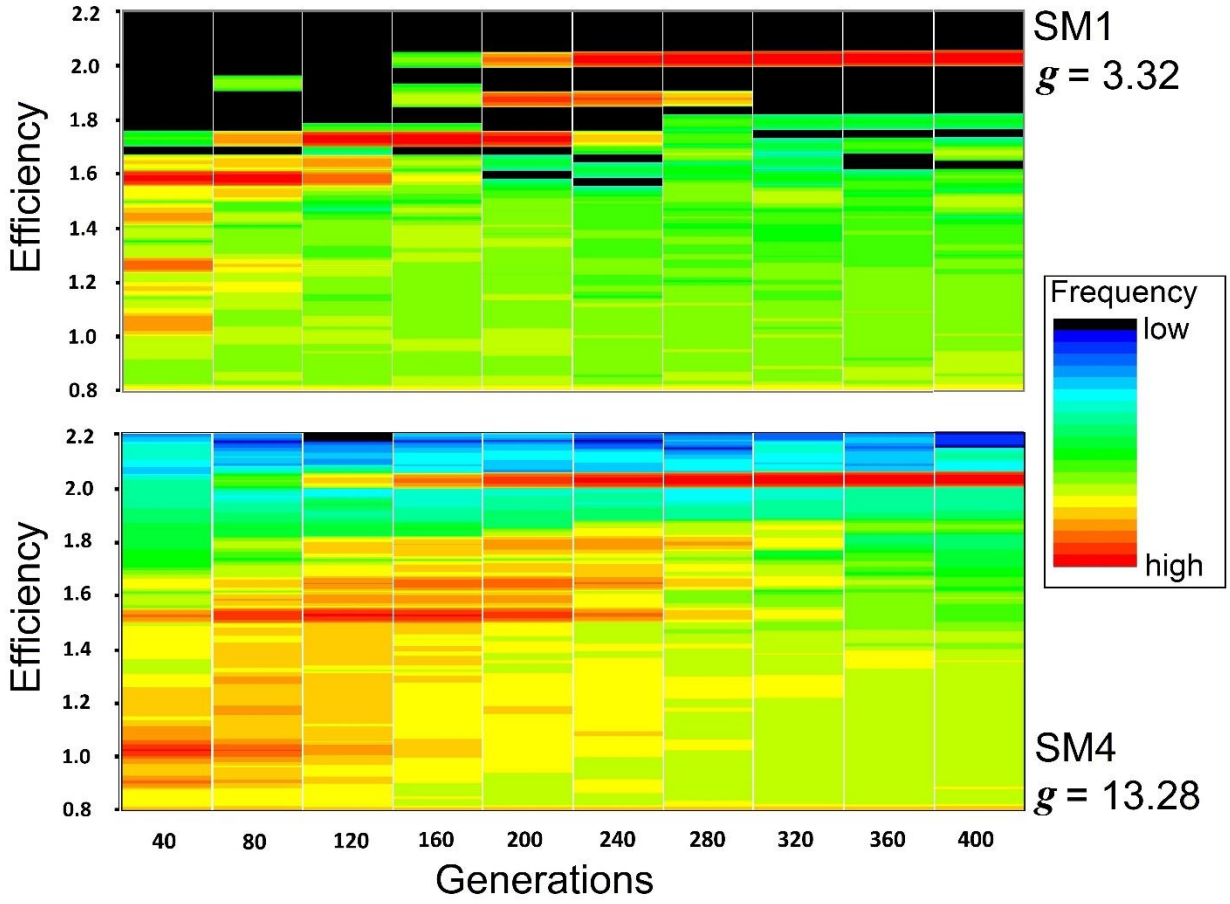

Fig. 2.15. Changes in fitness distributions during adaptation within two representative populations with similar N_0 but different g. The distributions plotted here represent the states of the populations just prior to the periodic bottleneck. Refer to Fig. 2.16-2.18 for adaptive dynamics over eight replicates.

mutations that were inaccessible to SM1 throughout the simulations (Fig. 2.15 and 2.16a). On the basis of these observations, the extent of adaptation can be expected to vary positively with g and thus SM4 was expected to be fitter than SM1 at a given point of time in general. However, counterintuitively, SM4 had a consistently lower extent of adaptation than SM1 (Fig. 2.14). Evidently, harsher periodic sampling impeded adaptation despite resulting in increased

substrate for selection. We also found that although higher N_f allowed SM4 to arrive at extremely rare mutations with very large benefits, these mutations failed to survive the harsh periodic bottlenecks by rising to large enough frequencies (Fig. 2.17a). In other words, SM4 typically wasted the best mutation explored by it but SM1 almost always conserved it. This explains why arriving at these rare mutations with very large benefits did not make SM4 adapt more than SM1 in a sustained manner. However, this does not explain why the extent of adaptation of SM4 was consistently lower than that of SM1.

The negative relationship between EoA and g can be explained in terms of the efficacy of selection

The efficacy of selection in eliminating deleterious mutations and spreading beneficial ones is an important factor that influences the increase of the extent of adaptation. We quantified the inefficacy of selection in increasing *EoA* using the Genetic Load, which was defined as: *Genetic Load = (Best Efficiency – Average Efficiency)/ Best Efficiency* (Crow, 1958; Rice, 2004). The term "Best Efficiency" refers to the highest efficiency value that succeeds in surviving the bottleneck. As discussed earlier, the magnitudes of the highest efficiency values explored by SM4 populations are much greater than those explored by SM1 (Fig. 2.16a). However, these high-fitness phenotypes of SM4 typically have such low frequencies that they almost always fail to survive bottlenecks and thus do not contribute significantly to the overall extent of adaptation (Fig. 2.17a). Therefore, we defined the genetic load only in terms of the phenotypes that survived the bottlenecks. We found that the Best Efficiency (after bottlenecks) for SM1 was very similar to that of SM4 (Fig. 2.17b). We note here that the phenotypes that are fitter than the wild type but less fit than the best phenotype also contribute to the genetic load. Thus, consistently higher genetic load entails lower contribution of the best phenotype to the *EoA*. Furthermore, if these best phenotypes (with respect to which genetic load is defined) are similar across populations being compared, consistently lower contribution of the best phenotype to *EoA* would in turn entail slower rise of the latter. We found that SM4 consistently experienced a heavier genetic load than SM1, particularly during the initial phases of evolution (Fig. 2.18a). This genetic load was constituted largely by phenotypes that are fitter than the wild type ancestor but less fit than the best phenotype (Fig. 2.19). We labelled the top five occupied fitness classes as the "nose" (*sensu* Desai and Fisher (2007)) and all the classes inferior to the nose as the "lagging chunk."

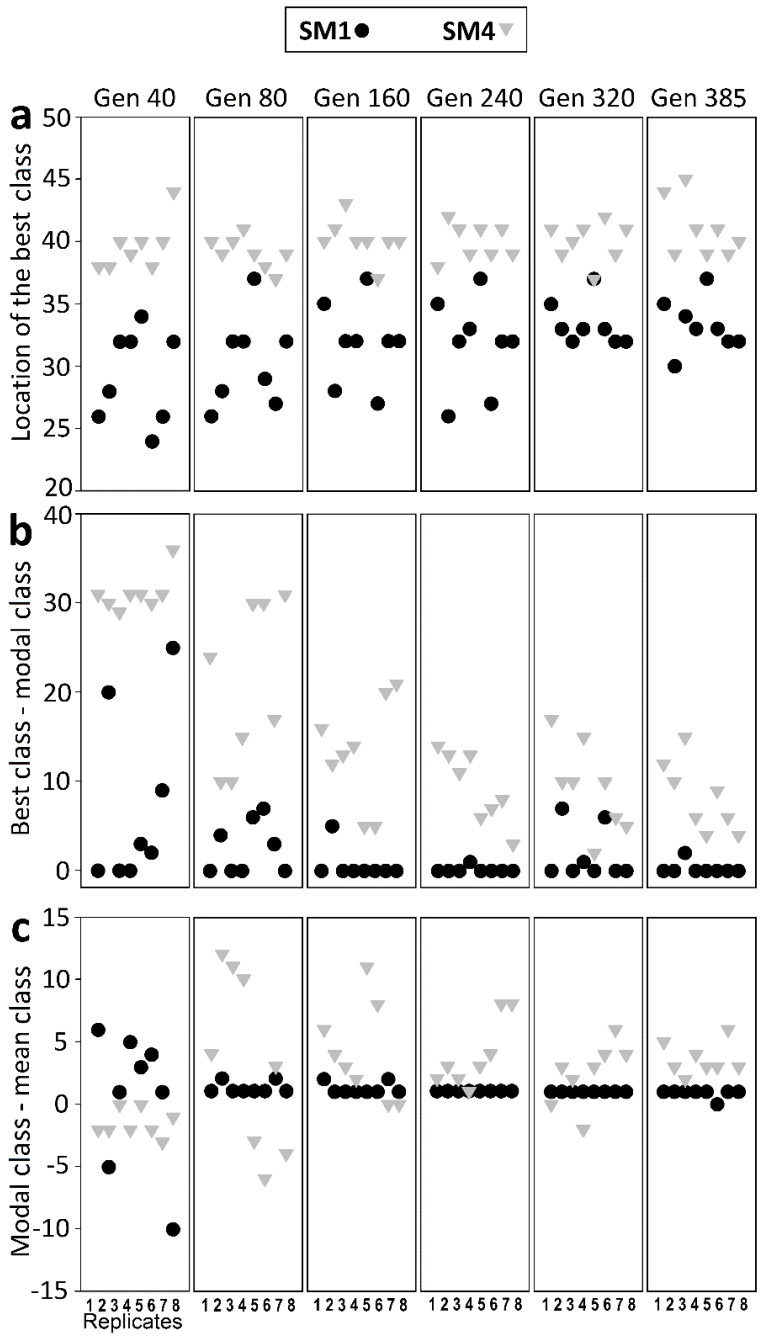

Fig. 2.16. Simulations: Distributions of phenotypic effects across individuals during adaptation in populations with similar N_0. The individuals of each simulated population (8 replicates each of SM1 and SM4) were classified into to a discrete frequency distribution of their efficiency values (50 static classes) prior to bottlenecks. Higher class indices correspond to higher efficiencies. (a) The best phenotype (in terms of efficiency) explored by SM4 was consistently fitter than the best phenotype explored by SM1. The modal phenotype quickly converged to the best available phenotype in all but one SM1 populations but failed to do so in all SM4 populations (b). The mean phenotype in SM1 approached the best phenotype very closely (b and c). However, there was a consistently larger gap between the best phenotype and the modal phenotype in SM4 (c) and an even larger one between its best and mean phenotype (b and c).

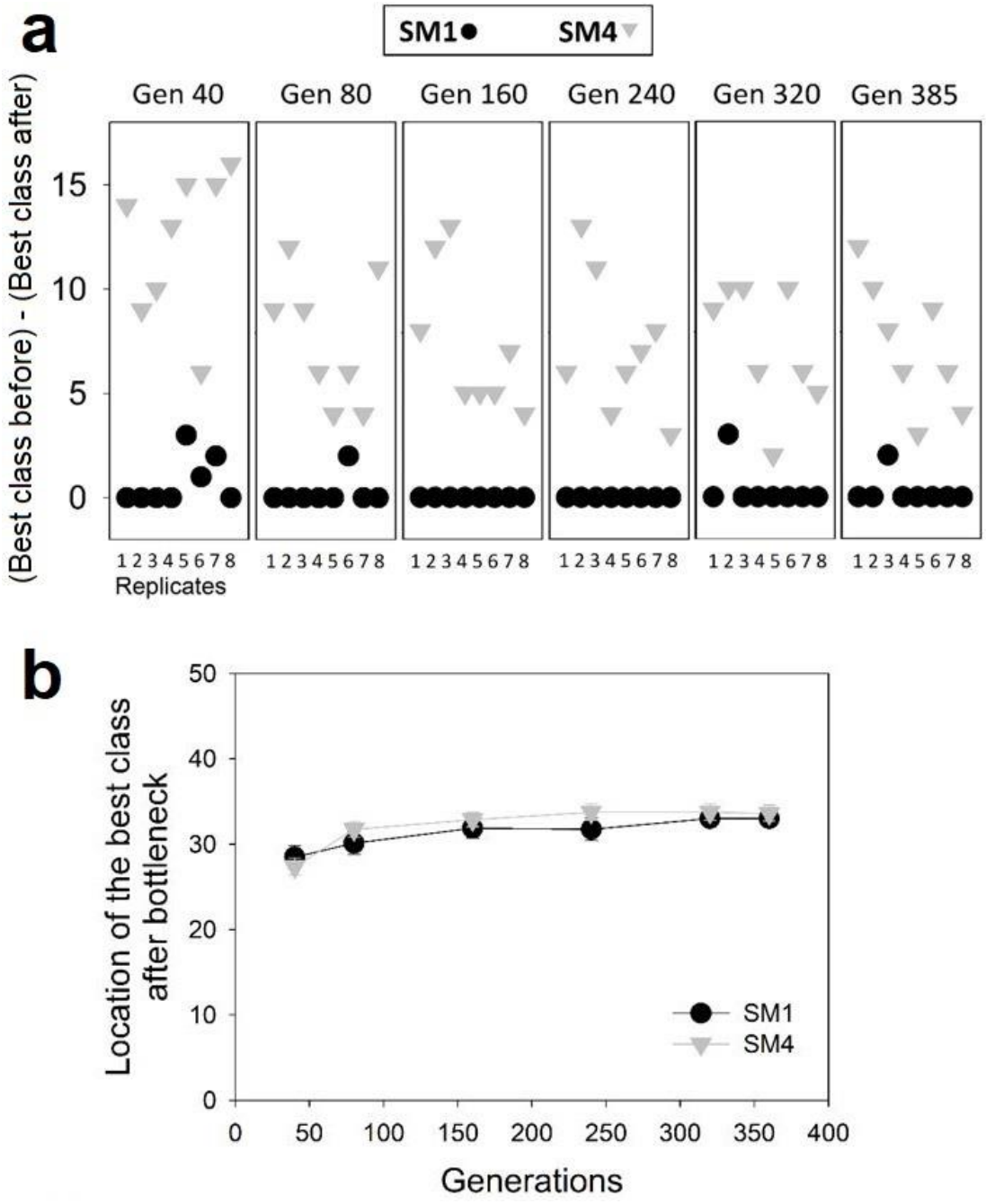

Fig. 2.17. Simulations: Locations of the best class before and after bottleneck. (a) Differences in the locations of the best class in the distribution of the efficeincy parameter before and after bottlenck in populations with similar N_0. The individuals of each simulated population (8 replicate populations each of SM1 and SM4) were classified into to a discrete frequency distribution of their efficiency values (50 static classes). While the best class of SM1 could survive the bottleneck in most cases (black circles), the best class of SM4 invariably failed to survive its harsh bottleneck (grey triangles). (b) The locations of the best class after bottlenecks were remarkably similar across SM1 and SM4. The data points show mean ± SEM (8 replicates; some error bars are smaller than the data symbols.

During the early phases of evolution, the relative contribution of the lagging chunk to the extent of adaptation was much higher in SM4 than in SM1 (Fig. 2.18b). In other words, the nose accounted for most of the *EoA* in SM1 but not in SM4 (shown schematically in Fig. 2.18c and 2.18d). Thus, compared to SM1, the best phenotype of a typical SM4 population needed to outcompete many more phenotypes (present in sizable frequencies) that were superior to the wild-type but inferior to itself. This suggests that the efficacy of selection was higher in SM1 than in SM4, which in turn explains the faster increase of *EoA* in the former. As selection proceeded, the genetic load of SM4 reduced greatly by generation 360 (Fig. 2.18a). This resulted in similar contributions of the respective noses to the overall *EoA* in SM1 and SM4 (Fig. 2.18e and 2.18f).

The above observations suggest that during the early phases of evolution, populations with higher g (here SM4) can face greater impediment (genetic load), which translates into a reduced *EoA*.

N_0/g is a better predictor of *EoA* than N_0g

Our simulations had suggested that the extent of adaptation has a positive relationship with N_0 and a negative relationship with g. Therefore, we went on to test if a mathematical function of N_0 and g that varies positively with N_0 but negatively with g could be a better predictor of the extent of adaptation (*EoA*). Since $N_0 >> g$ within the biologically relevant ranges of these quantities, functions like $N_0 - g$ would not be appropriate to predict EoA despite varying positively with N_0 but negatively with g. Therefore, we tested if N_0/g is a better predictor of adaptive trajectories than N_0g.

Indeed, we found that N_0/g is a much better predictor of adaptive trajectories than N_0g, not only in our simulations (Fig. 2.20 and 2.21; also see Fig. 2.5), but also in our experiments. Specifically, the N_0/g values of LL, SS and SL populations were approximately 3.01×10^9, 1.13×10^4, and 5.02×10^3, respectively. This led to a predicted *EoA* trend of LL>SS>SL, which was observed in the experiments in terms of both the fitness measures (K and R) (Fig. 2.1).

Interestingly, if several populations with identical N_f but different N_0 and g, both N_0/g and N_0g follow the same trends within the biologically relevant ranges of N_0 and g. Our simulations validated this expectation successfully (Fig. 2.22).

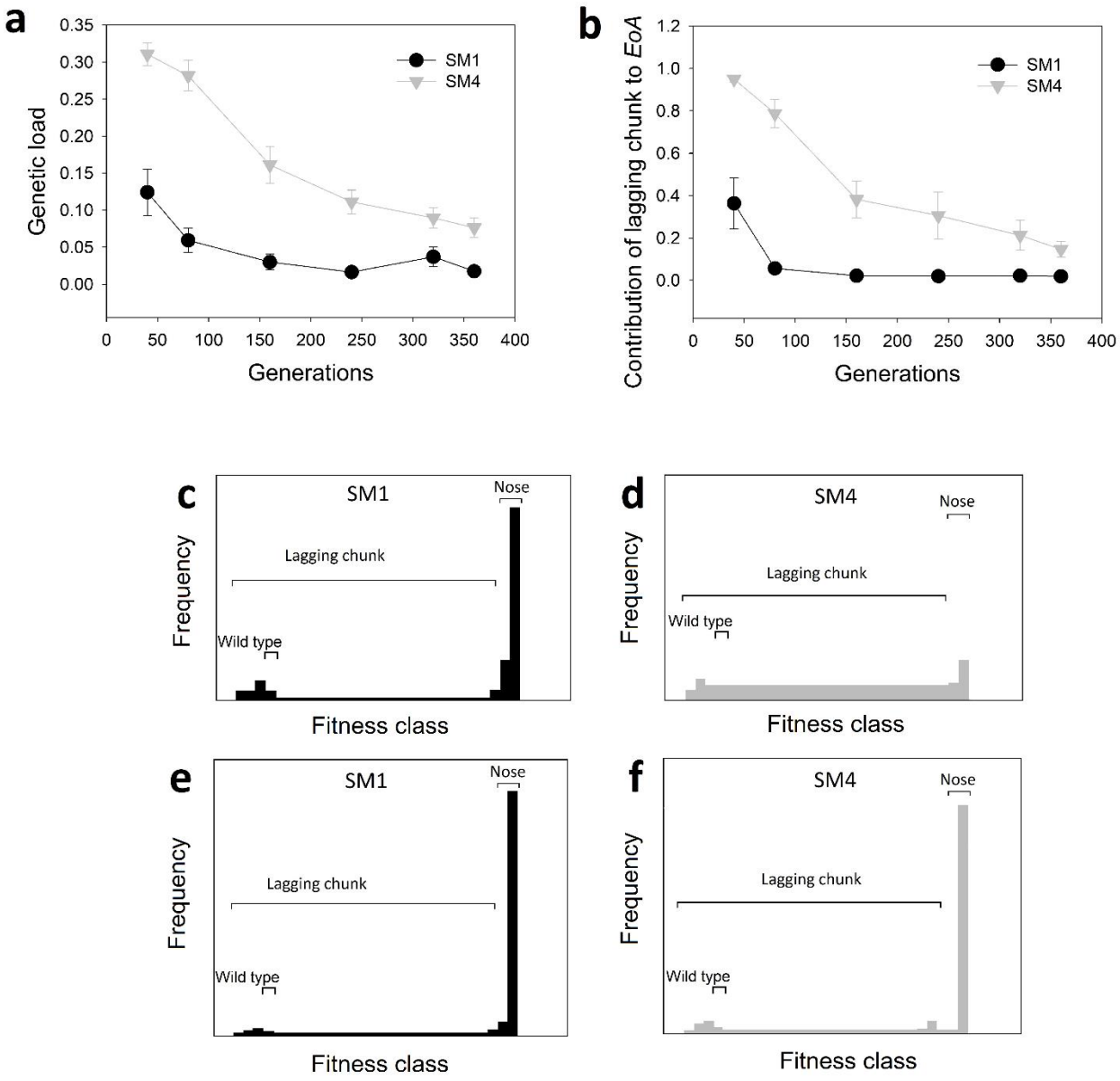

Fig. 2.18. The efficacy of selection in SM1 was more than that in SM4. (a) SM1 consistently experienced a much lower genetic load than SM1 (the error bars represent SEM (8 replicates)). (b) The lagging chunk was the major contributor to the Extent of Adaptation (*EoA*) in SM4 but not in SM1 (the error bars represent SEM (8 replicates)). This also means that the contribution of the nose to the *EoA* (which equals (1 – contribution of the lagging chunk)) in SM1 was much more than that of the lagging chunk. (c) and (d) Schematic representations of the distribution of efficiency across individuals during adaptation during the initial phases of evolution (before generation 80). Due to the high efficacy of selection in SM1, the majority of individuals were found in the nose (c). On the other hand, a relatively low efficacy of selection due to harsher bottlenecks in SM4 resulted in most individuals being found in the lagging chunk (please refer to the text for more details) (d). (e) and (f) During the later phases of evolution (around generation 360), the contributions of the nose to the overall *EoA* became relatively similar in SM1 and SM4.

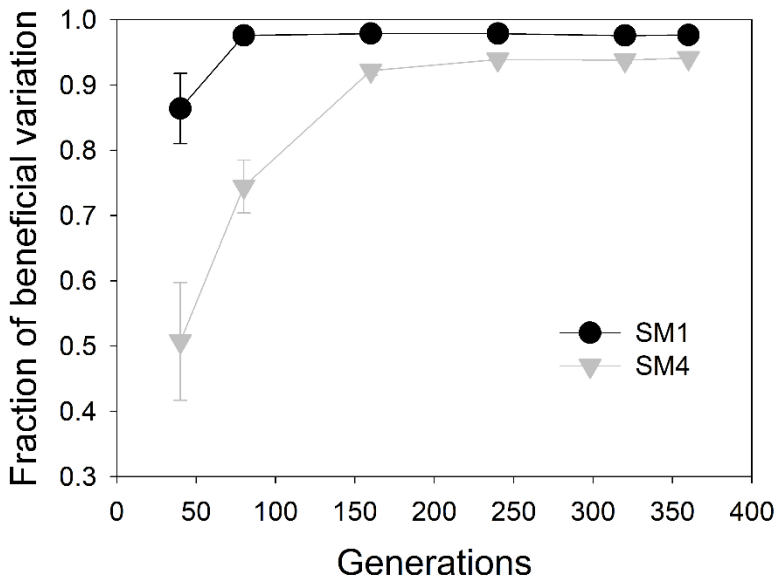

Fig. 2.19. The fraction of beneficial variation with respect to the ancestor (in terms of efficiency) in SM1 and SM4. The data points show mean ± SEM (8 replicates; some error bars are smaller than the data symbols. Beneficial variation is refers to the variation by the individuals that are fitter than the wild-type (ancestor).

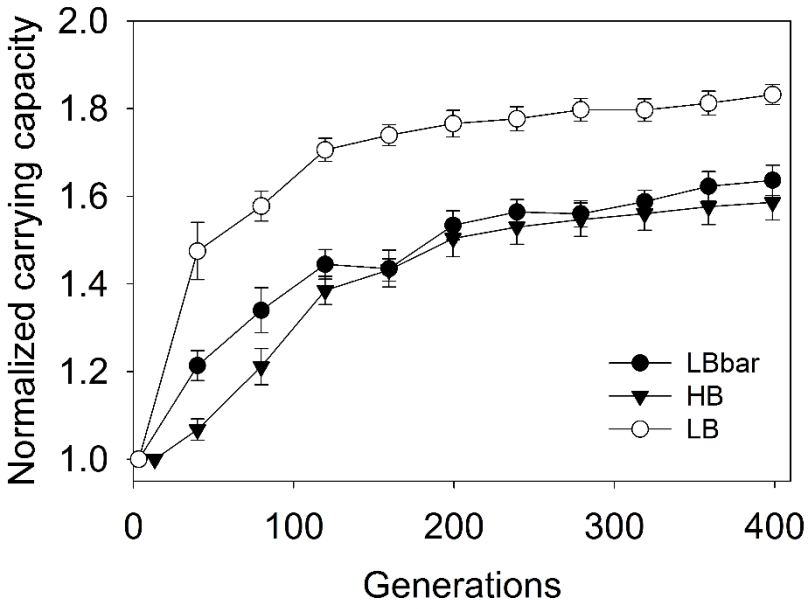

Fig. 2.20. Simulations: *EoA* trajectories in terms of *K*. Populations with similar N_0/g (LBbar and HB) match more closely in terms of mean adaptive trajectories than populations with similar N_0g (LB and HB). LB: $N_0 \approx 3600$, bottleneck ratio: 1/10; HB: $N_0 \approx 900$, bottleneck ratio: $1/10^4$; LBbar: $N_0 \approx 225$, bottleneck ratio: 1/10. N_0/g predicted the average fitness much better than N_0g.

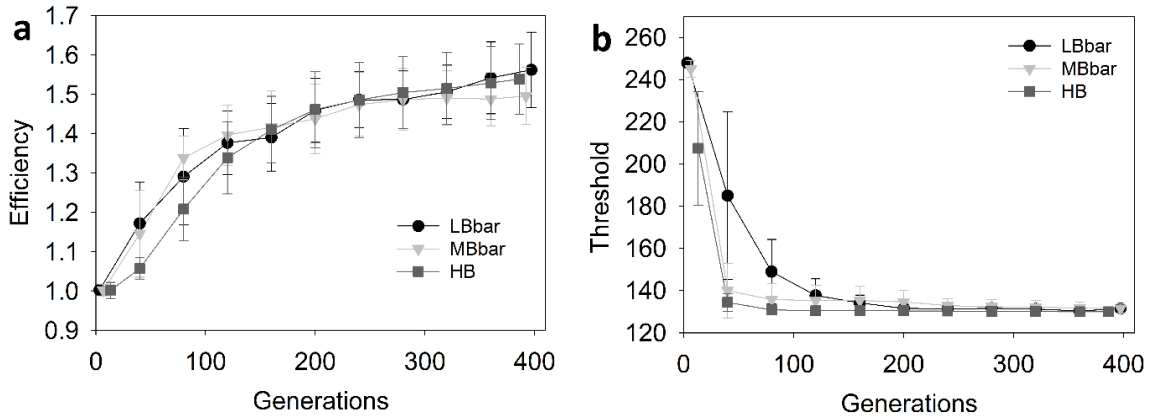

Fig. 2.21. N_0/g was a better predictor of *EoA* than N_0g. Adaptive trajectories in populations with similar N_0/g expressed in terms of individual-level fitness parameters: efficiency (a), threshold (b). The data points show mean ± SD (8 replicates) in (a) and (b). Populations with similar N_0/g (LBbar and HB) match more closely in terms of mean adaptive trajectories than populations with similar N_0g (LB and HB). LBbar: $N_0 \approx 225$; bottleneck ratio = 1/10; MBbar: $N_0 \approx 450$; bottleneck ratio = $1/10^2$; $N_0 \approx 900$; bottleneck ratio = $1/10^4$. HB: $N_0 \approx 900$, bottleneck ratio: $1/10^4$.

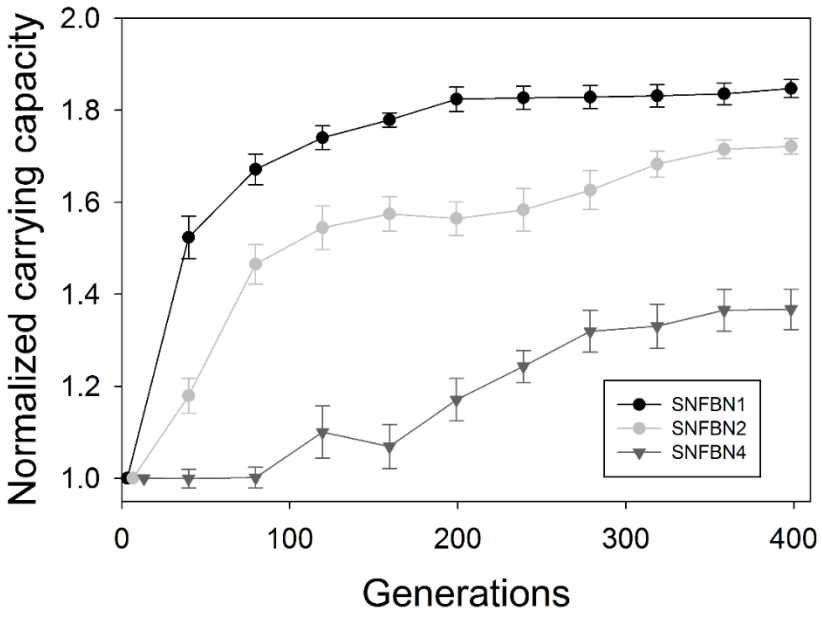

Fig. 2.22. *EoA* in populations with similar initial N_f, but different N_0g. When initial N_f is constant, populations with higher N_0g also have higher N_0/g, and thus show higher *EoA*. However, when N_f is not constant (e.g., see Fig. 2.5, 2.9b, and 2.14), higher N_0g does not lead to higher *EoA*. SNFBN1: $N_0 \approx 9000$; bottleneck ratio = 1/10; $N_0g \approx 29900$; $N_0/g \approx 2711$. SNFBN2: $N_0 \approx 900$; bottleneck ratio = $1/10^2$; $N_0g \approx 5975$; $N_0/g \approx 136$. SNFBN4: $N_0 \approx 9$; bottleneck ratio = $1/10^4$; $N_0g \approx 120$; $N_0/g \approx 0.68$.

2.4. Discussion

Overview of the main results and what they suggest

Most experimental studies with periodically bottlenecked asexual populations have used the harmonic mean as the measure of population size (de Visser and Rozen, 2005; Desai et al., 2007; Lachapelle et al., 2015; Lenski et al., 1991; Rozen et al., 2008; Samani and Bell, 2010; Vogwill et al., 2016) to investigate quantities akin to the extent of adaptation *(EoA)*. Desai et al. (2007) stated that the enhancement in mean population fitness with respect to time (a quantity equivalent to *EoA*) depends upon the harmonic mean of the population size in such populations (Desai et al., 2007). However, there has been no empirical or theoretical test for the validity of the harmonic mean as a predictor of the extent of adaptation. Therefore, as a starting point, we performed evolutionary experiments on *E. coli* populations to test if the harmonic mean of population size ($=N_0 g$) can predict *EoA*. Our experiments revealed that $N_0 g$ does not predict *EoA* (Fig. 2.1). This observation could be interpreted in two ways. Either there was something wrong with harmonic mean in terms of predicting *EoA*, or there were some idiosyncratic properties of our experimental system (e.g. different kinds of containers) that masks the relationship between harmonic mean and *EoA*. Apart from their different numbers (whose effect we study here) and the fact that the LL/SL treatments are grown in flasks while the SS treatment is grown in tissue culture plates, there are no differences between the three treatments, and hence the corresponding selection pressures. Since they are grown with continuous shaking, aeration is unlikely to be a significant issue. To account for the possibility that some idiosyncrasies of our experiments were responsible for our results, and to test if the results of our experimental case-study were generalizable, we simulated the adaptive evolution of asexual populations that grow via fission. For this purpose, we used a very generic model that did not contain any *E. coli* specific functions or parameters. The idea here was that if the outcomes of the simulations matched the experiments, we could be reasonably confident that the experimental results are not due to some peculiarities of the *E. coli* system or experimental protocols. The simulations also revealed no association between $N_0 g$ and *EoA* (Fig. 2.5) which strengthened the first interpretation that $N_0 g$ is not a good predictor of *EoA*.

It must be added here that conventionally, the harmonic mean has been treated as an evolutionarily relevant measure of population size only in terms of neutral mutations (Charlesworth, 2009; Kimura, 1983). However, at least in terms of fixation probabilities of beneficial mutations, it has been shown that population size measures similar to the harmonic

mean can act as the relevant measure of population size (Campos and Wahl, 2009, 2010; Heffernan and Wahl, 2002).

To investigate why N_0g is an inappropriate measure for predicting *EoA*, we used our model to test how *EoA* varied with N_0 and g independently and found the counter-intuitive result that *EoA* varies negatively with g (Fig. 2.9b, 2.11 and 2.12). To explain this result, we probed the composition of our simulated populations as they evolved (Fig. 2.14 – 2.18). We found that g plays a dual role in terms of determining *EoA.* Higher values of g positively affect *EoA* by increasing the supply of variation, but negatively affect *EoA* by decreasing the efficacy of selection, as reflected by a consistently greater genetic load. We found that this second effect of g on *EoA* overshadows the first, something that is underappreciated in the empirical literature. Since N_0 and g have positive and negative relationships respectively with *EoA*, intuition suggests that a good predictor of *EoA* should also do the same. One such expression (of the many possible, taking into account that in most evolutionary experiments $N_0 >> g$) is N_0/g. N_0/g indeed turns out to be a better predictor of *EoA* in our simulations than N_0g (Fig. 2.20 and 2.21). We show below how both measures, (i.e. N_0g and N_0/g) could lead to similar predictions about *EoA* under certain circumstances, and why is it important to consider the cases when this correspondence breaks down.

The rest of the discussion elaborates the various insights mentioned above (and some more) and their consequences.

Periodic bottlenecks lead to increased variation but reduced adaptation

The growth of many natural asexual populations is punctuated by episodic bottlenecks caused by, for example, abrupt dissociation from hosts or spread of infections across hosts (reviewed in (Abel et al., 2015)), etc. Moreover, periodic sampling during sub-culturing is a common feature of most asexual populations propagated during experimental evolution studies (Kawecki et al., 2012; Lenski et al., 1991). Therefore, it is important to appreciate the complex role played by periodic bottlenecks in the evolutionary dynamics of asexual populations.

Most experimental evolution studies with asexual microbes are started with either genetically uniform/clonal replicate populations or a mixed inoculum of relatively small number of genotypes. In such populations, *de novo* beneficial mutations are the principal basis of adaptation (Barrick et al., 2009; Kawecki et al., 2012). That is why populations that experience

greater number of binary fissions per generation are expected to generate more *de novo* beneficial variation and thus, to have a higher extent of adaptation. Now, the number of binary fissions per generation is given by $N_0(2^g-1)/g$. This quantity varies positively with the number of generations before a bottleneck (g) and also with the size of the population at the bottleneck (N_0). Thus, all else being equal, the harmonic mean ($N_0 g$) can be expected to be a good predictor of the extent of adaptation.

However, the above line of reasoning disregards the fact that there can be a significant loss of variation during periodic bottlenecks. As g increases, N_0 represents a smaller fraction of the final population size (N_f) before bottleneck, which in turn increases the chances of loss of variation. For example, assume that there are two bacterial populations that have the same value of N_0 (=10^2) but g values of 3.32 and 13.28, leading to N_f values of 10^3 and 10^6 respectively (Lenski et al., 1991). For a given value of N_0, increasing the value of g decreases the probability that a new beneficial mutation would survive the bottlenecks (Wahl and Zhu, 2015; Wahl et al., 2002). All else being equal, this should reduce the extent of adaptation.

Thus, increasing g has opposite effects on supply and survival of mutations in a population. Several theoretical studies have investigated which of these two effects is more important for adaptive evolution in asexual populations. For example, it has been suggested that increasing g increases the probability of fixation of a beneficial mutation (Heffernan and Wahl, 2002). This implies that the positive relationship between g and mutational supply can overcome the negative effect of increasing g on adaptation. Other theoretical studies have also shown a positive relationship between adaptively relevant population size and the product $N_0 g$ (Campos and Wahl, 2009, 2010). Unfortunately, this rich body of theoretical predictions are not in the context of quantities (like *EoA*) that are experimentally tractable, which was one of the motivations behind this study.

Our experiments (Fig. 2.1) and simulations (Fig. 2.5) showed that populations with similar values of $N_0 g$ can have very different adaptive trajectories, suggesting that $N_0 g$ is not a good predictor of *EoA*. Moreover, our simulations predicted the relationship between *EoA* and g to be negative (Fig. 2.9b, 2.11 and 2.12) and not positive. These two results disagree with a rather large body of existing literature, as outlined above. One way by which this can happen is if our model incorporates some atypical assumptions which lead to the observed counter-intuitive results. However, if that were to be the case, then one would also expect our model to show other unintuitive results. Therefore, we first investigated whether various other predictions of

our model matched those from the literature. Our model was able to replicate several intuitive theoretical predictions that had not been coded directly. Firstly, as expected (Elena et al., 1996; Sniegowski and Gerrish, 2010), very small populations showed discontinuous staircase-like (stepwise) trajectories of fitness increase whereas large populations showed smooth adaptive trajectories (Fig. 2.3). Secondly, *EoA* trajectories showed diminishing returns with time despite never hitting the explicitly coded wall of adaptive limit (Fig. 2.4 - 2.6, 2.9 - 2.14, 2.20, and 2.22) (Lenski et al., 1991; Tenaillon et al., 2016). Thirdly, as expected, we found a non-monotonous relationship between *EoA* and mutation rate (Fig. 2.13c) (Orr, 2000). Fourthly, *EoA* showed a positive but saturating relationship with N_0 (which is an unambiguous measure of absolute population size) (Fig. 2.9a) (Gerrish and Lenski, 1998; Sniegowski and Gerrish, 2010). All this was highly unlikely if our model incorporated unrealistic or atypical assumptions. Furthermore, for numerically similar populations (i.e. populations with similar N_0 and g) and identical time frames, the results from our simulations were a very close match to the results of our experiments in terms of both K and R trajectories (Fig. 2.2). This again suggests that the model captures at least some features of the *EoA* of our *E. coli* populations. Finally, the *EoA* rank predictions generated for the three experimental populations based on our model agreed well with the empirical data (LL>SS>SL, Fig. 2.1). Therefore, it is reasonable to state that our model was generic and a good descriptor of evolving bacterial populations.

EoA varies negatively with g because higher g makes selection less effective

In order to explain why *EoA* varies negatively with g, we simulated populations with similar values of N_0 (*i.e.*, bottleneck size) but different degrees of harshness of the bottlenecks, namely SM1 (lenient bottleneck (= 1/10), g =3.32) and SM4 (harsh bottleneck (= $1/10^4$), g = 13.28) (Fig. 2.14 and 2.18).

Our results demonstrate that higher g decreases the efficacy of selection in terms of spreading beneficial mutations and purging deleterious ones (Fig. 2.18, also see Fig. 2.16 and 2.17). As shown in Fig. 2.18, very high-efficiency classes rise to very high frequencies in SM1 populations by generation 80. However, such classes fail to do so in SM4 populations. Owing to lenient bottlenecks (lower g), selection operates so effectively in SM1 that its best efficiency class quickly converges with the modal class (Fig. 2.16b). This is also reflected by the proximity of the mean class with the modal class in SM1 (Fig. 2.16c). Thus, once a high-fitness class arises in an SM1 population, its rapid spread results in a steep increase in the population's

EoA. However, despite having the same bottleneck size (= N_0) as SM1, SM4 populations exhibit a much slower rise in their *EoA*. This happens due to two reasons. As opposed to SM1, high-fitness genotypes in SM4 need to rise to much higher frequencies to survive the harsh periodic bottlenecks. This results in the removal (due to sampling) of several high-fitness classes from SM4 during the bottleneck (Fig. 2.17a). More importantly, the higher mutational supply rate of SM4 increases the genetic load (Fig. 2.18), which ultimately results in a much slower rise in the extent of adaptation of SM4.

Evolution of carrying capacity can feedback into adaptive trajectories

Both our experiments and simulations showed that carrying capacity (*K*) can evolve during adaptation in asexual microbes (Fig. 2.1a and 2.5a respectively), which is consistent with previous results (Novak et al., 2006). Unfortunately, most models of asexual adaptation do not take into account such adaptive changes in the carrying capacity (Campos and Wahl, 2009, 2010; Gerrish and Lenski, 1998; Wahl and Gerrish, 2001). Most evolution experiments keep the bottleneck ratio (represented by *g*) constant (Kawecki et al., 2012; Lenski et al., 1991). This constancy of *g* ensures that any evolutionary change in carrying capacity would also change N_0. In other words, if *K* increases, a constant value of *g* throughout evolution would ensure an increase in N_0. Since higher values of N_0 accelerate adaptation (Fig. 2.9a), the regularity of bottlenecks introduces a positive feedback during evolution if *K* increases adaptively. Stated differently, a larger value of N_0 would make a population evolve higher *K*, which in turn would increase the next N_0, and so on. We think that this aspect of fitness should not be omitted from theoretical models of how microbes evolve, particularly under resource-limited conditions, which are a common feature of experimental evolution protocols (Kawecki et al., 2012; Lenski et al., 1991).

N_0/g is a better predictor of the extent of adaptation than N_0g

As shown in Fig. 2.9b, 2.14 and 2.11, when selection is at work, the extent of adaptation decreases with increasing *g*. This suggests that a population size measure which is an increasing function of N_0 but a decreasing function of *g* can be a better predictor of *EoA* than the conventional measure (N_0g). For example, as shown in Fig. 2.20, we found that N_0/g is a better predictor of *EoA* than the harmonic mean size (= N_0g). Admittedly, it is not possible to reason

from this that the expression N_0/g will always be a good predictor of *EoA*, and we make no such claims. We simply submit this expression as a potential candidate for this purpose and hope that future theoretical work will be able to validate this empirically derived quantity.

Implications

The adaptive dynamics of asexual populations depend on a delicate interplay of the rate at which variation is introduced in the population and the amount of variation lost periodically during bottlenecks. (Luria and Delbrück, 1943) showed that in periodically bottlenecked systems, each generation contributes equally to the total number of mutants, which, in turn, is proportional to $N_0(2^g)\mu$. Furthermore, ignoring the competition between distinct mutations, the per-generation rate of production of the mutants that would eventually survive the bottleneck is proportional to $(1/2^g)N_0(2^g)\mu$ $(= N_0\mu)$. Thus, the only population size parameter that would determine the supply rate of mutations in the absence of mutational competition is N_0. However, ignoring mutational competition inevitably overestimates the supply of variation in the population. Moreover, we have shown that populations with the same N_0 can have starkly different adaptive trajectories if they have different values of g, with the extent of adaptation varying negatively with g (Fig. 2.14 and 2.18). If N_0 is an overestimation of the mutational supply, N_0g (the harmonic mean size) is an even bigger overestimate. Our finding that N_0/g successfully predicts the adaptive trajectories of bottlenecked populations can thus potentially correct for such overestimations in the supply rate of mutations. However, it is possible to think of other theoretical expressions that can also capture the observed relationships between N_0 and *EoA* (positive) or N_0 and g (negative). A detailed theoretical investigation of what is the correct expression that incorporates these relationships will be the logical next step but is outside the scope of the current study.

Most theoretical studies assume that the final population size attained in their study systems (N_f) is constant (Campos and Wahl, 2009, 2010; Gerrish and Lenski, 1998; Heffernan and Wahl, 2002; Wahl and Gerrish, 2001; Wahl et al., 2002). Interestingly, if the experimental populations that are being compared have similar values of N_f (Desai et al., 2007; Raynes et al., 2014; Vogwill et al., 2016), then the populations with larger values of N_0g will typically also have larger values of any quantity that is an increasing function of N_0 but a decreasing function of g. This is because of two reasons. First, if N_f is held constant, since $N_f = N_02^g$, increasing N_0 necessarily decreases g. Second, in most empirical studies, $N_0>>g$.

Consequently, if N_f is assumed to be the same across the populations being compared, any prediction based on the relative values of N_0g will typically be similar to predictions based on N_0/g (Fig. 2.22). However, whenever N_f is not held constant (e.g., Fig. 2.5, 2.9, 2.14, 2.20 and 2.21, and studies like (Lachapelle et al., 2015; Ramsayer et al., 2013; Raynes et al., 2014; Rozen et al., 2002; Samani and Bell, 2010), N_0/g predicts *EoA* much better than N_0g. The above observations can explain why N_0g has been widely used across several empirical studies despite failing to capture the effects of g on *EoA* accurately.

At very long time-scales, the high-fitness mutations accessible only to SM4 (but not to SM1) may end up surviving a harsh periodic bottleneck. A post-facto analysis of our SM4 simulations shows that mutations of this kind rise to a frequency between 10^{-7} and 10^{-8} in a typical growth phase just prior to bottlenecks in SM4. Since N_0 is close to 10^3 in these populations, the above high-quality mutations would survive one bottleneck in every 10^4 to 10^5 growth phases which roughly amounts to 1.3×10^5 to 1.3×10^6 generations. However, to this date, there are no reported experimental evolution studies over this long a time-span. Therefore, we conclude that the observation that increasing g decreases *EoA* should be relevant for the time-scales most commonly employed in experimental evolution studies.

Our results can be used to compare the extents of adaptation in independent evolution experiments with similar environments but dissimilar demographic properties (differences in terms of N_0 and/or g and/or N_f). Such studies, which compare populations evolving in similar environments but with dissimilar demographic properties, are reasonably common in the field of experimental evolution (Desai et al., 2007; Lachapelle et al., 2015; Raynes et al., 2014, 2014; Rozen et al., 2002; Samani and Bell, 2010; Vogwill et al., 2016).

Our study shows that in serially bottlenecked asexual populations, the destructive aspect of bottlenecks (reduction in efficacy of selection by harsher bottlenecks) can overshadow their constructive aspect (increase in supply of variation in harsher bottlenecks). This calls for a change in perspective about periodic bottlenecks and a substantial re-evaluation of the role of population size as a predictor of adaptive evolution.

Chapter 3

Adapting in larger numbers can increase the vulnerability of *Escherichia coli* populations to environmental changes

Highlights

- We studied the effects of historic population size on the vulnerability of asexual populations to sudden environmental changes.
- We subjected replicate *Escherichia coli* populations of different sizes to experimental evolution in an environment containing a cocktail of three antibiotics. In this environment, the ability to actively efflux molecules outside the cell is expected to be a major fitness-affecting trait.
- We found that all the populations eventually reached similar fitness in the antibiotic cocktail despite adapting at different speeds, with the larger populations adapting faster. Surprisingly, whereas efflux activity enhanced in the smaller populations, it decayed in the larger ones.
- The evolution of efflux activity was largely shaped by pleiotropic responses to selection and not by drift.
- The larger populations also showed inferior fitness upon sudden exposure to several alternative stressful environments.
- Our study provides a novel link between population size and the vulnerability to environmental changes.

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3.1. Introduction

Population size is a key ecological parameter that influences the rate at which asexual populations evolve (Desai and Fisher, 2007; Desai et al., 2007; Gerrish and Lenski, 1998; Wilke, 2004). All else being equal, larger populations are supposed to evolve faster as they are expected to have access to greater variation (Desai and Fisher, 2007; Desai et al., 2007; Orr, 2000; Sniegowski and Gerrish, 2010; Wilke, 2004). Moreover, the efficiency of natural selection, in favoring beneficial mutations and keeping out deleterious ones, increases with increasing population size (Chavhan et al., 2019a; Petit and Barbadilla, 2009), which is also expected to increase the rate of adaptation. However, little is known about how evolving large asexual populations fare when their environment changes abruptly. Are their performances comparable with smaller populations that have evolved in the same environment?

Consider a clonally derived large asexual population that has evolved in a constant environment for an extended period. The ability of such a population to face sudden environmental changes would be determined by the variation accumulated during evolution in the constant environment. However, the population size experienced during evolution will influence variation in two contrasting ways. On the one hand, larger asexual populations are expected to stumble upon more mutations during adaptation (Desai and Fisher, 2007; Desai et al., 2007; Sniegowski and Gerrish, 2010). On the other hand, since natural selection is more efficient in larger populations, it can lead to a rapid increase in the average fitness and severe reduction in the genetic variation of such populations (Chavhan et al., 2019a; Desai and Fisher, 2007; Sniegowski and Gerrish, 2010). Such reduction in variation can potentially be detrimental if the environment changes suddenly, particularly if high fitness in the old environment is correlated with low fitness in the new one (antagonistic pleiotropy *sensu* (Cooper, 2014; Cooper and Lenski, 2000). Thus, the actual amount of variation available to the population would be determined by an interaction between these two opposing aspects.

Asexual populations of very different sizes also have markedly different accessibilities to beneficial mutations in identical environments (Chavhan et al., 2019a; Desai and Fisher, 2007; Sniegowski and Gerrish, 2010; Wilke, 2004). This is because beneficial mutations that confer higher fitness gains are generally rarer (Eyre-Walker and Keightley, 2007; Kassen and Bataillon, 2006; Neher, 2013; Perfeito et al., 2007; Sniegowski and Gerrish, 2010)). Consequently, whereas adaptation in very large populations is driven predominantly by rare large-effect beneficial mutations, small populations typically adapt via relatively common

small-effect beneficial mutations (Sniegowski and Gerrish, 2010). The ability of a small population to face environmental changes is also expected to be different from that of a larger one. This notion stems primarily from theoretical studies which predict that the pleiotropic effects of large- and small-effect beneficial mutations should be very different (Lande, 1983; Orr and Coyne, 1992). For example, Lande's model for studying the response to selection on beneficial mutations of varying sizes assumed that major beneficial mutations have substantial pleiotropic costs while minor beneficial mutations have none (Lande, 1983). It has been suggested that a better assumption would be that the deleterious pleiotropic effects of a beneficial mutation are proportional to the size of the benefit it confers (Orr and Coyne, 1992). Furthermore, recent empirical investigations have found that deleterious mutations that confer larger fitness deficits also tend to have more pleiotropic effects (Cooper et al., 2007). Overall, the extant literature suggests larger beneficial mutations may have greater deleterious pleiotropic effects. Given that large populations adapt primarily via rare large-effect mutations and small populations via relatively common mutations of small effect (Sniegowski and Gerrish, 2010), it is expected that larger populations would suffer heavier pleiotropic disadvantages. Thus, if asexual populations of different sizes adapt to the same constant environment for an extended period, larger populations can become inferior to smaller ones in terms of their immediate response to environmental changes.

In this study, we used experimental evolution to examine the above notion. Specifically, we propagated replicate *Escherichia coli* populations of different sizes in a constant environment for ~ 380 generations. This constant environment contained an unchanging sub-lethal cocktail of three antibiotics, namely, norfloxacin, rifampicin, and streptomycin. When all the populations reached similar fitness in this environment, we estimated their ability to face sudden changes in environmental conditions using two different approaches. First, we studied the evolution of energy-dependent efflux activity (EA), which represents the generic capacity of bacteria to actively transport unwanted molecules out of their cells and is a critical component of xenobiotic metabolism (Sun et al., 2014). EA is known to be one of the broad-based mechanisms in bacteria for fighting multiple stresses including antibiotics (Kumar and Schweizer, 2005), heavy metals (Nies, 2003; Poole, 2005), bile salts (Thanassi et al., 1997), organic solvents (Fernandes et al., 2003), intercalating mutagens (Ma et al., 1993; Nishino et al., 2009), etc. This makes EA a good candidate character to study the ability of bacterial populations to thrive in the face of sudden environmental stress (Karve et al., 2015). Second, we directly tested the fitness of our populations in several alternative environments which are

known to affect *E. coli* very differently as compared to the three antibiotics in the selection environment.

The three antibiotics used in our selection environment had very different mechanisms and sites of action (Campbell et al., 2001; Drlica and Zhao, 1997; Sharma et al., 2007). Evolution in this environment is expected to favour the presence of EA. However, we found that whereas larger populations undergoing fast adaptation experienced decay of EA, smaller populations undergoing slow adaptation experienced enhanced EA. These results were attributable to correlated responses to selection rather than the accumulation of contextually neutral mutations via genetic drift. The larger population also had lower fitness upon exposure to four different alternative environments. This demonstrates that highly efficient selection during rapid adaptation in large populations can render them vulnerable in terms of their response to environmental changes.

Adaptation to a given environment is expected to result either in enhancement/maintenance or in decay of a biological character (but not both). To the best of our knowledge, this is the first study to show that even in the absence of major effects of drift, a biological character under selection can decay or enhance depending on the size of the adapting populations.

3.2. Materials and methods

Experimental evolution and measurement of adaptive dynamics

The maintenance protocol of these selection lines has been previously described in another study (Chavhan et al., 2019a). We derived 24 microbial populations from a single *Escherichia coli* MG 1655 colony and randomly distributed them among three population size treatments, namely LL, SL, and SS (refer to the next paragraph for the details of this nomenclature), leading to 8 independently evolving replicate populations per treatment. The populations evolved in a constant environment made of nutrient broth containing a sub-lethal cocktail of three antibiotics (henceforth called 'selection environment') under batch culture for ~380 generations (see Supplementary Methods for the detailed composition of the nutrient broth). The three antibiotics used were norfloxacin (0.015 μg/ml), rifampicin (6 μg/ml) and streptomycin (0.1 μg/ml). These antibiotics target different cellular mechanisms: norfloxacin interferes with DNA replication (Drlica and Zhao, 1997), rifampicin affects RNA transcription (Campbell et al., 2001), while streptomycin affects protein translation (Sharma et al., 2007).

We propagated the three population types at different population sizes. The size of a typical periodically bottlenecked asexual population depends on three interdependent parameters: N_0 (the number of individuals in the bottleneck), N_f (the number of individuals before the bottleneck), and g (the number of generations between successive bottlenecks). Since these populations grow via binary fissions, $N_f = N_0 \times 2^g$ (Lenski et al., 1991). The conventional measure of size in bottlenecked populations is the harmonic mean of population size (HM = $N_0 \times g$) (but also see (Chavhan et al., 2019a) for a measure of population size relevant for predicting the extent of adaptation in such systems). Thus, bottleneck properties are instrumental in shaping the size of such populations. Our experiment had three different population size treatments, called LL, SL, and SS. The first letter of a population type's name represents a relative measure of the harmonic mean size ($L \approx 3.3 \times 10^{10}$; $S \approx 2.0 \times 10^6$) and the second letter represents a relative measure the culture volume (L refers to 100 ml and S refers to 1.5 ml). The density of individuals (number of individuals per unit volume) was identical across the three population types at the beginning of the experiment. Moreover, whereas LL faced lenient bottlenecks (1/10; g = 3.3), the bottleneck ratios were much harsher in SS ($1/10^4$; g = 13.3) and SL ($1/10^6$); g = 19.9). This ensured that LL >> SL = SS in terms of HM.

We computed the speed of adaptation (SoA) of the three population types using the fitness trajectories over ~380 generations reported in an earlier study (Chavhan et al., 2019a). The

fitness trajectories of all three population types had displayed diminishing returns, which is a common observation in evolution experiments with microbes (Cooper and Lenski, 2000; Couce and Tenaillon, 2015; Schoustra et al., 2009). Therefore, we quantified SoA as the maximum slope of fitness trajectories observed during the evolution experiment. SoA was quantified in terms of two measure of fitness, namely, carrying capacity (K, the maximum optical density reached in a growth assay) and maximum growth rate (R, the maximum slope of the growth curve during the assay).

The experimental design of our study is shown schematically in Fig. 3.1.

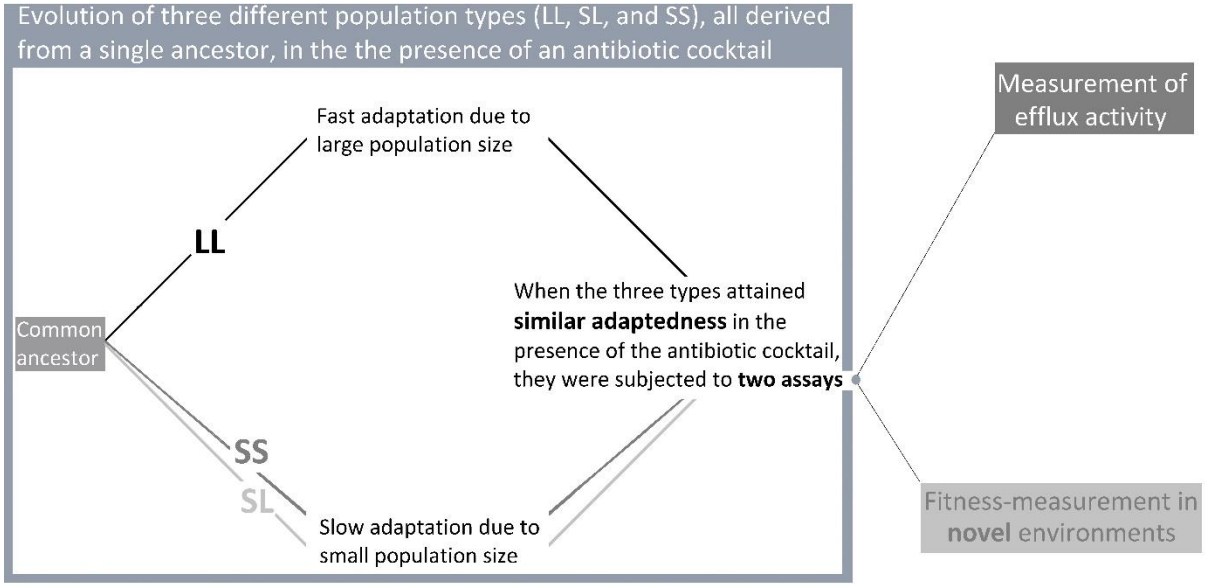

Fig. 3.1. A schematic representation of our study. Note that the assays reported here had been carried out when the three population-types (LL, SL, and SS) did not have significantly different fitness in the presence of the antibiotic cocktail, i.e. they were at similar levels of adaptedness.

Measurement of efflux activity (EA)

We measured the generic EA of the three population types at the beginning and the end of the above evolution experiment using a previously established protocol (Karve et al., 2015; Webber and Coldham, 2010). Specifically, we measured the efficiency with which bacteria could transport a small, foreign molecule out of their cells (see Appendix 5).

We used one-sample t-tests to determine if the population types had evolved significantly different EA than the ancestor. We also used a one-way ANOVA with population type (LL,

SL, or SS) as the categorical predictor and efflux activity (EA) as the dependent variable to determine the statistical significance of EA differences between the three types. We made sure that the EA assays were carried out when the fitness of the three types were statistically indistinguishable in their selection environment (Fig. 3.1). Furthermore, all the three population types were derived from a common ancestor. Therefore, the results of these assays can only be attributed to differences in their population sizes. We further used Cohen's *d* for comparing the significance of differences in the EA of the three population types in terms of effect sizes (Cohen, 1988). We also tested if the variation across replicates was significantly different for the three population types. To this end, we compared the variances of LL, SL, and SS lines in a pairwise manner using the Fligner-Killeen test for homogeneity of variances (Donnelly and Kramer, 1999; Fligner and Killeen, 1976).

Fitness assays in alternative environments

We quantified the fitness of the three population types in four distinct alternative environments at the end of our evolution experiment (the same time-point at which EA was measured). The design of our study demanded that each alternative environment must impose a challenge that is known to be different from the one imposed by the antibiotic cocktail. Otherwise, the fitness of a population in an alternative environment could be trivially predicted from its fitness in one of the antibiotics in the selection environments. We used Ampicillin as an alternative stress because it has a different site and mechanism than all the three antibiotics used in the cocktail (ampicillin is a β-lactam antibiotic which inhibits cell-wall synthesis) (Waxman and Strominger, 1983). Similarly, we used high concentrations of copper (heavy metal stress) as another alternative environment. At high concentrations, the incompletely filled d-orbitals of Cu^{2+} ions form unspecific complex compounds which are toxic to the cellular physiology (Nies, 1999). Further, we also used two nutritionally challenging minimal media based on sorbitol and urea as the only carbon sources, respectively.

We revived the endpoint cryostocks from our selection-experiment and grew them in nutrient broth (without antibiotics) for 12 hours, which represents ~ 6.6 doublings. Thus, any lingering physiological effects of stress due to antibiotics were ameliorated. Since all the population types had evolved in the same environment, the effects of the historic environment were not an issue in our study. We carried out automated growth-assays on these populations in the alternative environments using 96-well tissue culture plates in a well-plate reader (Synergy HT,

BIOTEK ® Winooski, VT, USA). We used OD at 600 nm as the measure of bacterial density, and assayed growth from each cryostock-derived population in three measurement-replicates. The 96-well-plate was incubated at 37^{O}C and shaken continuously at 150 rpm. The culture volume in each well was 180 µl. The reader took OD readings every 20 minutes, which gave rise to high resolution sigmoidal growth-curves. We used two measures of fitness: (1) Carrying capacity (*K*, the maximum OD achieved during the growth curve) and (2) Growth rate (*R*, the highest slope of growth curve measured over a dynamic window of ten OD readings).

The fitness trends in alternative environments were analyzed in two ways. First, we performed a pooled analysis using a mixed-model ANOVA with population type (three levels: LL, SL, and SS) and alternative environment (four levels: presence of ampicillin, urea as the sole carbon source, sorbitol as the only carbon source, and presence of high [Cu^{2+}]) as fixed factors crossed with each other, and replicate number (1-8) as a random factor nested in population type.

Since differences in performance within any one of the four alternative environments could have driven the results of the pooled analysis on their own, we also performed a separate analysis for each alternative environment. Each of these tests involved a nested-design ANOVA with population type (LL, SL, and SS) as the fixed factor, and replicate number (1-8) as a random factor nested in population type. We controlled for false discovery rates (FDR) in these individual ANOVAs using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). We used a stringent set of four conditions to determine the significance of the tests done for each environment individually: (1) The ANOVA done over the triplet of LL, SL, and SS should have *P* smaller than the corresponding Benjamini-Hochberg critical value. (2) The difference within the population type triplet has large effect size (partial $\eta^2 > 0.14$) (Cohen, 1988). (3) Tukey's HSD should reveal significant pairwise differences ($P < 0.05$). (4) The pairwise differences should have medium or large effect sizes (Cohen's *d*). We counted the results of a test as significant only when all four of these conditions were met simultaneously.

3.3. Results

LL populations had greater speed of adaptation (SoA)

There was a significant effect of population size on SoA, both in terms of K (Fig. 3.2a; $P = 1.505\times10^{-8}$; $F_{(2,21)} = 47.870$) and R (Fig. 3.2b; $P = 2.73\times10^{-6}$; $F_{(2,21)} = 25.070$) (one-way ANOVA, N = 8). Tukey's HSD (pair-wise post-hoc) suggested the following relationships for K: LL > SL ($P = 1.501\times10^{-4}$), LL > SS ($P = 1.403\times10^{-4}$), SL > SS ($P = 0.004$), and R: LL > SL ($P = 1.55\times10^{-4}$), LL > SS ($P = 1.45\times10^{-4}$) and SL ~ SS: ($P = 0.907$). Taken together, this suggests that LL populations adapted faster than both SL and SS.

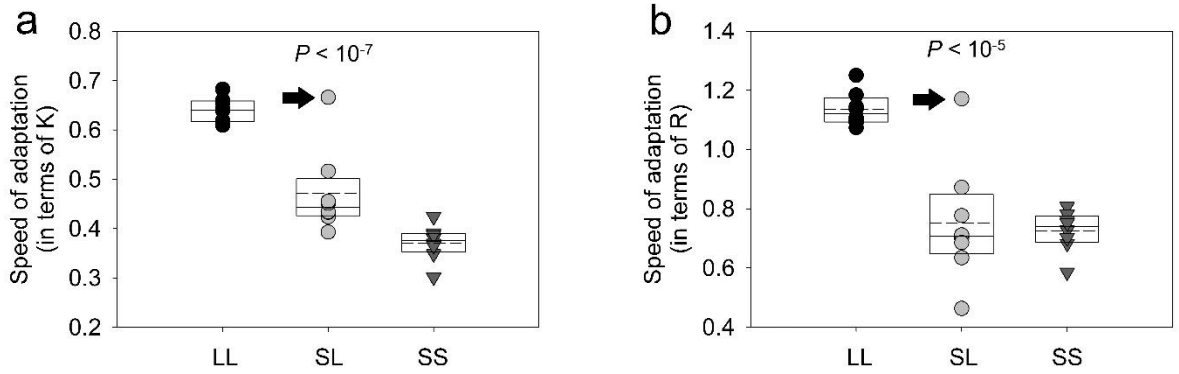

Fig. 3.2. Speed of adaptation during evolution to the antibiotic cocktail. The solid lines in the box plots mark the 25th, 50th, and 75th percentiles; the dashed lines within the box plots represent means (N = 8). (a) Speed of adaptation in terms of K. (b) Speed of adaptation in terms of R. The grey data points marked with an arrow represent the only non-LL population that lost the ancestral efflux activity (see the text for details). Note that it was the same replicate population which was an outlier in terms of both K and R in the SL treatment.

LL populations evolved reduced efflux activity (EA)

We found that whereas LL lost the efflux activity (EA) with respect to the common ancestor, SL and SS gained it (Fig. 3.3a) (one sample t-test against the ancestral efflux activity: $P = 0.003086$, Cohen's $d = 1.26$ (large effect) (LL); $P = 0.029800$, Cohen's $d = 1.25$ (large effect) (SL); $P = 0.000823$, Cohen's $d = 2.28$ (large effect) (SS). One-way ANOVA (N=8) across the three population types revealed a significant main effect of population type ($P = 2.055\times10^{-6}$; $F_{(2,21)} = 26.044$) and Tukey's HSD for the pairwise comparisons showed LL < SL ($P = 2.710\times10^{-4}$), LL < SS ($P = 1.411\text{x}10^{-4}$) and SL ~ SS ($P = 0.155$). Furthermore, the statistically significant pairwise differences (LL-SL and LL-SS) also had very large effect sizes: Cohen's

d = 2.512 for LL-SL; Cohen's d = 3.53 for LL-SS). We also found that the variation across replicates was not significantly different across the three population types (pairwise analysis using the Fligner-Killeen test: P = 0.576 (LL-SL); P = 0.644 (LL-SS); P = 0.762 (SL-SS). Taken together, it is clear that EA, a major fitness-affecting trait in the presence of multiple antibiotics, had diminished in LL but enhanced in SL and SS.

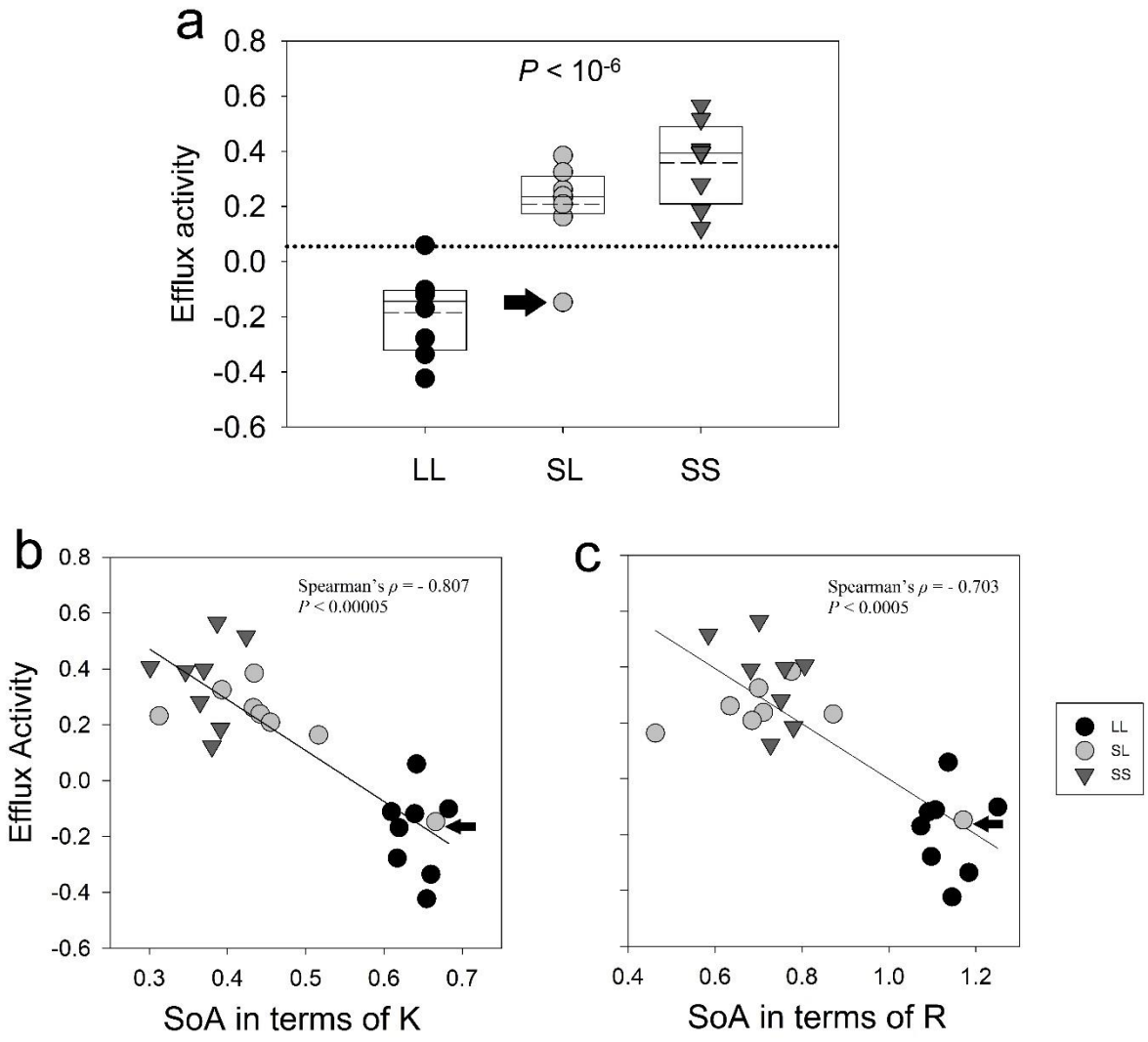

Fig. 3.3. Evolved efflux activity and its correlation with the speed of adaptation. (a) EA in the three population-types after evolution in the presence of the antibiotic cocktail. The solid lines in the box plots mark the 25th, 50th, and 75th percentiles; the dashed lines within the box plots represent means (N = 8). The black dotted line represents the ancestral efflux activity. Each data point represents the average of two independent efflux measurements. The grey data point marked with an arrow in each of the three panels represents the only non-LL population that lost the ancestral efflux activity (see the text for details) and is the same replicate that was an outlier in Fig 3.2. EA had a strong negative correlation with SoA, expressed in terms of (b) carrying capacity (K) and (c) maximum growth rate respectively.

EA was negatively correlated with SoA

A corollary of the above observations was a strong negative correlation between EA and SoA, both in terms of *K* (Fig. 3.3b; Spearman's ρ = -0.807; $P = 1.898\times10^{-6}$) and *R* (Fig. 3.3c; Spearman's ρ = -0.703; $P = 1.256\times10^{-4}$). Since the three population types differ in terms of their population sizes, we also checked if EA was also negatively correlated with the harmonic mean population size and found the same (Spearman's ρ = -0.766; $P = 1.274\times10^{-5}$).

LL populations fare worse in alternative environments

In the alternative environments, we found a significant population type × environment interaction, both in terms of carrying capacity (K, mixed-model ANOVA: $P = 3.206\times10^{-13}$; $F_{6,255} = 13.42$; partial $\eta^2 = 0.24$ (large effect)) and maximum growth rate (R, mixed-model ANOVA: $P = 3.61\times10^{-9}$; $F_{6,255} = 9.244$; partial $\eta^2 = 0.178$ (large effect)). As the interaction terms were significant, we chose not to interpret the main effects.

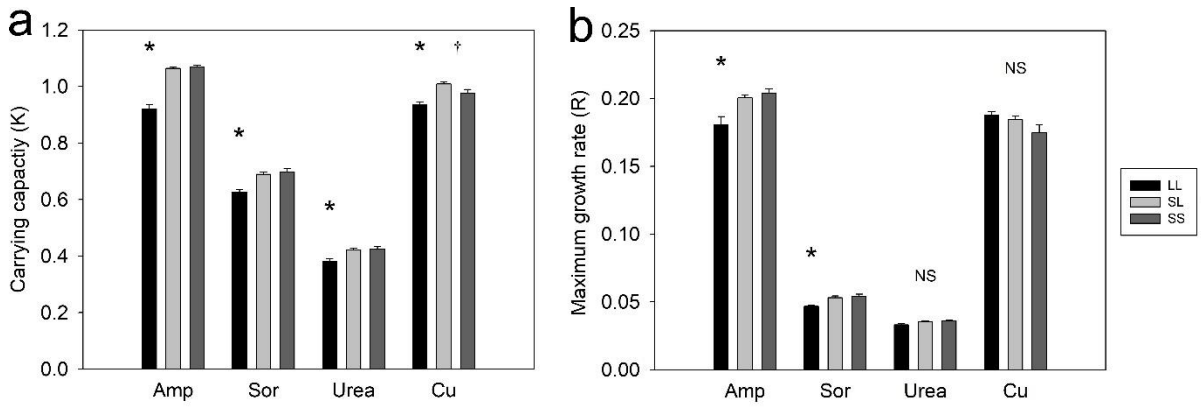

Fig. 3.4. LL had significantly lower fitness than SL and SS in alternative environments. (a) Fitness expressed in terms of K (mean ± SEM; N = 8). (b) Fitness expressed in terms of R (mean ± SEM; N = 8). * refers to cases where the following four conditions are met simultaneously: (1) The ANOVA for the population-type triplet reveals significant differences after the Benjamini-Hochberg procedure. (2) The difference within the population-type triplet has large effect size (partial η^2). (3) The pairwise differences between LL-SL and LL-SS are significant (Tukey's HSD (post-hoc)). (4) The pairwise differences between LL-SL and LL-SS have large or medium effect sizes (Cohen's d). 11 out of 12 pairwise differences marked by * had large effect sizes. † refers to the only case where SL was significantly different from SS. 'NS' refers to cases where the ANOVA for the population-type triplet reveals no significant differences after Benjamini-Hochberg procedure. See Table A6.1 and A6.2 (Appendix 6) for detailed statistical results. See Table A7.1 (Appendix 7) for the ancestral fitness values.

To determine the effects of population type on fitness in alternative environments, we performed a separate analysis for each environment, and subjected it to a stringent set of conditions before establishing statistical significance (see Materials and methods). We found that amongst the three population types, LL had the lowest fitness in alternative environments. In terms of K, LL had the lowest fitness in all four alternative environments (Fig. 3.4a). In terms of R, LL had the lowest fitness in two alternative environments (Fig. 3.4b). Importantly, there was no environment in which LL had significantly higher fitness than SL or SS. As with EA, the observations made in Fig. 3.4 can only be attributed to differences in the population sizes of LL, SL, and SS during evolution in the antibiotic cocktails.

3.4. Discussion

Large populations pay a cost for adapting faster

The three population types (LL, SL, and SS) had experienced identical selection environments containing the antibiotic cocktail. An earlier study had reported that there was no significant difference in their fitness in the selection environment at the same time point at which EA was measured in this study (Chavhan et al., 2019a). Moreover, all the three population types had descended from the same ancestral colony. Therefore, the observations in Fig. 3.2 and Fig. 3.3 can only be attributed to the differences in their population sizes experienced during their selection history.

The extant literature shows that increased EA can improve the performance of *E. coli* in the presence of the antibiotics used in our study (Morita et al., 1998; Nikaido and Pagès, 2012; Nishino et al., 2009). Hence, the presence of the antibiotics in the selection environment would intuitively suggest that EA should either be conserved or enhanced during adaptation to this environment. This is consistent with the increase in EA in the SL and SS lines (Fig. 3.3a). However, the decay of EA in LL lines demonstrates that even under the same selection environment, whether a fitness-related trait will enhance or decay, can depend on the population size faced during selection.

The role of population size in affecting the evolution of a trait is extremely well studied since the days of Sewall Wright (Charlesworth, 2009; Goodhart, 1963; Wright, 1984). All else being equal, for a given magnitude of stress, larger populations entail reduced effects of drift and therefore, stronger effects of selection (Charlesworth, 2009). All the experimental populations in our study were large enough for their evolutionary dynamics to be driven primarily via selection and not by drift (Cooper, 2018; Desai et al., 2007; Sniegowski and Gerrish, 2010).

Loss of efflux activity is primarily due to pleiotropic response

Evolutionary changes in a biological character like EA can be explained by two mechanisms that need not be mutually exclusive (Cooper and Lenski, 2000; Dorken et al., 2004; Hall and Colegrave, 2008; Maughan et al., 2006). The first of these is the accumulation of mutations that are neutral to fitness in the selection environment but non-neutral to the biological character in question (conventionally known as mutation accumulation (MA) (Cooper and Lenski, 2000; Kawecki et al., 1997; Kimura, 1983). The other mechanism is pleiotropy, in

which the adaptive variation which gets selected in the selection environment affects the biological character in question non-neutrally (Cohan et al., 1994; Cooper, 2014; Holt, 1996; Rose and Charlesworth, 1980). However, MA is unlikely to play a significant role in the evolution of characters that undergo experimentally detectable phenotypic changes within a few hundred generations (Cooper, 2018; Kassen, 2002). Specifically, all three population types were derived from the same ancestor. Importantly, all the three types had such large population sizes that drift over a few hundred generations is not expected to produce phenotypically detectable changes (Cooper, 2018; Desai and Fisher, 2007; Sniegowski and Gerrish, 2010). In other words, a time period of approximately 380 generations is too short to observe significant effects of MA in our experiments. However, some previous studies have attributed phenotypic changes in trait values to MA over similar timescales in similarly sized populations (Hall and Colegrave, 2008). Therefore, we briefly examined whether MA can have a significant effect on the evolution of efflux activity in our populations.

Mutation accumulation (MA) and pleiotropy have contrasting dependencies on a variety of population genetic parameters. MA is positively related with the rate of spontaneous mutations per individual per generation (μ) (Hall and Colegrave, 2008; Kimura, 1983), but is independent of the population size (N) (Kimura, 1983) and the speed of adaptation (Cooper, 2014; Cooper and Lenski, 2000; Hall and Colegrave, 2008). On the other hand, pleiotropic responses are influenced by both N and μ (Hall and Colegrave, 2008; Kimura, 1983), and are expected to be correlated with the speed of adaptation (SoA) (Cooper, 2014; Cooper and Lenski, 2000).

We found strong negative correlations between EA and SoA (Fig. 3.3 (b and c)), as well as between EA and the harmonic mean of population size. Furthermore, we found that the only non-LL population that lost its ancestral EA (SL - replicate 4, the outlier marked with an arrow in Figs. 2 and 3) was also the only outlier in terms of SoA. This outlier was similar to LL populations in terms of adaptation-speed. In other words, SL – replicate 4 was an outlier in terms of EA and SoA. However, it was not an outlier in terms of the negative correlations shown in Fig 3, making the correlations stronger. Thus, our experimental design enabled us to attribute the differences in population size across our treatments to the differences in the pleiotropic responses which shaped the evolution of EA (antagonistic pleiotropy in LL but synergistic pleiotropy in SL and SS).

If the LL populations had attained much higher mutation rates than SL and SS during ~ 380 generations of evolution in identical environments, then they could be expected to show the

lowest EA due to heaviest MA. However, any mutation that could increase μ in LL would have to appear *de novo* with the very small frequency of 1/N. Since selection does not act directly on mutation-rate-altering loci (Chao and Cox, 1983; Gentile et al., 2011; Orr, 2000; Sniegowski et al., 1997), μ-altering mutations spread via hitchhiking with mutations that are direct targets of selection. Thus, a mutation that increases μ (henceforth referred to as 'mutator') can only rise via random drift before an extra non-neutral mutation happens in its lineage. Since the occurrence of two mutations within a lineage is a highly improbable event (the maximum probability being μ^2), the mutator needs to rise to large frequencies before it can start hitchhiking with a non-neutral mutation.

The above argument makes the establishment of a mutator genotype highly unlikely if N is large. It has been demonstrated that mutators rise successfully via hitchhiking only if their initial frequency is larger than a threshold (Chao and Cox, 1983). Indeed, *de novo* mutators have been shown to go to extinction in most replicate populations (de Visser and Rozen, 2005; Giraud et al., 2001; Raynes and Sniegowski, 2014; Sniegowski et al., 1997, 2000; Taddei et al., 1997). Therefore, MA is unlikely to explain the fact that almost all replicates of SL and SS increased EA while all LL replicates had evolved reduced EA.

Furthermore, among populations that are large enough to experience clonal interference, the evolution of high mutation rate, leading to greater MA, is more likely to happen in smaller populations and not in larger ones (de Visser and Rozen 2005; Desai and Fisher 2007; Raynes et al. 2012). Indeed, recent experimental studies have demonstrated that despite starting with initial frequencies as high as 30%, the variants with higher mutation rates go to extinction in large asexual populations but hitchhike to fixation in small ones (Gentile et al., 2011; Raynes et al., 2012). Thus, even in the unlikely scenario of MA influencing efflux over a timescale of a few hundred generations, efflux was more likely to decay in SL and SS, not in LL. Since we observed the decay of efflux in LL and not SL or SS (Fig. 3.3), MA is unlikely to be an explanation for the observed EA patterns.

Table 3.1 presents a summary of the contrasting expectations of MA and pleiotropy with respect to the evolution of EA. Overall, the evolution of EA in our experiments was explained much better by correlated response to selection (pleiotropy) and not by random accumulation of conditionally neutral mutations.

	Predictions based on MA	Predictions based on pleiotropy
Correlation between SoA and EA	No correlation	Strong correlation *
Correlation between population size and EA	No correlation	Strong correlation *
The population-type most likely to lose EA	SL and/or SS	LL *
Convergence between replicates	Low	High *

Table 3.1. A summary of experimental predictions of the evolutionary fate of efflux activity (EA) based on mutation accumulation (MA) and correlated pleiotropic response. The asterisks denote the results that were observed in our experiments.

Quantitative differences in population size can lead to qualitative differences in the result of selection on characters

Biological characters can be lost over evolutionary time if they are unessential or disadvantageous (Fong et al., 1995; Jeffery, 2005; Porter and Crandall, 2003; Visser et al., 2010). The fate of the character in question (whether it decays, gets maintained, or enhances) during evolution is expected to be determined by the environment in which evolution occurs (Cooper, 2014). For example, extremely dark environments have been invoked to explain the loss of eyes in multiple systems (Jeffery, 2005; Protas et al., 2011). Similarly, the metabolic erosion observed over >50,000 generations in the Lenski Long-Term Evolution Experiment (LTEE) has been linked to the presence of only one usable carbon source throughout evolution (Leiby and Marx, 2014). Moreover, the environment in which evolution happens is conventionally assumed to be the major factor in determining the utility of the biological character in question (Hall and Colegrave, 2008). If in a given environment, the evolution of the biological character is largely affected by selection and not by drift, it is expected to result either in enhancement/maintenance or in decay of the character (but not both). It has been demonstrated empirically that if the selection-environments are different, the same biological character can decay by disparate evolutionary mechanisms (MA versus pleiotropy) (Hall and Colegrave, 2008). Our study adds to the understanding of evolutionary decay of characters by

showing how the character in question can decay or enhance in the same environment based on population size (Fig 3). In other words, our study shows that the selection-environment cannot always explain divergent evolutionary fates of a biological character. An important goal of experimental evolution is to understand how quantitative differences in population genetic parameters can lead to qualitatively different evolutionary outcomes. Our study takes a step in this direction by demonstrating how quantitative differences (in population size) can translate into qualitative differences (decay or enhancement) in a fitness-related trait during evolution.

One important question to ask here is why did the efflux activity decline in the LL populations? Although it is not possible to answer this question definitively from our data, we provide a brief speculation in this regard. Efflux is known to be an energetically expensive process (Nikaido 1994). In the presence of mutations that directly reduce the effects of antibiotic(s), efflux enhancing mutations are expected to be deleterious. However, in the absence of such mutations, efflux enhancing mutations are expected to be beneficial. Hence, once mutations that directly make the antibiotic ineffective arise, decay of efflux could be beneficial to fitness. Owing to their large population size, the LL populations could have accessed rare large-effect beneficial mutations for loci that directly render the antibiotic(s) ineffective. Therefore, once such mutations arose, the LL populations could increase their fitness by the decay of efflux. On the other hand, Inaccessibility to such rare mutations in the small populations (SL and SS) could have led to positive selection for efflux, which manifested as enhanced EA levels in SL and SS after ~380 generations. We note that recent advances in whole-genome whole population sequence analysis coupled with appropriate genetic manipulation can potentially be used to validate these speculations (Anand et al., 2016; Cooper, 2018; Long et al., 2015). However, such an analysis is outside the scope of the present study.

Large populations had lower fitness in alternative environments

The LL populations not only evolved reduced EA (Fig. 3.3a), they also had the lowest fitness among the three types in alternative environments (Fig. 3.4). It should be noted that the reduced EA of LL can be linked to low fitness only in two of the four alternative environments (ampicillin and high [Cu^{2+}]). This is because the other two alternative environments challenged the bacterial population with nutrient-poor conditions (not with xenobiotic chemicals), where EA is not expected to directly provide a fitness advantage.

In this study, we use random samples of potentially heterogeneous populations for assaying fitness in the alternative stressful environments. Such assays can potentially be driven by rare outliers with very high fitness values under these unexplored conditions, leading to inflated estimates of mean population fitness. We find that in the alternative stressful environments, the larger populations (LL) had lower fitness than the smaller ones (SL and SS). If the observed population-level fitness of LL was driven largely by the rare high fitness genotypes, when we remove the effects of these outliers, the robust (i.e., outlier-removed) value of the mean phenotype of the LL populations would be even lower than what is currently reported. This implies that our estimates about the reduction in fitness of LLs (vis-à-vis the SL and SS populations) is conservative. Technically, the same argument could work for the SL and SS populations too. However, since the size of these populations is ~16,500 times less than the LL populations, theoretically one expects the SL and SS populations to have relatively milder outliers owing to their low supply of variation. Thus, our estimates of fitness are expected to be more inflated in the larger populations as compared to the smaller ones. Importantly, it is highly unlikely that the inflation is higher for the smaller populations. Thus, our observations regarding fitness in the alternative environments are likely to be robust to the potential effects of rare highly fit outliers.

The fitness trajectories of asexual populations are known to show a decrease in the rate of fitness-increase as adaptation progresses (Chavhan et al., 2019a; Cooper and Lenski, 2000; Couce and Tenaillon, 2015; Elena and Lenski, 2003; Tenaillon et al., 2016). If pleiotropy plays a major role in shaping fitness in alternative environments, periods of faster adaptation are known to cause greater loss of unused functions within in a given population (Cooper and Lenski, 2000). This leads to the expectation that larger populations (which adapt faster) should have lower fitness in alternative environments. We found that this indeed was the case as the LL populations had lower fitness in the alternative environments in general. Our results are applicable across populations of different sizes and are congruent with those of Cooper and Lenski (2000) whose study was applicable within single populations at different times during their evolution. However, since the three population types in our experiments eventually reached similar fitness in the selection-environment despite initially adapting at different speeds, theory predicts that simple pleiotropic responses arising from beneficial mutations should lead to similar fitness across the three types in the alternative environments. Since the

three population types had significantly different fitness in the alternative environments, such simple pleiotropy cannot possibly explain our results.

The theory of asexual adaptive dynamics predicts that in a population of effective size 'N_e' and rate of spontaneous beneficial mutations (per genome per cell division) 'U_b', the number of beneficial mutations that can potentially compete with a beneficial mutation of selection coefficient 's' on its way to fixation is given by $2N_eU_b\ln(N_es/2)$ (Sniegowski and Gerrish 2010). For all the three population types, even with highly conservative estimates of $U_b = 0.0001$ and $s = 0.01$, this number amounts to more than a thousand competing beneficial mutations (the harmonic mean sizes of our experimental populations were close to 10^{10} for LL, and 10^6 for SL and SS). Moreover, both the measures of fitness (growth rate and carrying capacity) had increased by more than 1.5-fold in all the experimental populations by the end of the experiment (Chavhan et al. 2019). This suggests that the reference value of 's' should be much larger than 0.01, making the number of competing beneficial mutations even larger. A previous ~500 generations long experimental evolution study had found that asexual yeast populations adapted via dynamics that were best explained by the multiple-mutations paradigm (Desai et al. 2007). This paradigm implies that multiple beneficial mutations occurring within a lineage can simultaneously rise to high frequencies (Desai and Fisher 2007; Desai et al. 2007). The effective size (i.e., harmonic mean ~10^6) of even the smallest populations in our study (SL/SS) were similar to the largest population in Desai et al. (2007). Thus, for the given evolutionary time scale and population sizes, the adaptive events in our experimental populations are likely to be based on multiple mutations. However, the observations regarding fitness in the alternative environments cannot still be explained using a simple (i.e. linear / additive) combination of pleiotropic effects of multiple beneficial mutations. This is because if pleiotropic effects of multiple mutations just combined additively then, given that all three population types (i.e., LL/SL/SS) had the same fitness in the selection environments, they would have shown similar fitness values in the alternative environments too. However, this was not the case (Fig 4). Thus, to explain the fitness patterns in alternative environments, one requires further assumptions about how the pleiotropic effects of multiple mutations interact with each other.

One such assumption can be that the pleiotropic effects of beneficial mutations increase more rapidly than linear (say exponential or any other similar non-linear function) with the magnitude of direct effects. This is similar to the key assumption made by previous studies that large beneficial mutations have substantial pleiotropic effects while small beneficial mutations

show negligible pleiotropy (Lande, 1983). Moreover, the pleiotropic effect of a combination of multiple mutations can be smaller than the sum of their individual pleiotropic effects, as found by a previous study on *Escherichia coli* populations (Bohannan et al., 1999). Finally, we note that these assumptions/possibilities are not mutually exclusive, and their simultaneous action can also explain our observations.

Unfortunately, few studies have rigorously investigated the pleiotropic effects stemming from a combination of multiple mutations (Flynn et al., 2013; Schick et al., 2015). A recent study demonstrated that although the direct fitness effects of combinations of mutations consistently showed diminishing returns in the selection-environments, the pleiotropic fitness effects of such mutational combinations were highly variable in alternative environments (Schick et al., 2015). In other words, the pleiotropic fitness effects of combination of mutations were less than the sum of individual pleiotropic effects in some cases but more than the sum in others. (Schick et al., 2015). Unfortunately, we are not in a position to ascertain the exact nature of the relationship between pleiotropy and fitness, a predicament succinctly summed up by Cooper (2014): "The uncertainty of the form of pleiotropic effects reflects a general lack of understanding of how mutations interact to affect fitness, particularly over the long term."

In summary, our observations regarding the performance in alternative environments suggest that pleiotropy can potentially explain the link between SoA and preparedness for alternative environmental conditions, and mechanisms like EA need not always be invoked.

The idea that large populations can adapt so effectively and rapidly to their constant environment that they can be rendered vulnerable to future environmental change is new and counterintuitive. Our results could be relevant for understanding evolution in asexual populations experiencing changes between environments with fitness trade-offs (Andersson and Hughes, 2010; Bahri et al., 2009; de Roode et al., 2008). Our study shows that large populations can have lower fitness in alternative environments (Fig. 3.4). In such populations, adaptation is expected to be driven by mutations with large fitness benefits (Sniegowski and Gerrish, 2010), which are typically assumed to be associated with heavier pleiotropic disadvantages (Lande, 1983; Orr and Coyne, 1992) that can lead to greater fitness trade-offs. Thus, a logical next step would be to test a putative relationship between population sizes and fitness-tradeoffs using reciprocal selection experiments in multiple environments. This can lead to a better understanding of the population genetics of fitness trade-offs and ecological

specialization (Cooper and Lenski, 2000; Fry, 1996; Kassen, 2002, 2014; Rodríguez-Verdugo et al., 2014; Schick et al., 2015).

It is a well-established notion that very small population sizes can lead to such strong effects of genetic drift that the latter can overshadow selection and preclude adaptation (Charlesworth, 2009). Here we show that very large population sizes (as in LL), while leading to rapid adaptation in the current environment, can also render populations vulnerable to sudden environmental changes. Taken together, these insights point to a trade-off between maximizing adaptation rate and avoiding becoming vulnerable to environmental changes. Thus, populations that are small enough to avoid pleiotropic disadvantages but large enough to adapt (albeit slowly) to the current conditions (like SL and SS) can face environmental changes better than very large populations (like LL). We have used periodically bottlenecked bacterial populations to demonstrate the above trade-off. Therefore, our counterintuitive results can potentially have important implications for the fate of naturally occurring microbial populations that face periodic bottlenecks (e.g., host-to-host transfer of gut microbiota or pathogens), particularly if their environment changes in bouts. By demonstrating a novel link between population size and the immediate response to sudden environmental changes, our study thus adds to the prospering field of the evolution of evolvability (Carter et al., 2005; Crombach and Hogeweg, 2008; Jones et al., 2007; Wagner, 2013).

Chapter 4

Minimal requirements for divergent character fates in populations adapting to the same environment at different sizes

Highlights

- We used Wright Fisher simulations to investigate the minimal set of conditions that can translate differences in the sizes of asexual populations adapting to the same environment into antagonistic fates of an important fitness-affecting character.
- We studied the interactive effects of sign epistasis and differential mutational supply on the dynamics of evolution in asexual populations of different sizes.
- We found that the simultaneous presence of sign epistasis and differential mutational supply are essential to obtain divergent evolution of a fitness-affecting character during adaptation to the same environment. Importantly, the removal of any one of these two conditions results in convergent (and not divergent) character evolution.
- Our results have important implications for understanding how selection manifests itself in asexual populations of different sizes, particularly in the presence of sign epistasis.

Chavhan, Y.D., Shah, S., and Dey, S. (2019). Minimal requirements for divergent character fates in populations adapting to the same environment at different sizes (Manuscript under preparation).

4.1. Introduction

Random genetic drift and natural selection, two of the major mechanisms of evolution, affect the variation in biological traits in fundamentally different ways (McShea and Brandon, 2010). Genetic drift leads to stochastic changes in the population-wide distributions of trait values regardless of how they affect fitness (Futuyma, 2005). On the other hand, natural selection causes non-random changes in trait-distributions based on how individual trait values map to fitness in the given environmental context (Rice, 2004). Thus, when genetic drift (and not natural selection) is the primary mechanism driving the evolution of a biological character, the average trait value may decrease over time (i.e., decay) or enhance merely by chance (Hall and Colegrave, 2008). Here the term 'character' refers to a phenotypic attribute of an organism that is heritable; the term 'trait' represents a variant of the character in question. For example, plant height can be thought of as a biological character and 'tall' or 'short' as two distinct traits in this regard. Thus, genetic drift can manifest itself as the decay of the biological character in question in some replicate populations and its enhancement in some others. However, when the evolution of the character in question is driven largely by natural selection (and not genetic drift), it is expected to result either in the decay of the character or in its enhancement (but not both). Hence, when natural selection overwhelms genetic drift, different replicate populations are expected to result in convergent evolutionary changes in the biological character in question. Moreover, when selection shapes evolution more than drift, the fates of biological characters are conventionally assumed to be determined by the environment in which evolution takes place (Hall and Colegrave, 2008). Thus, characters can be lost during the course of evolution if they are detrimental to fitness (they carry a fitness cost) or are unessential in the environment in which evolution happens (Fong et al., 1995; Jeffery, 2005; Porter and Crandall, 2003; Visser et al., 2010). For instance, dark environments (where photoreception is expected to be unessential or costly) have been invoked to explain the loss of eyes in multiple taxa (Jeffery, 2005; Protas et al., 2011). Similarly, the metabolic erosion documented in Richard Lenski's famous long-term evolution experiment has been linked to the presence of only one usable source of carbon (glucose) (Cooper and Lenski, 2000). Thus, the environment in which evolution happens has conventionally been expected to determine the utility of biological characters. Is it possible for a biological character to have divergent utilities (and therefore divergent adaptive fates) in the same environment?

As discussed in the previous chapter (published as Chavhan et al., 2019b), we have found empirical evidence that adaptation to the same environment can result in divergent fates (decay versus enhancement) of an important fitness-associated character based on differences in population size. Specifically, while adapting to an environment with a cocktail of three antibiotics, the efflux activity enhanced in the small bacterial populations but decayed in the larger ones.

Here we use individual-based Wright-Fisher simulations to determine the population-genetic requirements that can reproduce such divergent evolution of a fitness-affecting biological character in adapting asexual populations of different sizes undergoing selection. Thus, our aim is to simulate the process of evolution in a single environment while meeting the following requirements: (1) All populations under consideration must adapt to the environment in question (the average fitness must increase). (2) In populations of a given size, the character in question must have convergent evolutionary fate across replicates. In other words, the evolution of the character in question (henceforth referred to as the 'focal character') must be driven largely by selection and not by random genetic drift.

The observations of Chapter 3 in terms of the evolution of efflux activity indicate that mutations that enhanced efflux were beneficial in the small populations but deleterious in the larger ones. Thus, simulating the empirical observations of Chapter 3 while adhering to the two conditions listed above requires that mutations which enhance the trait value of the focal character are expected to be deleterious in larger populations but beneficial in the smaller ones. Along similar lines, mutations that result in the decay of the focal character are expected to be beneficial in larger populations but deleterious in the smaller ones. Thus, the mapping between the expression of our focal character and fitness in the environment needs to be variable (not unique). In principle, such variable mapping between our focal character and fitness can potentially occur in two contexts: (i) mutations causing high character expression can lead to high or low fitness, depending upon the environment in question (Cooper, 2014; Kassen, 2002); (ii) mutations that enhance the expression of the focal character can be beneficial in some genetic background but deleterious in others (Weinreich et al., 2005). Since we consider only a single environment here, differences in environments cannot provide the basis for the variability in the mapping required by the current study. Hence, the genetic background can be the only potential source of such variability. In other words, simulating the empirical results of Chapter 3 requires that mutations that enhance the focal character are beneficial on some genetic backgrounds but deleterious on others. The phenomenon where the selective effect of

a mutation depends upon the genetic background is conventionally referred to as epistasis (Cordell, 2002; Fenster et al., 1997).

Epistasis is an umbrella term that encompasses all the cases where alleles at two or more loci interact with each other to affect the phenotype (Lehner, 2011; de Visser et al., 2011). Statistically speaking, epistasis occurs when the interactions between the effects of alleles at different loci are more important than their main effects (Fenster et al., 1997). Thus, epistasis makes combinations of multiple loci deviate from the expectation of additivity in their in individual effects (Cordell, 2002). In its simplest form, epistasis makes the effect of a combination of distinct loci deviate from their additive effects only in terms of magnitude (and not sign). Such epistasis is conventionally categorized as 'magnitude epistasis' (Remold, 2012), and is commonly observed in combinations of multiple beneficial mutations (Chou et al., 2011; Kryazhimskiy et al., 2014). In extreme cases, the sign of the effect of a mutation on fitness in the given environment can itself be under epistatic control, making a mutation beneficial in the given environment on some genetic backgrounds but deleterious on others. Such epistasis is conventionally known as 'sign epistasis' (Weinreich et al., 2005), and has been observed in multiple studies in asexual systems (Carroll et al., 2014; Szendro et al., 2013a; Zee et al., 2014).

As described above, our simulations require that the sign of the fitness effects of individual mutations that enhance the focal character is contingent on the genetic background. Therefore, such mutations are expected to exhibit sign epistasis. Moreover, since all the populations in the evolution experiment pertaining to Chapter 3 had a common ancestor, all the populations in our simulations also need to start with the same ancestor. Thus, all the asexual populations in our simulations need to originate from the same genetic background, regardless of their size. Since we aim to simulate that the focal character decays during adaptation in large populations but enhances during adaptation in smaller ones, our simulations would require that populations of vastly different sizes diverge genotypically with respect to each other as they adapt to their common environments. What could be the source of such genotypic divergence in asexual populations of different sizes originating from the same ancestor and adapting to the same environment? As discussed in the previous chapter, adaptation in extremely large asexual populations is primarily driven by beneficial mutations of large effect sizes (Sniegowski and Gerrish, 2010). Since mutations of larger benefit sizes are generally expected to be rarer (Eyre-Walker and Keightley, 2007; Kassen and Bataillon, 2006; Neher, 2013), mutations that confer large benefits can be too rare to be accessible to small populations (Desai and Fisher, 2007;

Sniegowski and Gerrish, 2010). In the absence of such large effect beneficial mutations, adaptation is expected to occur slowly in small populations, driven largely by relatively common mutations that carry small benefits. Thus, if populations of vastly different sizes adapt to the same environment, the range of beneficial mutations that rise in frequencies within the smaller populations is predicted to be different from that in the larger ones. Such differences can be a potential source of variation in the genetic backgrounds across populations of different sizes originating from the same ancestor; epistasis can come into action once such variation in genetic background arises.

We constructed a Wright Fisher model incorporating the features described above to study the interactive effects of sign epistasis and differential mutational supply on the dynamics of evolution in asexual populations of different sizes. Next, we relaxed the above conditions to arrive at the minimum framework required to simulate divergent evolution of a fitness-affecting focal character in populations of different sizes adapting to the same environment. We show that differential per generation mutational supply across fitness affecting loci and sign epistasis across these loci are the minimum requirements for reproducing the observations of Chapter 3 in a generalizable manner.

4. 2. Methods

Model 1

We constructed an individual-based model of asexual Wright-Fisher populations with discrete generations (Charlesworth, 2009; Fisher, 1931; Rice, 2004). The populations were composed of haploid individuals and the size of any given population remained constant across generations. The genome of each individual was conceptualized as a combination of two fitness-affecting loci (A and B), each with three discrete allelic states (L (low gene expression), O (intermediate gene expression), and H (high gene expression)). This gave rise to a discrete genotype space containing nine different genotypes. Each locus underwent random mutations independent of the other locus. We categorized all mutations that reduced gene expression as deleterious and mutations that enhanced gene expression as beneficial. In agreement with theoretically and empirically established trends of distributions of mutational effects (Eyre-Walker and Keightley, 2007; Fisher, 1930; Kassen and Bataillon, 2006; Neher, 2013), deleterious mutations were much more probable than beneficial mutations in our model. The occurrence of two simultaneous mutations in an individual was so unlikely that such an event can be ignored given the range of population sizes used in our simulations. We used a landscape with arbitrarily assigned fitness values (Jain et al., 2011), whose shape changed according to the population genetic treatment in question (see below). The fitness value of each individual was taken as the number of offspring produced by it in each generation (e.g., the fitness values of HL and LL are 5 and 1, respectively (Fig. 4.1)). We used a two-letter notation for the genotypes in this model, with the first and second letters representing the allelic states of loci A and B, respectively. We arbitrarily assigned OO as the wild-type genotype and started all our simulations using clonal population composed completely of OO individuals. Every generation, each population grew in size based on the fitness of its constituent individuals and was bottlenecked down to its original size via random sampling. We let all our simulated populations evolve for one thousand generations and tracked the frequencies of all the nine genotypes within a given population throughout this duration. We also determined the average population fitness after each generation and used this information to reconstruct trajectories of fitness increase during evolution. We carried out these simulations at a wide range of population sizes, spanning from 10^3 to 10^7 individuals.

There were two key readouts from our simulations: (1) How much a given population has adapted during the 1000 generations of evolution. (2) What is the composition of the population

at any given time. We used this information to determine whether the two loci evolved towards opposite (divergent) allelic states in our simulations.

Treatment: Sign Epistasis

In all the two-locus fitness landscapes throughout our study, higher expression of locus B always led to higher fitness values, regardless of the allelic state at locus A (Fig. 4.1). In our principal treatment, locus A showed sign epistasis on locus B backgrounds such that higher expression of gene A was beneficial on the BL or BO backgrounds but deleterious on the BH background (Fig. 4.1a).

We also conducted control simulations where such sign epistasis was removed such that higher expression of gene A was always beneficial, regardless of the allelic state at locus at locus B (Fig. 4.1b).

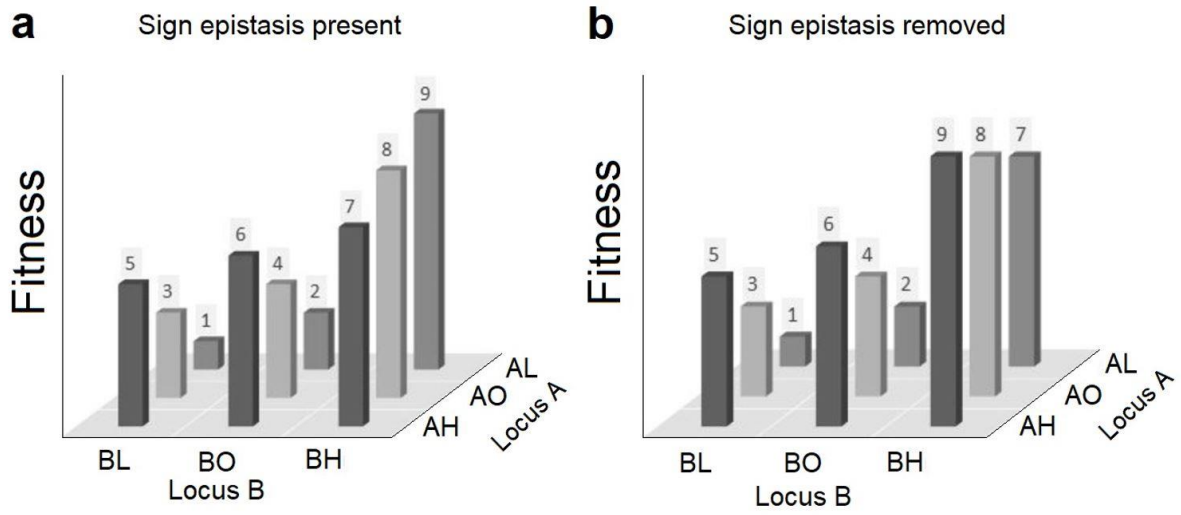

Fig. 4.1. Two-locus three-allele fitness landscapes. The values on top of each genotype represent its fitness. (a) Landscape with locus A showing sign epistasis on locus B backgrounds. (b) Landscape without any sign epistasis.

Treatment: Differential mutational supply across loci

In our principal treatment, locus A and locus B mutated with different rates (μ). Specifically, mutations on locus A were more common than mutations on locus B. This gave rise to the trend of mutation rates across shown in Fig. 4.2. The exact values of the mutation rates used in our simulations are given in Table 4.1.

We also conducted control simulations where such differences in mutational supply across the two loci was removed. Such control simulations were of two kinds, one in which both the loci followed the mutational tendencies belonging to locus A in Fig. 4.2, and the other in which both the loci mutated like locus B in Fig. 4.2.

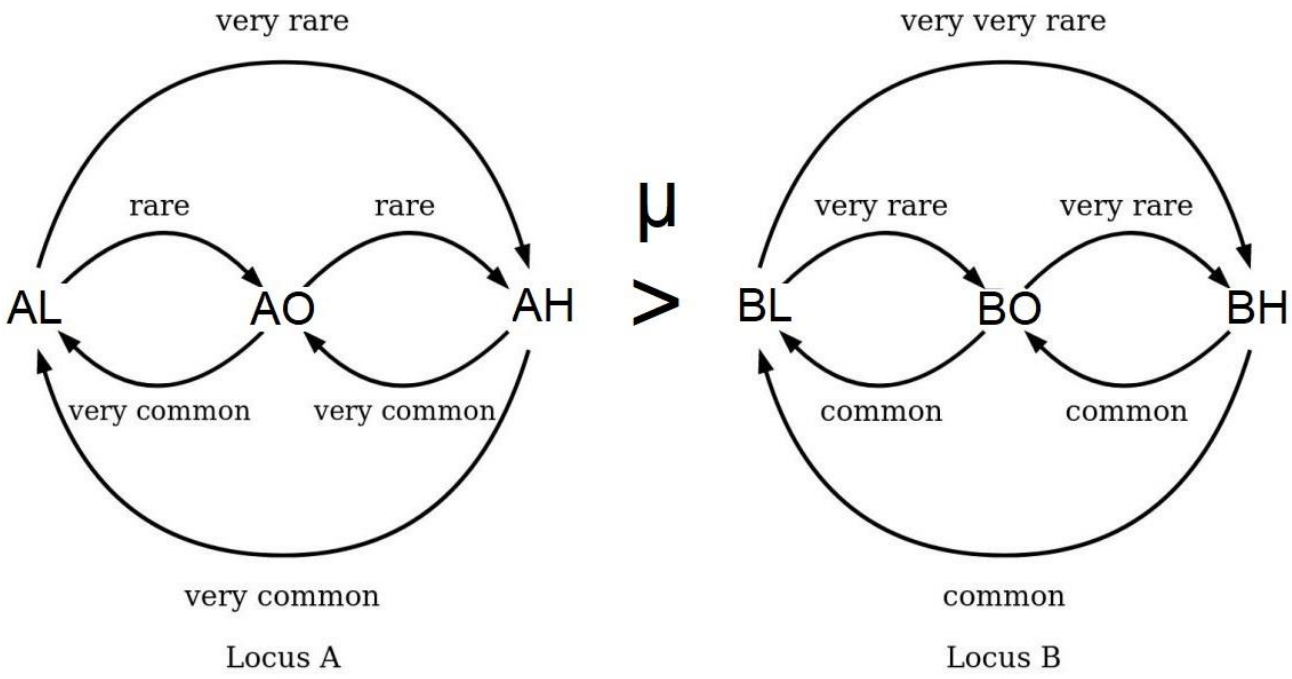

Fig. 4.2. Differential mutation rates across loci A and B. Locus A mutated much more frequently than locus B.

Mutation rate category	Value
Very common	10^{-3}
Common	5×10^{-4}
Rare	5×10^{-5}
Very rare	5×10^{-8}
Very very rare	10^{-9}

Table 4.1. Mutation rate values corresponding to the differential mutational supply across loci A and B (see Fig. 4.2).

Model 2

We carried out a separate set of simulations with a three-locus three-allele framework. To this end, we added one extra locus (locus C) to Model 1 and used the same three allelic states as before (L, O, and H). Here the character in question was controlled by the expression of both loci A and C, with higher expression leading to greater trait value. Since locus C was equivalent to locus A, the two loci had identical mutational tendencies, and thus mutated much more frequently than locus B (see Fig. 4.2 and Table 4.1). Similar to locus A of Model 1, loci A and C of Model 2 showed sign epistasis on locus B backgrounds (their higher expression being beneficial on BL and BO backgrounds, but deleterious on the BH background).

Our experiments (Chapter 3) had revealed that the small and large populations had eventually reached similar fitness despite evolving divergent fates in terms of efflux activity. This suggests that the small and large populations would have taken different mutational paths, eventually reaching different destinations with similar fitness. This indicates the existence of multiple global peaks in our experiments, with at least one peak being mutationally accessible to the small populations. However, the mutational tendencies of Model 1 entailed that the global peak can be reached only via large-effect beneficial mutations that are too rare to be accessible to small populations. Interestingly, small asexual populations can accrue multiple small-effect mutations and eventually reach the same fitness as large populations (Cvijović et al., 2018; Desai, 2013). Furthermore, as shown in Fig. 4.3, if loci A and C show such dependence on locus B and the latter's fitness effects are independent of the former two, the resulting landscape cannot have two distinct global fitness peaks.

We created a fitness landscape with two global peaks by allowing the expression of locus B to be influenced by the A and C backgrounds to a very small degree. In this fitness landscape, loci A and C still show sign epistasis on locus B backgrounds in most regions of the genotypic space. To this end, we first combined two different two-locus fitness landscapes similar to the landscape of Model 1 (one with loci B and A and the other with loci B and C) to obtain a three-locus landscape where A and C showed sign epistasis on B backgrounds. Since this three-locus landscape had only one peak (LHL), we tweaked the landscape to introduce an extra global fitness peak with high expression of both A and C. The resulting 4d fitness landscape comprising 27 different genotypes and adhering to the above conditions is shown in Fig. 4.4.

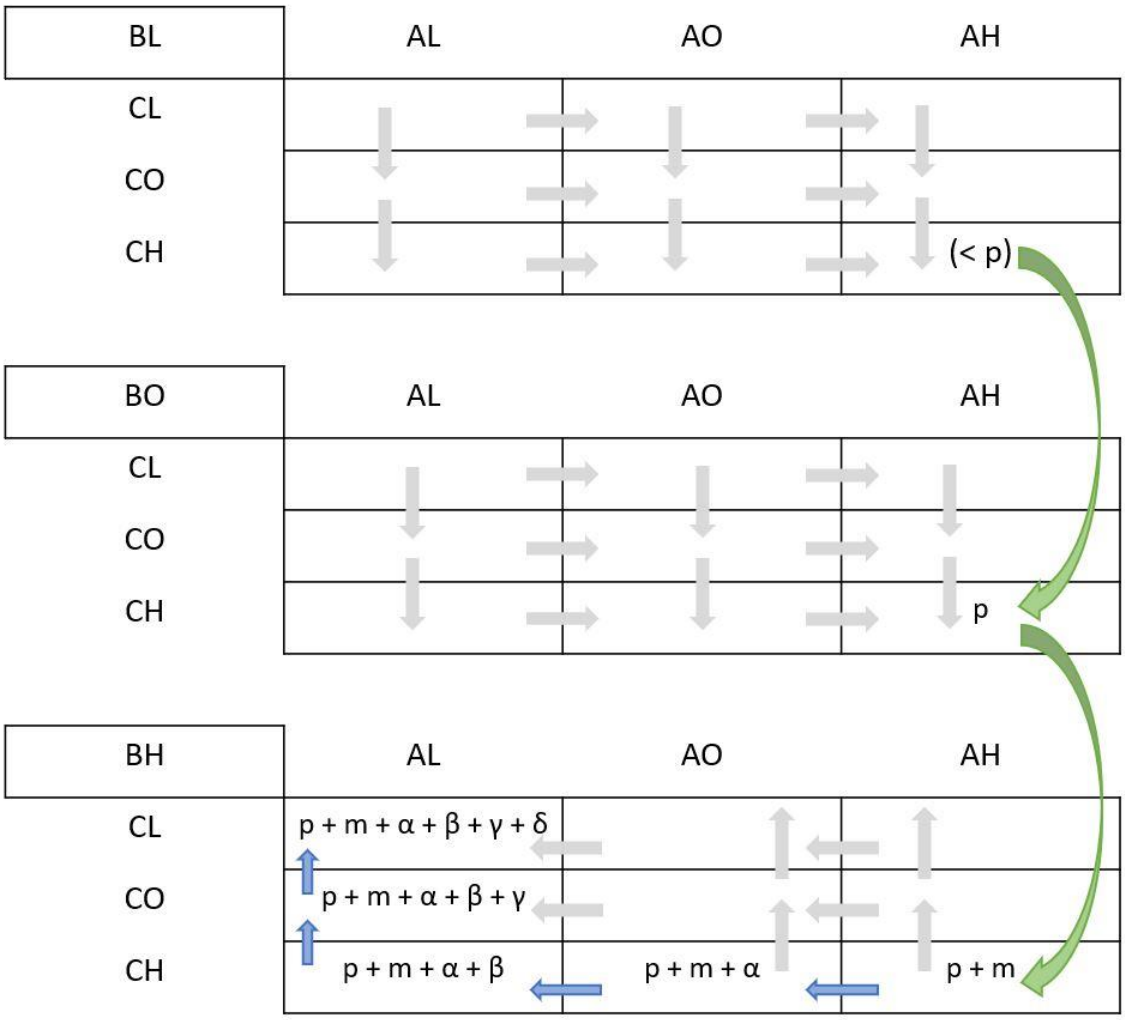

Fig. 4.3. The figure shows 27 boxes, each corresponding to one genotype on a three-locus three-allele landscape. All pairwise arrows point towards genotypes of higher fitness, regardless of their color. The green arrows depict that in terms of fitness, BH > BO > BL, regardless of the allelic states of loci A and C. All the lowercase English and Greek letters have positive values. English letters represent fitness changes due to changes on locus B; Greek letters represent fitness changes due to changes on loci A and C. The grey arrows show the pairwise fitness trends across the allelic states of loci A and C. The HOH genotype is assumed to have fitness p. Since BH > BO in terms of fitness, HHH will have a higher fitness than HOH, depicted as p + m. Sign epistasis demands that on the BH background, AH < AO < AL in terms of fitness. The same logic applies to CH < CO < CL on the BH background. We follow the blue arrows to compare the fitness of the LHL genotype with that of HHH. It is clear that LHL must be fitter than HOH (and all other genotypes). Thus, LHL also represents the global fitness peak. Therefore, a fitness landscape where two loci (A and C) individually show sign epistasis on the background of a third locus (B) cannot have multiple global peaks with opposite allelic states of loci A and C if the fitness effects of locus B are independent of the other two loci.

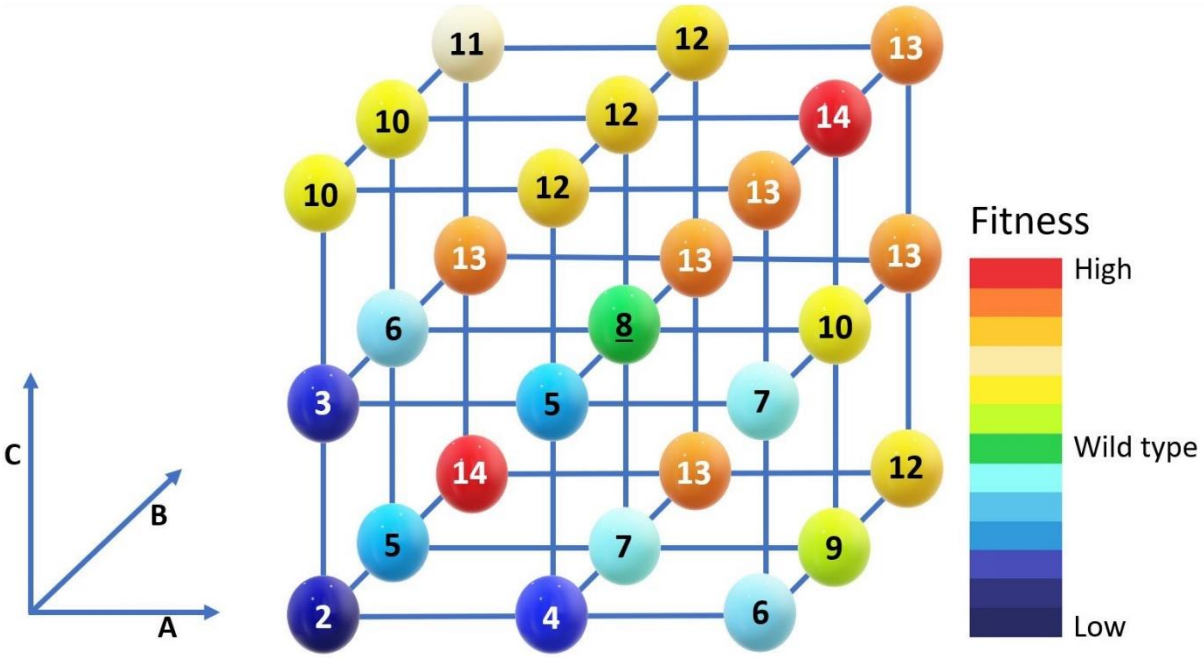

Fig. 4.4. The three-locus three-allele fitness landscape used in Model 2. The genotypic space is shown here as a cube with 27 distinct nodes, where each node represents one genotype. The fitness of each genotype is represented by the number (and also by its color, following the code shown on the right). The three axes shown on the left correspond to the three loci (A, B, and C), with the origin at LLL and the arrows pointing towards direction of allelic states with higher gene expression. The wild type (OOO) has a fitness of 8 on this landscape and is shown in green.

4.3. Results and Discussion

Model 1

Large populations reached the global fitness peak, but small populations could not

In our principal treatment (with both sign epistasis and differential mutational supply across loci A and B), we observed different dynamics of fitness increase in populations of different sizes. Specifically, the larger populations not only adapted much faster than the smaller ones, they also adapted to a greater extent (Fig. 4.5).

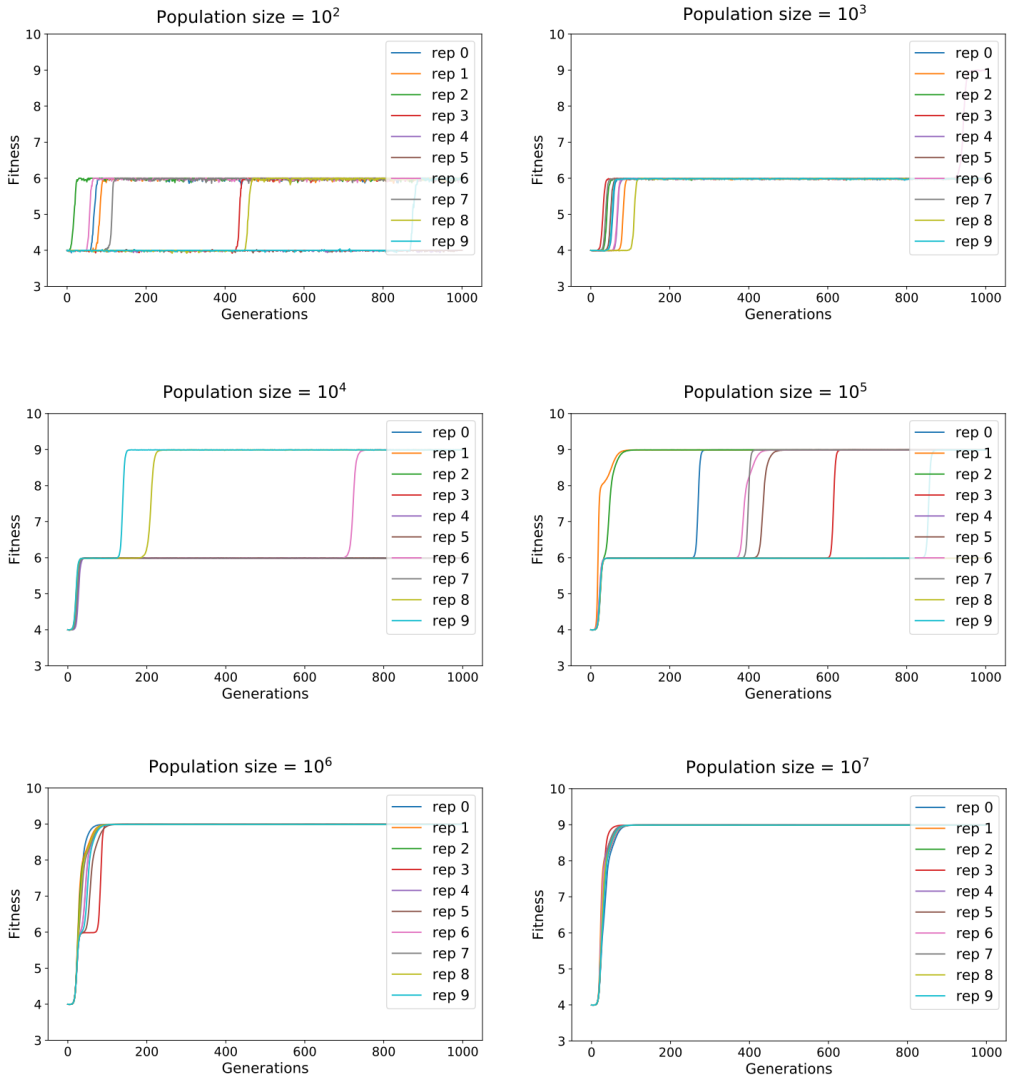

Fig. 4.5. Trajectories of average fitness on the two-locus landscape with sign epistasis in populations of different sizes when there was differential mutational supply across the two loci. The wild-type fitness was 4; the global fitness peak corresponded to 9.

Most populations with a size of at least 10^5 individuals succeeded in reaching the global fitness peak. On the other hand, most populations with $\leq 10^4$ individuals could not reach the global fitness peak (Fig. 4.5). The smallest path between the wild-type genotype and the global fitness peak involved two mutational steps on the two-locus landscape (Fig. 4.1a). One of these mutations (BO to BH) had an extremely small probability of occurrence (5×10^{-8}), making it inaccessible for populations of small sizes ($< 10^5$ individuals). However, such small populations could readily arrive at the AO) to AH mutation. Since this mutation was beneficial on the BL and BO backgrounds, even the small populations could readily attain a 50% fitness increase by converging on the HO genotype (Fig. 4.5, 4.6 and 4.7). Thus, populations of both small and large sizes were successful in increasing their fitness on the two-locus fitness landscape.

Locus A evolved divergent fates in populations adapting on the same landscape at different sizes

We found that most populations with 10^4 or lesser individuals evolved enhanced expression of gene A during adaptation while populations with 10^5 or more individuals adapted by reducing the expression of gene A (Fig. 4.6 and 4.7). Thus, a combination of sign epistasis and differential mutational supply across loci can successfully lead to divergent fates of fitness-affecting characters in populations adapting to the same landscape at different sizes. Hence, the fitness landscape of Fig. 4.1a and the mutational tendencies of Fig. 4.2 can be a potential explanation of the empirical observations regarding the divergent evolution of efflux activity in populations of different sizes as reported in the previous chapter.

Interestingly, the populations that were successful in reaching the global fitness peak (LH) showed two distinct kinds of adaptive trajectories. Among such populations, most of the smaller ones (10^4 and 10^5) showed staircase like trajectories of fitness increase (with long stretches of no fitness increases) while most populations with $\geq 10^6$ individuals showed smooth trajectories (Fig. 4.5).

Staircase-like trajectories occur when populations wait for new beneficial mutations to arrive and rise via natural selection (Sniegowski and Gerrish, 2010). Most of the staircase-like trajectories showed a two-step fitness increase punctuated by a flat region at an average fitness of 6. By probing population compositions in the flat regions, we confirmed that the HO genotype (fitness = 6) had risen to very high frequencies in most populations that displayed a

staircase. Curiously, the distance between HO and the global peak (LH) is larger than the distance between the wild-type (OO) and the global peak. Moreover, the mutation rates in our study (Fig. 4.2 and Table 4.1) are low enough to disregard the probability of multiple mutations happening in the population sizes under consideration. Taken together, the genotype that initially rose to high frequencies (i.e., HO) was leapfrogged by a mutation originating on another genetic background (Gerrish and Lenski, 1998) (this was confirmed using high-resolution time-series of genotypic distributions).

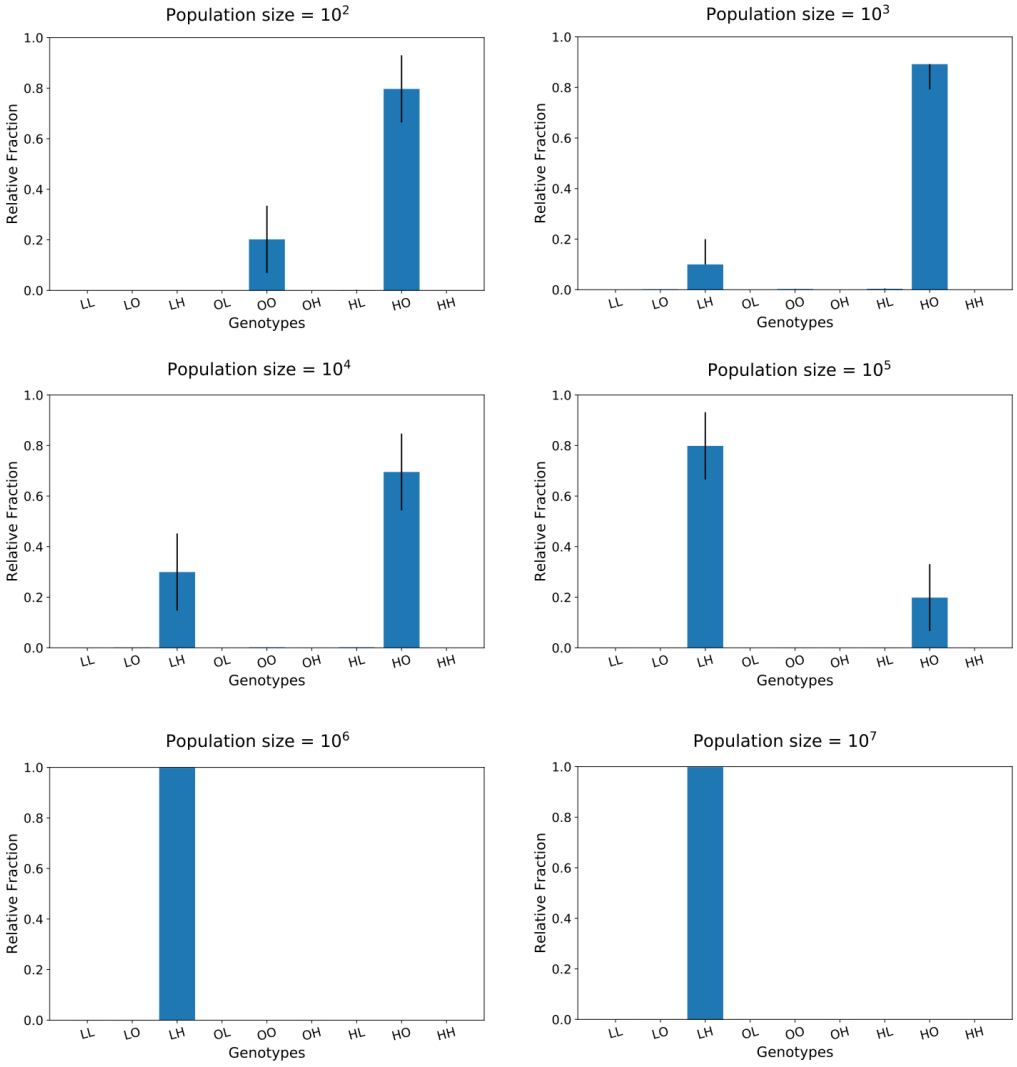

Fig. 4.6. The composition of simulated populations after 1000 generations of evolution on the two-locus fitness landscape with epistasis and differential mutational supply across the two loci. The error bars represent SEM (N=10). The small populations ($\leq 10^4$) had largely converged on the HO genotype (which had fitness = 6) while the large populations ($\geq 10^5$) had primarily converged on the LH genotype (which had fitness = 6). Overall, while both small and large populations adapted on the two-locus fitness landscape, the expression of gene A enhanced in relatively smaller populations but decayed in relatively larger ones. Also see Fig. 4.7.

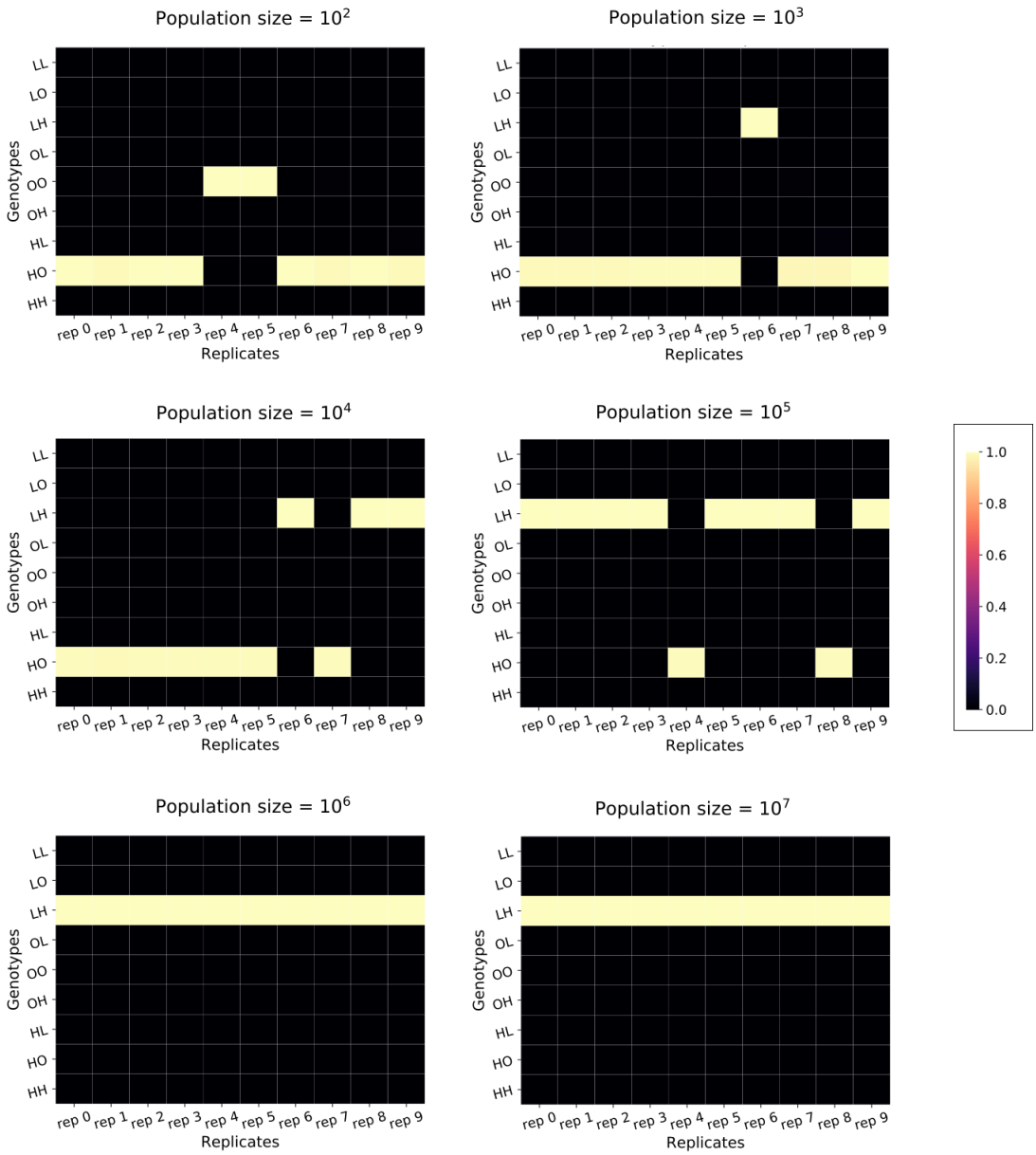

Fig. 4.7. Frequency distributions within populations of different sizes after 1000 generations of evolution on the two-locus fitness landscape with sign epistasis and differential mutational supply across the two loci. The color scale shown on the right refers to within population frequencies. Almost all populations showed fixation of a single genotype after 1000 generations of evolution. Whereas the small populations ($\leq 10^4$) had largely fixed on the HO genotype, the larger ones ($\geq 10^5$) had primarily fixed on the LH genotype.

Taken together, the simultaneous presence of sign epistasis and differential mutation supply across loci can translate quantitative differences in population sizes to qualitative differences (enhancement versus decay) in the fate of a fitness-affecting character during adaptation in the same environment. Next, we eliminated sign epistasis and differential mutational supply, both

one-by-one and in combination, to determine the minimal conditions for evolving divergent character fates in large versus small populations.

Removal of sign epistasis led to convergent character fates across large and small populations

We used the fitness landscape shown in Fig. 4.1b to remove sign epistasis from our control simulations while still using the mutational tendencies shown in Fig. 4.2 and Table 4.1. We found that in the absence of sign epistasis, populations of several different sizes ranging from 10^3 to 10^7 individuals evolved convergent fates of the character in question (controlled by locus

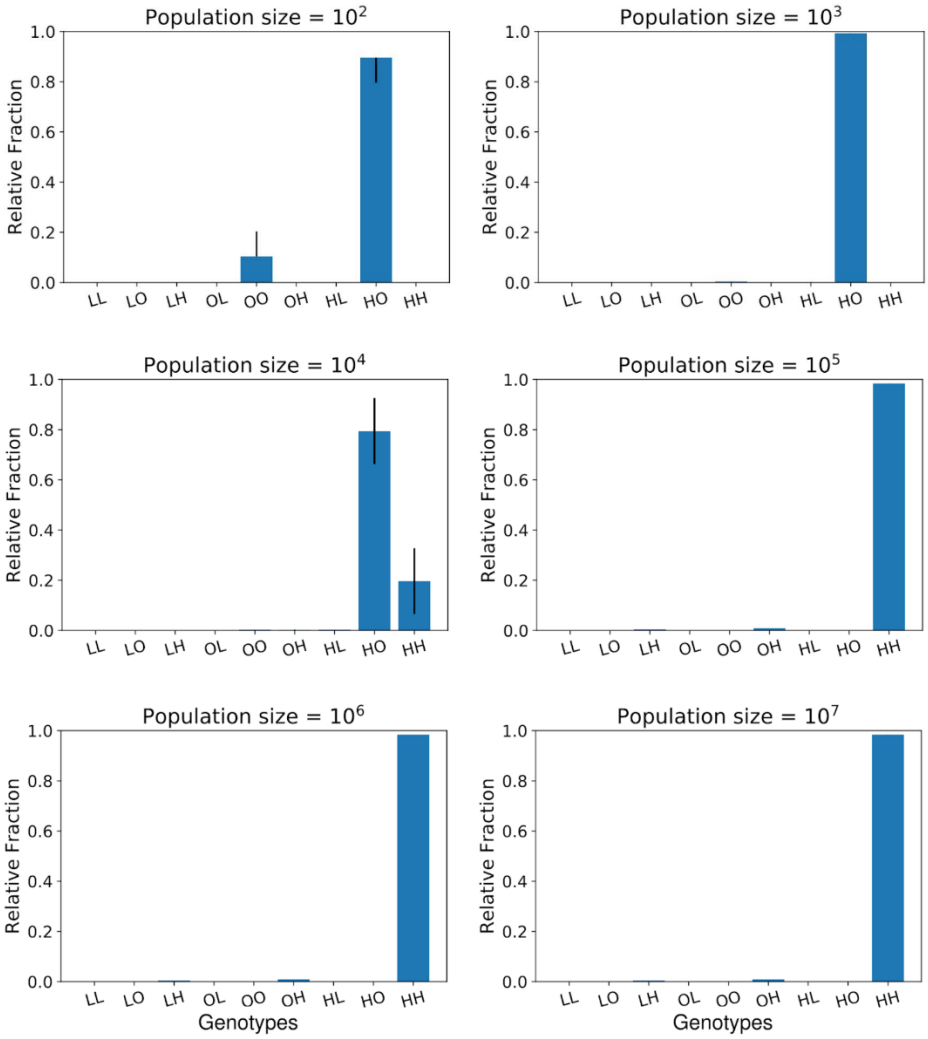

Fig. 4.8. The composition of simulated populations after 1000 generations of evolution with differential mutational supply across the two loci but without sign epistasis. The error bars represent SEM (N=10). All the simulated populations evolved enhanced expression of gene A, regardless of the population size.

A) (Fig. 4.8). Specifically, adaptation on the two-locus fitness landscape without sign epistasis resulted in enhanced expression of gene A in both large and small populations despite the presence of differential mutational supply across the two loci (Fig. 4.8). Thus, sign epistasis was an essential requirement for divergent evolution of character fates across populations adapting to the same environment at different sizes.

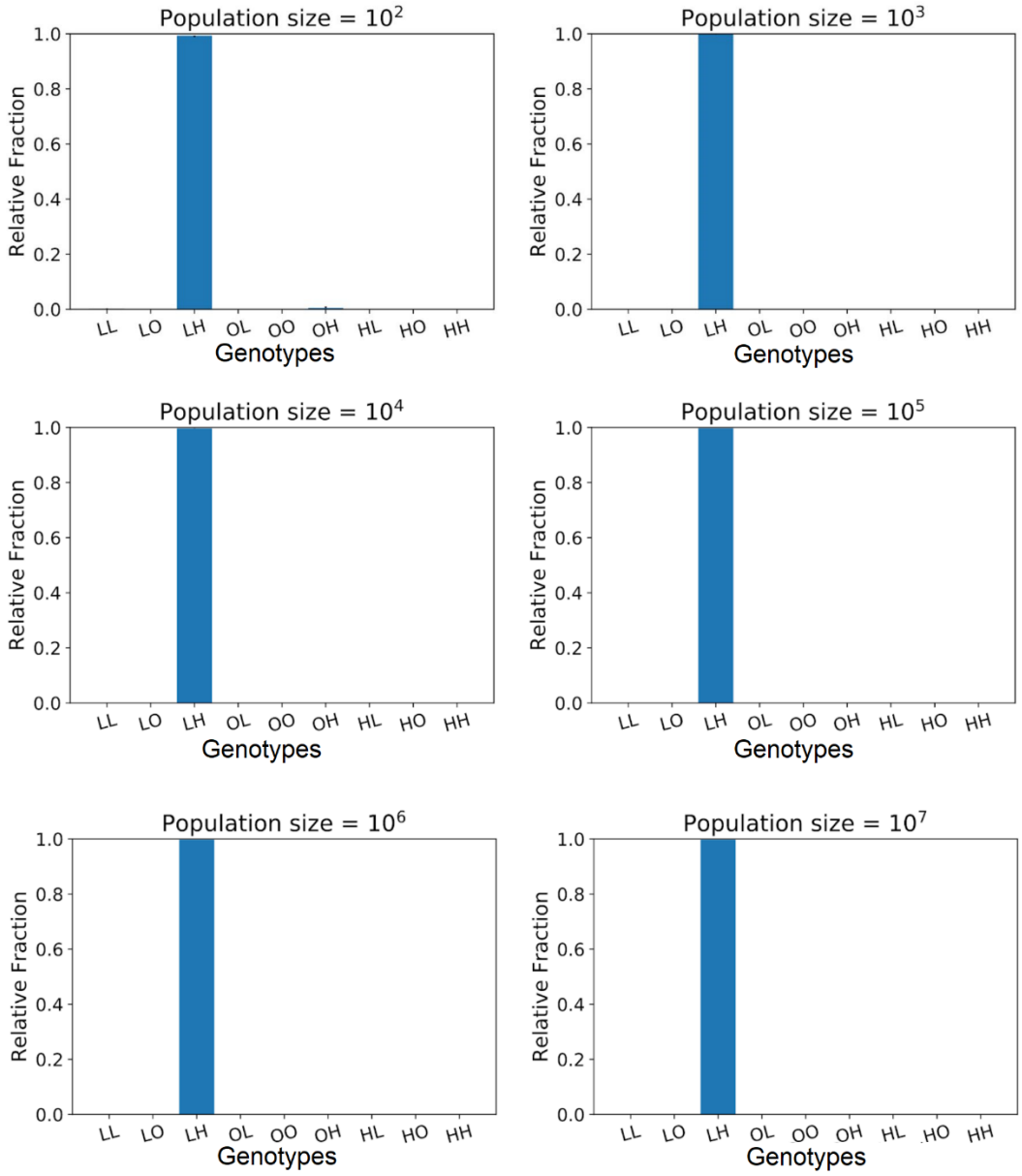

Fig. 4.9. The composition of simulated populations after 1000 generations of evolution with sign epistasis but without differential mutational supply across the two loci. The mutational tendencies of locus B were made identical to those of locus A in these simulations, and both the loci had mutational tendencies as shown in the left half of Fig. 4.2. The LH genotype was the global maximum. The error bars represent SEM (N=10). All the simulated populations evolved decayed expression of gene A, regardless of the population size.

Identical mutational supply at the two loci prevented the divergence of character fates across large and small populations

In another set of controls, we simulated evolution in the presence of sign epistasis but without differential mutational supply across the two loci. The differences in mutational supply across loci A and B can be removed in two distinct ways. First, by making both the loci follow the mutational tendencies of locus A. Second, by making them follow the mutational tendencies of locus B. We conducted both kinds of simulations and determined the evolution of character fates across populations adapting to the same environment at different sizes.

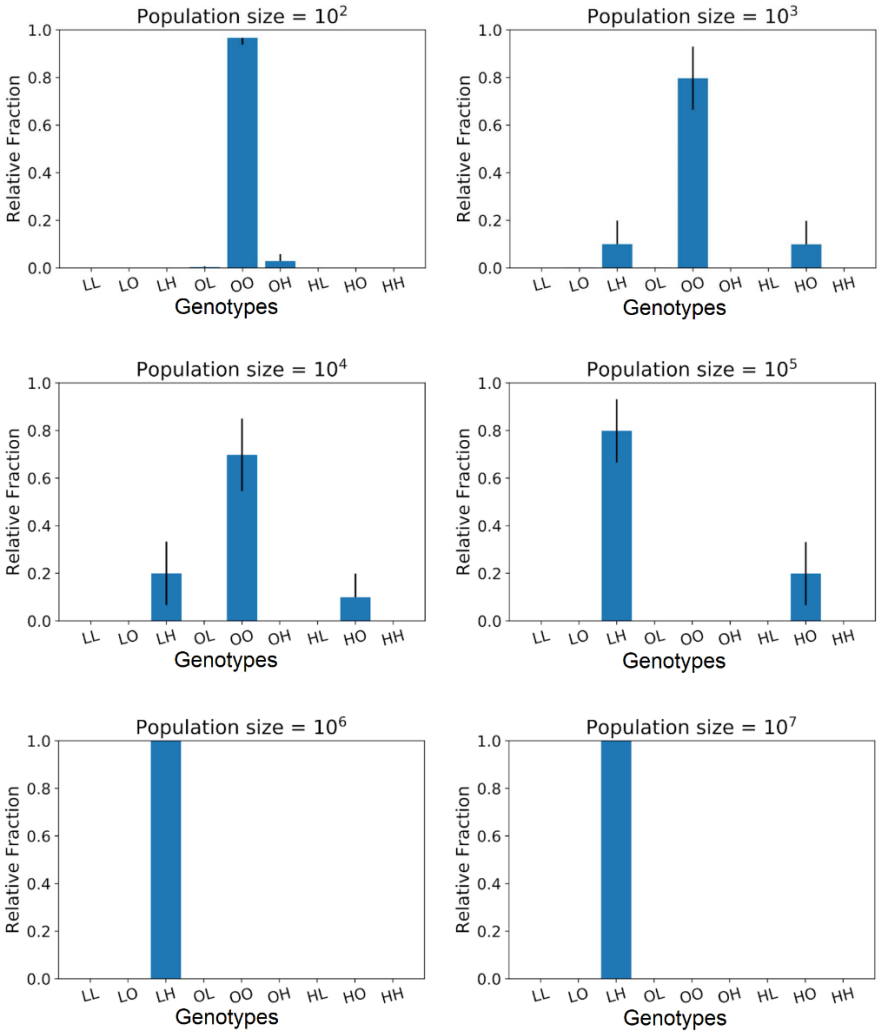

Fig. 4.10. The composition of simulated populations after 1000 generations of evolution with sign epistasis but without differential mutational supply across the two loci. The mutational tendencies of locus A were made identical to those of locus B in these simulations, and both the loci had mutational tendencies as shown in the right half of Fig. 4.2. The error bars represent SEM (N=10). There was no divergence of character fates (decay versus enhancement) across populations of different sizes. The small populations ($\leq 10^4$) largely remained on the ancestral OO genotype; the larger populations ($\geq 10^5$) primarily converged on the LH genotype.

In the treatment with differential mutational supply across the two loci, the BO to BH mutation was inaccessible to populations of sizes smaller than 10^5 individuals. However, when both the loci followed the mutational tendencies of locus A in the control simulations, the BO to BH mutation became readily accessible, even to populations with only 10^2 individuals. This led to highly convergent adaptation where populations of all sizes successfully fixed on the global fitness peak within 1000 generations (Fig. 4.9).

The AO to AH mutation was readily accessible to populations of 10^2 individuals in the treatment with differential mutational supply across the two loci. However, when both the loci followed the mutational tendencies of locus B in the control simulations, the probability of an AO to AH reduced by three orders of magnitude and became identical to the probability of a BO to BH mutation (Fig. 4.2 and Table 4.1), rendering both these mutations largely inaccessible to populations with $\leq 10^4$ individuals. In the absence of these mutations, the populations with $\leq 10^4$ individuals largely remained at the wild-type genotype, even after 1000 generations of evolution (Fig. 4.10). Contrastingly, most populations with $\geq 10^5$ individuals could access the global fitness peak, and thus converged on the latter within 1000 generations (Fig. 4.10). Most importantly, there was no divergence (decay versus enhancement) in the fate of either of the two characters (controlled by loci A and B, respectively).

Taken together, Fig. 4.9 and Fig. 4.10 show that differential mutational supply is also essential for divergent character evolution across populations adapting to the identical environmental conditions at different sizes.

We also conducted double-control simulations in which populations evolved in the absence of both sign epistasis and differential mutation supply across loci. As described earlier, there are two distinct ways of making the mutational supply uniform across the two loci. Therefore, we conducted two different sets of double-control simulations. In the absence of sign epistasis, when both the loci followed the mutational tendencies of locus A, all the simulated populations (with sizes ranging from 10^2 to 10^7) converged on the global fitness peak (Fig. 4.11). When both the loci followed the mutational tendencies of locus B, in the absence of sign epistasis, the populations with $\leq 10^3$ individuals either remained at the wild-type genotype or evolved enhanced expression at one of the two genes (Fig. 4.12). On the other hand, most populations with $\geq 10^5$ individuals converged the global fitness peak (which was shifted from LH to HH by the removal of sign epistasis) (Fig. 4.12). Importantly, there was no character divergence across adapting populations of different sizes (ranging from 10^2 to 10^7 individuals). Overall, the

simultaneous absence of sign epistasis and differential mutational supply across loci failed to lead to qualitative changes in character fates across populations adapting to the same environment at different sizes.

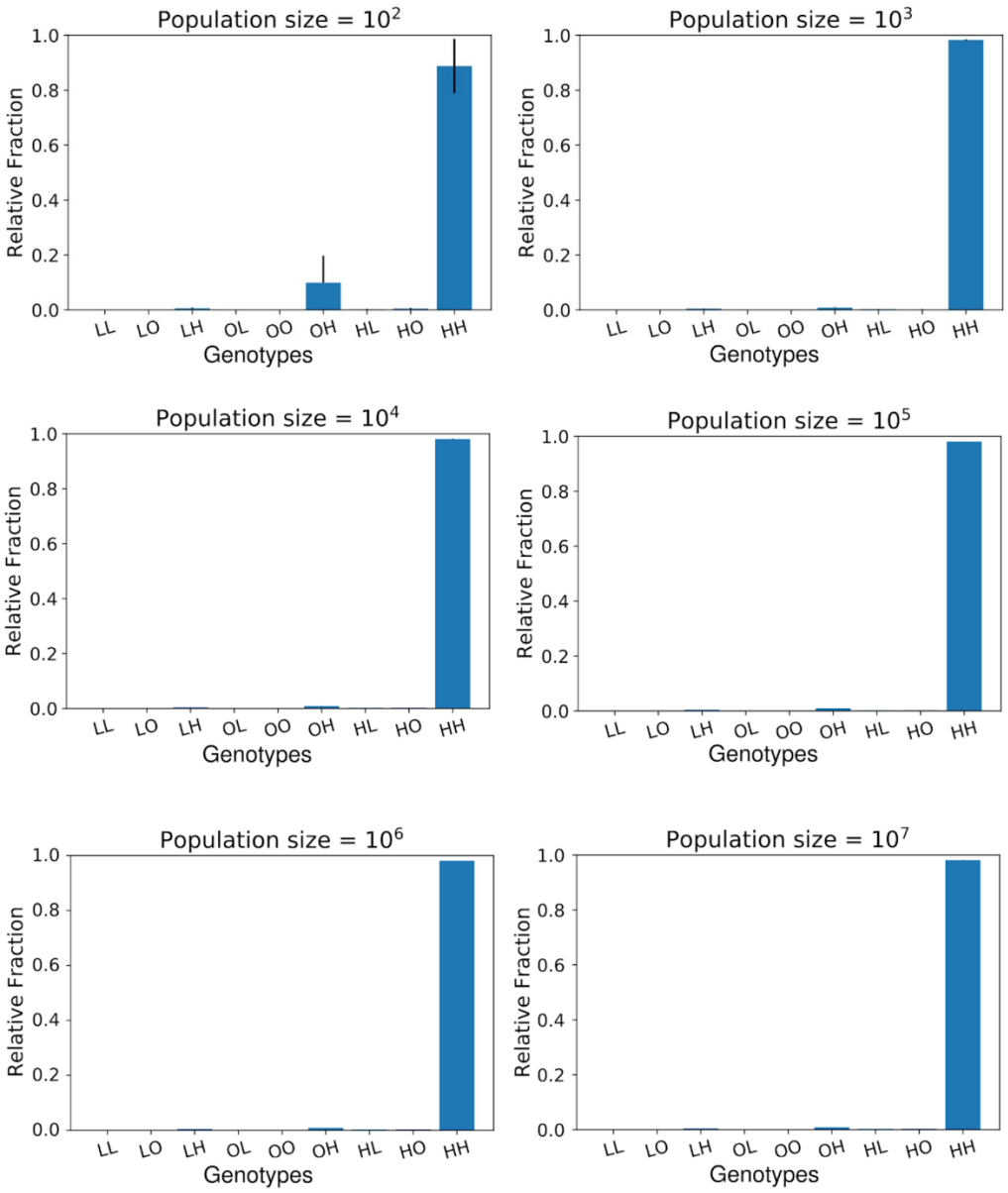

Fig. 4.11. The composition of simulated populations after 1000 generations of evolution without both sign epistasis and differential mutational supply across the two loci. The mutational tendencies of locus B were made identical to those of locus A in these simulations, and both the loci had mutational tendencies as shown in the left half of Fig. 4.2. The error bars represent SEM (N=10). Convergent (and not divergent) character evolution was observed.

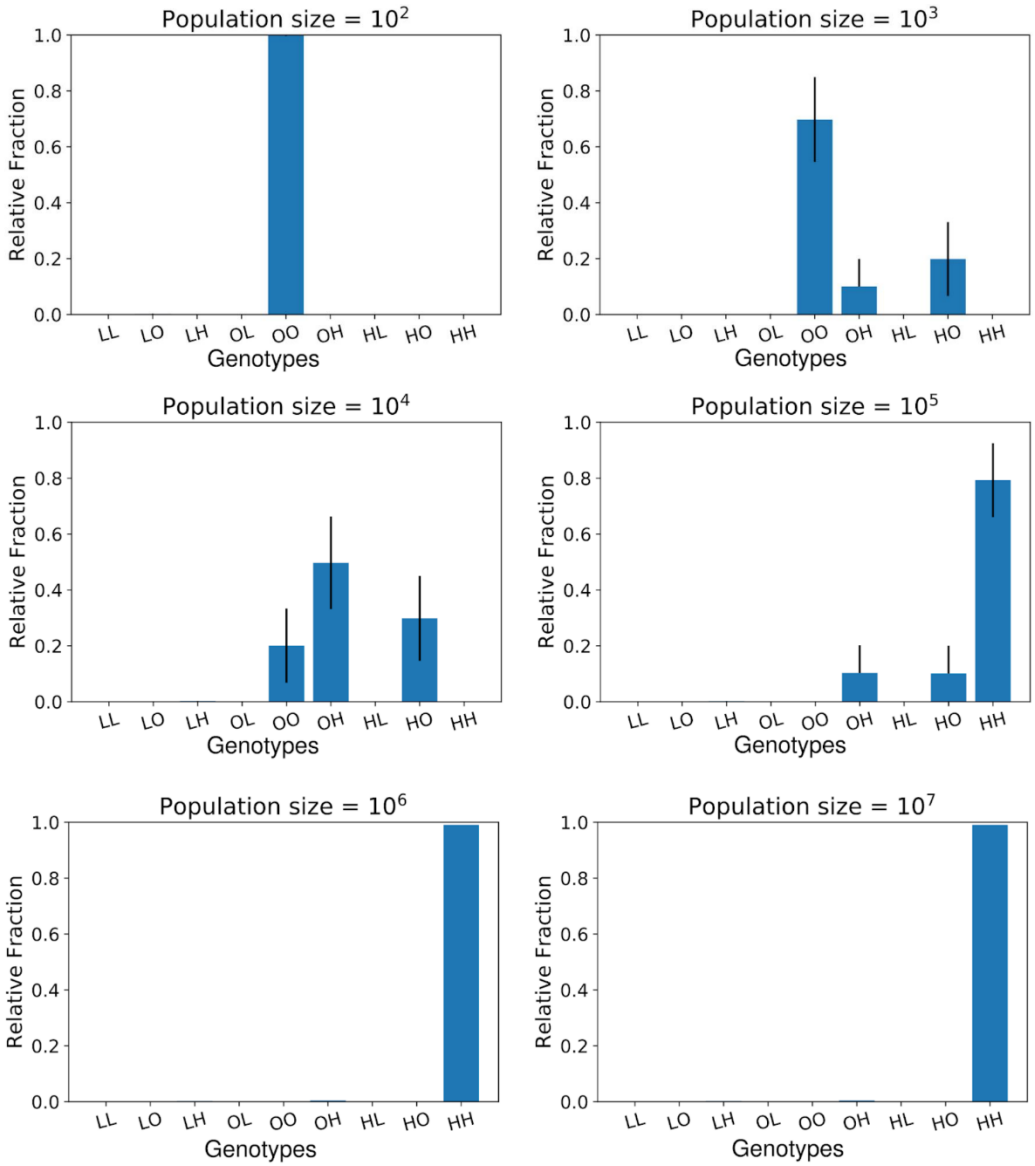

Fig. 4.12. The composition of simulated populations after 1000 generations of evolution without both sign epistasis and differential mutational supply across the two loci. The mutational tendencies of locus A were made identical to those of locus B in these simulations, and both the loci had mutational tendencies as shown in the right half of Fig. 4.2. The error bars represent SEM (N=10). Divergent character evolution was not observed.

Thus, we reached the conclusion that an interplay of sign epistasis and differential mutational supply is essential for reproducing the empirical observations regarding the evolution of efflux activity reported in the previous chapter (Chavhan et al., 2019b). To begin with, like efflux activity, the biological character controlled by locus A decayed in the larger populations but enhanced in the smaller ones. The decay of this character in the simulated populations was contingent on the chance arrival of a BO to BH (beneficial) mutation, which was more likely in the larger populations than the smaller ones. Indeed, we found that most populations ranging

in size between 10^3 to 10^4 individuals could not arrive at this mutation and hence the gene expression from locus A did not decay in them. Interestingly, the rare (outlier) populations of sizes between 10^3 to 10^4 individuals that arrived at the BO to BH mutation eventually experienced a decay in the A gene expression similar to the large populations ($\geq 10^5$) (Fig. 4.6 and 4.7). This is similar to the empirical observation of the previous chapter that one out of eight replicates of the small (SL) populations experienced a decay in efflux activity similar to the large populations.

However, not all aspects of the empirical results were thus reproduced. Specifically, an important aspect of the empirical observations of Chapter 3 that could not be mimicked by Model 1 is that the fitness of the small and large populations was statistically indistinguishable at the end of the experiment. Thus, despite evolving divergent fates of efflux activity (enhancement versus decay), small and large populations had reached similar fitness. In contrast to our experiment, in Model 1, the populations that experienced expression decay at locus A were much fitter than populations that evolved enhanced expression of locus A (Fig. 4.5). Two different possibilities can account for this discrepancy between our experiment and Model 1. First, in our experiments, the large and small populations had reached different fitness, but our assays failed to establish these differences statistically. Second, there were multiple fitness peaks in our experiment, one with enhanced efflux activity and the other with reduced efflux. We investigated the second possibility by making another model (Model 2) based on a three-locus three-allele framework (see Methods).

Model 2

Populations of different sizes can eventually reach similar fitness peaks via different mutational paths

We made asexual populations of several different sizes evolve on the three-locus fitness landscape of Fig. 4.4 with loci A and C mutating much more frequently than locus B. We found that although all simulated populations with sizes ranging from 10^2 to 5×10^5 individuals adapted to some extent, all populations of sizes $\geq 5 \times 10^5$ individuals could attain the highest possible average fitness within 1000 generations, albeit at different speeds (Fig. 4.13). Moreover, most populations of sizes $\leq 10^4$ individuals failed to fix on any of the two fitness peaks on the three-locus landscape (Fig. 4.13).

Interestingly, amongst the populations that succeeded in attaining the highest possible average fitness, the smaller ones evolved enhanced expression of loci A and C while the larger ones evolved decayed expression of the focal trait. Specifically, whereas most populations of size 10^7 individuals became fixed on the LHL genotype, most populations of size 5×10^5 individuals became fixed on the HOH genotype (Fig. 4.14 and Fig. 4.15). These genotypes correspond to the two global fitness peaks (shown in red in Fig. 4.2). This is similar to the empirical observation made in Chapter 3 regarding the divergent evolution of efflux activity in populations of different sizes despite the latter attaining similar average fitness.

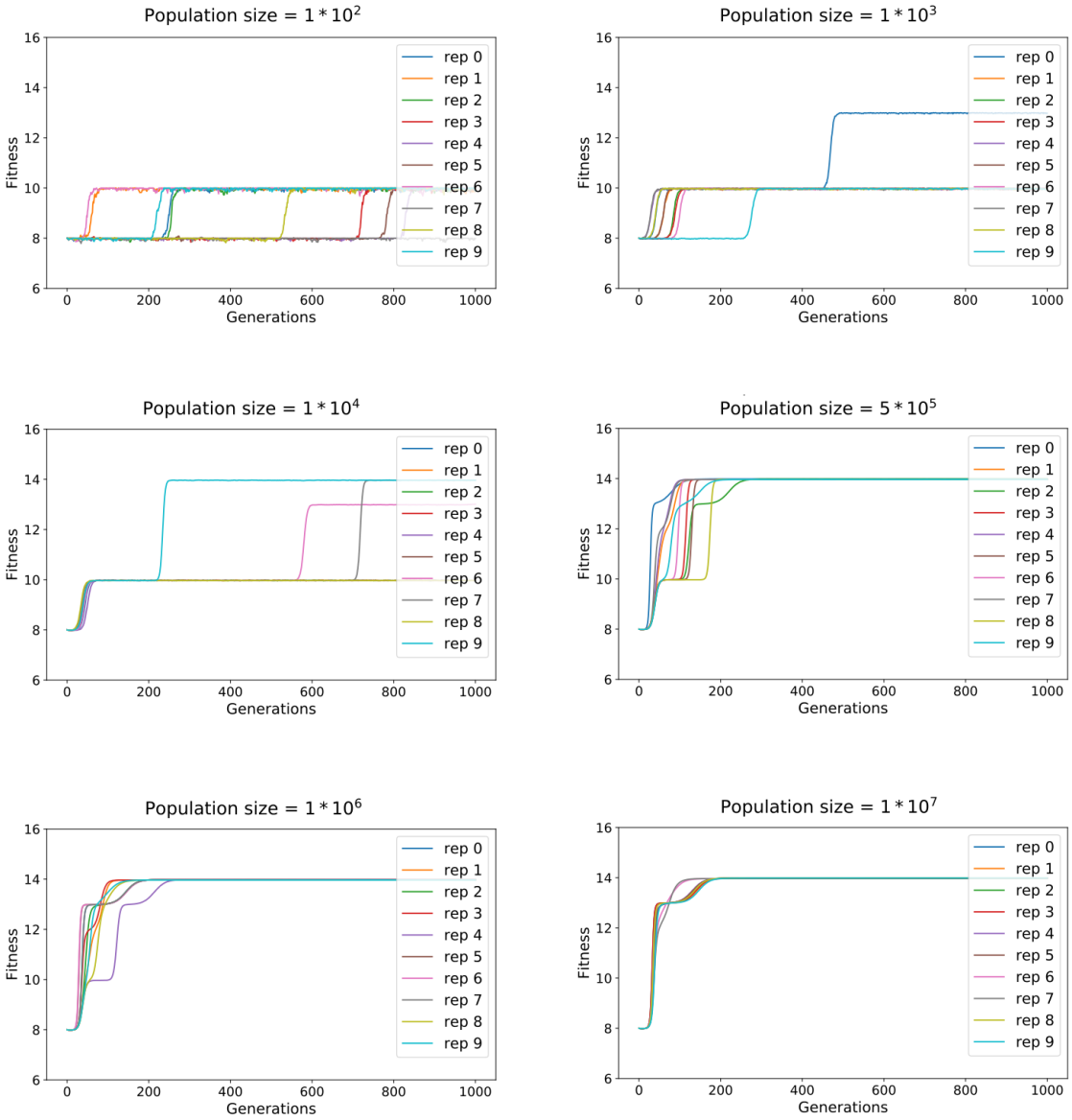

Fig. 4.13. Trajectories of average fitness on the three-locus landscape with sign epistasis in populations of different sizes when there was differential mutational supply across loci. The wild-type fitness was 8; the global fitness peak corresponded to 14.

Locus A of Model 1 and loci A and C of Model 2 can be imagined as genes whose expression requires significant resources. Therefore, if their expression is rendered unessential in some environmental and/or genetic background, alleles that show reduced expression would be beneficial to fitness while alleles that enhance expression would be selected against. In our study, the allelic state BH provides the genetic background in which higher expression at loci A and C becomes largely deleterious. In the context of the experiment reported in Chapter 3, Locus B can be imagined to code for a specific structural target of a xenobiotic drug. On the

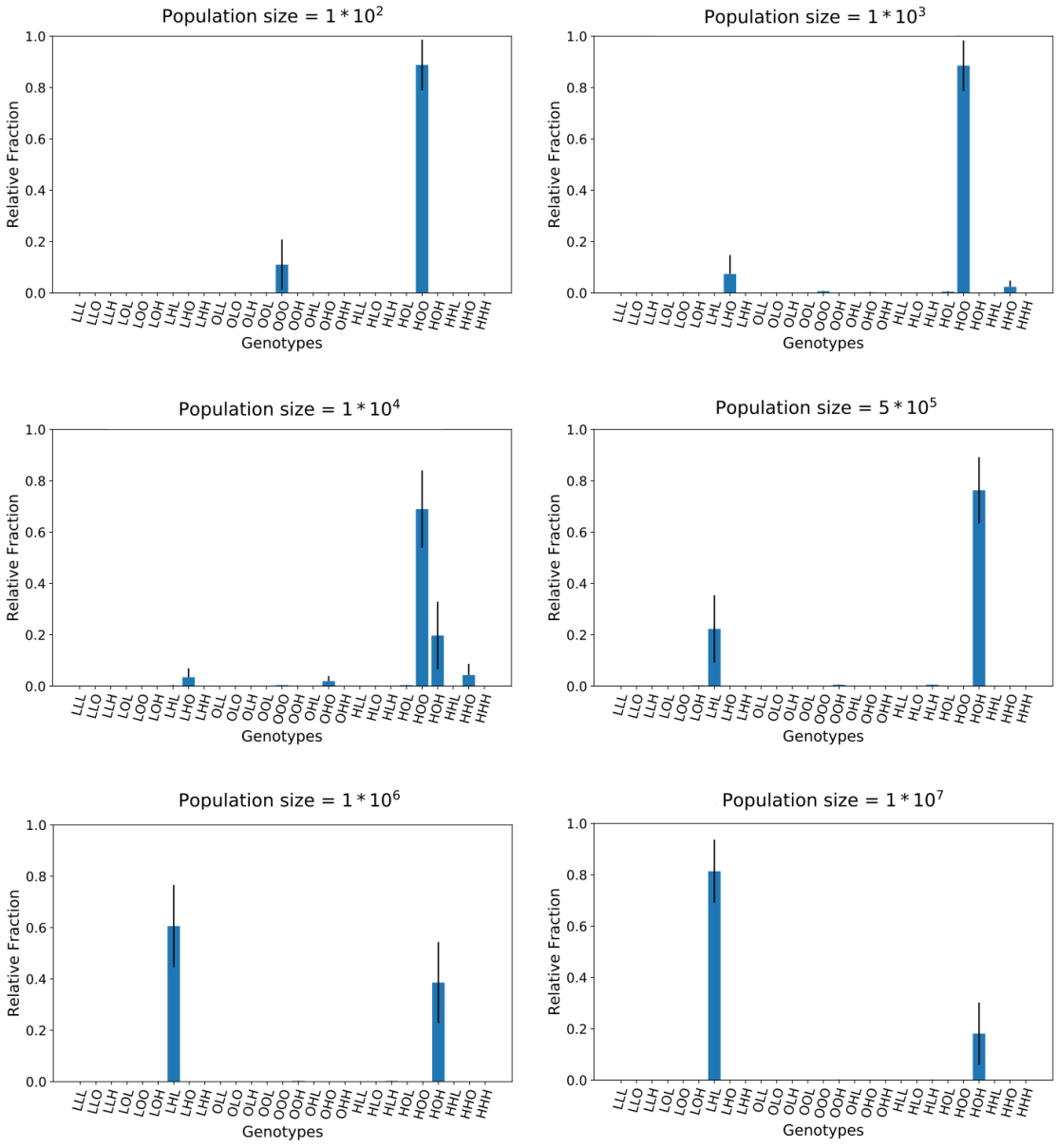

Fig. 4. 14. The composition of simulated populations after 1000 generations of evolution on the three-locus fitness landscape with epistasis and differential mutational supply across loci. The error bars represent SEM (N=10). The expression of genes A and C enhanced in relatively smaller populations but decayed in relatively larger ones. Also see Fig. 4.15.

other hand, locus A in Model 1 and loci A and C in Model 2 can be imagined to code for a variety of efflux pumps that can throw the drug out. If locus B can acquire a structural mutation (BH) that inhibits the binding of the drug, the energy intensive pumps encoded by loci A and

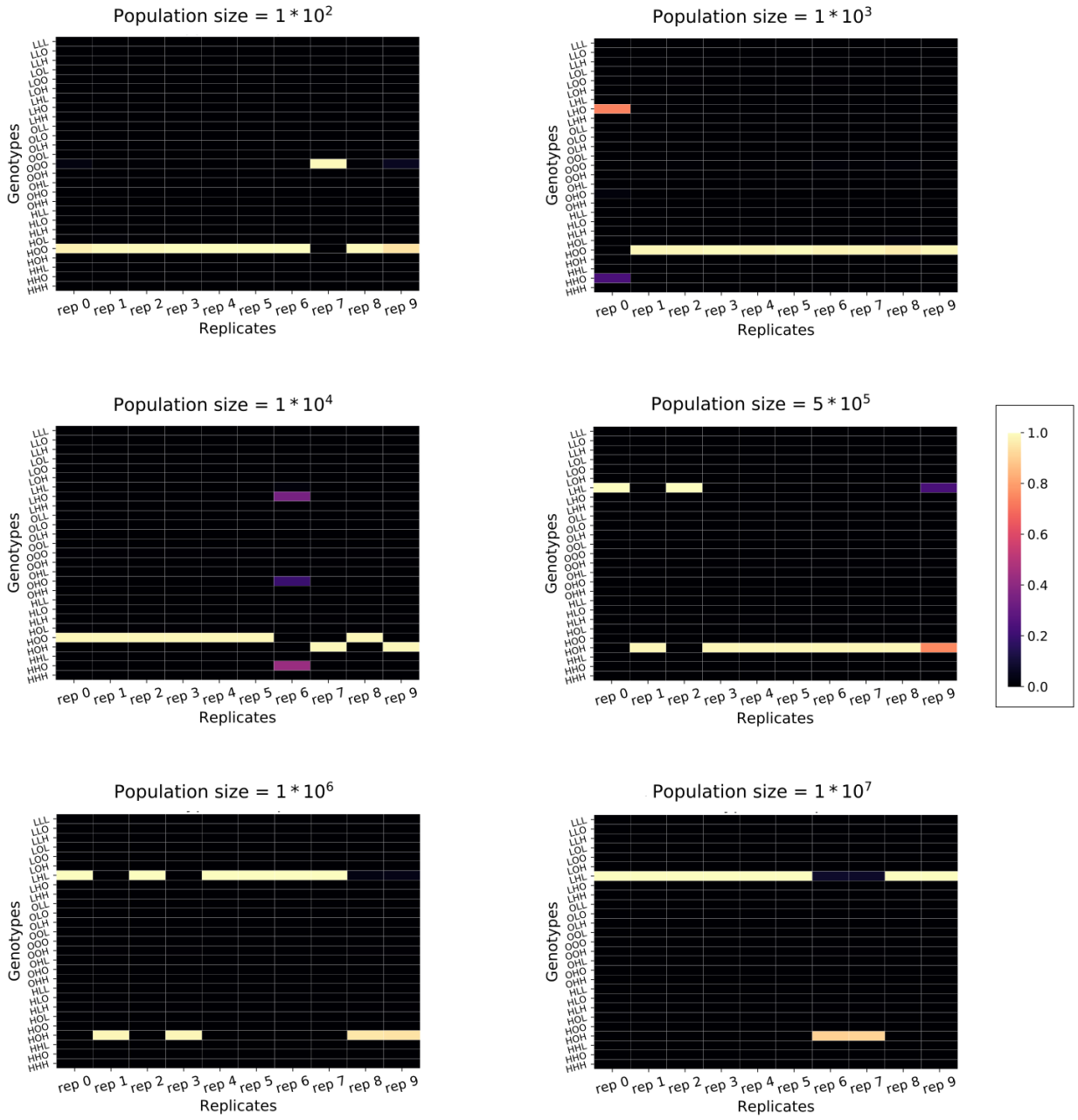

Fig. 4.15. Frequency distributions within populations of different sizes after 1000 generations of evolution on the three-locus fitness landscape with sign epistasis and differential mutational supply across the loci. The color scale shown on the right refers to within population frequencies. Most populations showed fixation of a single genotype after 1000 generations of evolution. Amongst populations that reached the highest possible average fitness (see Fig. 4.13), while the small populations ($\leq 5 \times 10^5$) largely fixed on the HOH genotype, the large ones ($\geq 10^6$) primarily fixed on the LHL genotype.

C become deleterious to fitness. Since xenobiotic drugs tend to have highly specific targets (Bakheet and Doig, 2010; Kohanski et al., 2010), mutations like BO to BH are expected to occur at very low rates. On the other hand, bacteria are known to have several promiscuous efflux pumps with overlapping targets (Nikaido and Takatsuka, 2009; Piddock, 2006; Vargiu and Nikaido, 2012; Yu et al., 2003). Therefore, mutations that can potentially increase the overall efflux activity (AO to AH and CO to CH) are expected to have relatively higher rates than BO to BH mutations. In this context, when an individual acquires the BO to BH mutation, the xenobiotic drug cannot bind to its target. Thus, even if such a drug remains in the cell, it would be rendered harmless by a BO to BH mutation. Moreover, any mutation that inhibits the binding of this drug to its target it would always be a beneficial, regardless of whether the drug remains within the cell or is thrown out of the cell by active efflux. This means that a BO to BH mutation would be beneficial regardless of the efflux activity (controlled by the allelic states at loci A and C). In contrast to this, the utility of efflux activity depends on whether the xenobiotic drug can bind to its target or not. This is because efflux is an energy intensive process (see Chapter 3), and its decay would be favored if the xenobiotic drug cannot bind its target (in the presence of a BO to BH mutation). However, when this mutation is absent, the drug succeeds in binding to its target, and the only way to combat it is through active efflux. Thus, in the absence of a BO to BH mutation, mutations that enhance the gene expression from loci A and C should be beneficial. This explains the unidirectionality of the sign epistasis used in Model 1 (and also found over the majority of the landscape in Model 2).

Taken together, evolution on the fitness landscapes of Fig. 4.1a and 4.4 can mimic the divergent evolution of efflux in populations adapting to the same environment at different sizes, the key observation of Chapter 3. While the landscape of Fig. 4.4 also gives rise to similar eventual fitness in small and large populations (matching the corresponding observation of Chapter 3), it must be emphasized that locus B has a sign epistatic control over the expression of loci A and C over most (but not all) regions of this landscape. As shown in Fig. 4.3, a strict sign epistatic control of loci A and C by locus B (with the latter's fitness effects being independent of the former two) can only result in a single global fitness peak. While it is possible for small and large populations to have divergent character fates on such a landscape with strict sign epistasis, populations of different sizes cannot reach the global fitness peak while evolving divergent characters (Fig. 4.3).

Overall, whereas the simultaneous presence of sign epistasis and differential mutational supply is adequate for divergent character evolution in populations adapting to the same environment

at different sizes, it cannot lead all populations of different sizes to similar eventual adaptedness (Chavhan et al., 2019b).

Previous studies have argued that populations of vastly different sizes can follow starkly different evolutionary trajectories, particularly if the fitness landscape involves sign epistasis (Ochs and Desai, 2015; Pál and Papp, 2017; Rozen et al., 2008; Schoustra et al., 2009; Szendro et al., 2013b). A recent simulation study has demonstrated that although both small and large (but not intermediate-sized) populations evolve increased biological complexity, they do so via different evolutionary paths (LaBar and Adami, 2016). Moreover, it has been predicted that the probability of crossing a fitness valley depends non-monotonically on populations size, with populations of intermediate sizes being the likeliest to be stuck at local fitness peaks (Ochs and Desai, 2015). This is expected because small populations can cross fitness valleys by sequentially fixing deleterious 'valley' mutations, eventually drifting towards the global peak. On the other hand, very large populations can undergo 'stochastic tunnelling,' in which double mutants can take genotypes directly to higher fitness peaks, bypassing the need to cross valleys (Ochs and Desai, 2015; Vahdati and Wagner, 2017). Although our study also links population size to differences in evolutionary paths, it is different from the above studies on three key axes. First, our observations are not explicable by drift—even the smallest populations (10^2 individuals) in our principal treatment increased their fitness by 50% (Fig. 4.5). Second, our observations cannot be explained by stochastic tunnelling as the probability of a double mutant carrying a genotype directly close to the global fitness peak is vanishingly low, making such an event virtually impossible even in the largest populations in our study (10^7). Third, unlike any of the above studies, our study deals with populations that evolve a fitness-affecting trait in opposite directions while adapting to the same environment, depending on their size. Thus, our results not only provide a population genetic explanation for the empirical observations of the previous chapter, but also have important implications for understanding how selection manifests itself in populations of different sizes, particularly in the presence of sign epistasis.

Chapter 5

Larger *Escherichia coli* populations suffer greater fitness trade-offs and undergo more ecological specialization

Highlights

- We conducted experimental evolution with *Escherichia coli* populations of two different sizes in two distinct nutritionally limited homogenous environments and studied fitness trade-offs from three different perspectives.
- We found that larger populations evolved greater fitness trade-offs, regardless of how trade-offs were conceptualized.
- Although larger populations adapted more to their selection conditions, they also became more maladapted to other environments, ultimately paying heavier costs of adaptation.
- To enhance the generalizability of our results, we further investigated the evolution of ecological specialization across six different environmental pairs and found that larger populations specialized more frequently and evolved consistently steeper reaction norms of fitness.
- This is the first study to demonstrate a relationship between population size and fitness trade-offs and the results are important in understanding the population genetics of ecological specialization and vulnerability to environmental changes.

Adapted from '**Chavhan, Y.**, Malusare, S., and Dey, S. (2019). Larger Escherichia coli populations suffer greater fitness trade-offs and undergo more ecological specialization (Under review).'

5.1. Introduction

Adaptation of a biological population to a given environment may not concomitantly increase its fitness in other environments (Anderson et al., 2013; Bono et al., 2017; Cooper, 2014; Kassen, 2002, 2014). In extreme cases, adaptation to one environment can also lead to maladaptation to others (Andersson and Hughes, 2010; Bataillon et al., 2011; Cooper and Lenski, 2000; Kassen, 2002; Remold, 2012). Such incongruity in fitness changes across environments forms the basis of ecological specialization, which happens when the fittest type in one environment cannot be the fittest type in another environment (Fry, 1996), and tends to restrict the niche breadth of populations to a narrow range of environments (Agrawal et al., 2010; Futuyma and Moreno, 1988; Levins, 1962, 1968). Studies of ecological specialization routinely invoke trade-offs in fitness across environments (Levins, 1962), with the latter leading to the intuitive and widespread assumption that the jack-of-all-trades is a master-of-none (MacArthur, 1984). Such fitness trade-offs and ecological specialization underlie the evolution of a wide range of biological properties and processes, including (but not restricted to) the composition of ecological communities (Farahpour et al., 2018; Kneitel and Chase, 2004), host specificity in several systems (Bruns et al., 2014; Joshi and Thompson, 1995; Messina and Durham, 2015; Rausher, 1984), virulence (Messenger et al., 1999), resistance to a variety of agents like herbivores (Koricheva, 2002), parasites (Boots, 2011), parasitoids (Gwynn et al., 2005), antibiotics (Andersson and Hughes, 2010; MacLean et al., 2010), etc.

The term ‘trade-off’ has been used in a variety of contexts in evolutionary studies (Agrawal et al., 2010), with the following three main usages: (1) when a trait that is adaptive in a given environment is costly (maladaptive or detrimental to fitness) in others (Bataillon et al., 2011; Bell and Reboud, 1997; Kassen, 2014; Rodríguez-Verdugo et al., 2014; Sane et al., 2018); (2) unequal adaptation to alternative environments, wherein populations become adapted to all the environments under consideration but no single genotype can be the fittest one in all the environments (Bell and Reboud, 1997; Kassen, 201a; Remold, 2012); (3) life-history trade-offs across traits that arise within a single environment due to the systemic properties and constraints of organismal features (Knops et al., 2007; Prasad et al., 2001; Stearns, 1989). The first two of the above usages pertain to trade-offs in fitness across environments, which can directly give rise to ecological specialization. Although the physiological and molecular mechanisms of trade-offs and specialization are difficult to decipher in multicellular organisms (Agrawal et al., 2010), there is a fairly detailed understanding of such mechanisms in microbes (reviewed in Ferenci, 2016). However, the role of a key parameter like population size in

determining fitness trade-offs, and the resultant specialization across a pair of environments, remains relatively unclear, even in asexual microbes.

Population size is known to shape a large variety of evolutionary phenomena and properties, including rate and extent of adaptation (Chavhan et al., 2019a; Desai and Fisher, 2007; Desai et al., 2007; Sniegowski and Gerrish, 2010), repeatability of adaptation (Lachapelle et al., 2015; Szendro et al., 2013b), biological complexity (LaBar and Adami, 2016), efficiency of natural selection (Chavhan et al., 2019a; Ohta, 1992; Petit and Barbadilla, 2009), etc. Numerous theoretical and empirical results have *indirectly* linked population size with the extent of ecological specialization. For example, multiple theoretical and empirical studies have established that larger populations generally adapt faster (Chavhan et al., 2019a; Desai and Fisher, 2007; Desai et al., 2007; Gerrish and Lenski, 1998; Sniegowski and Gerrish, 2010). Moreover, larger populations are expected to adapt primarily via rare large effect beneficial mutations while relatively smaller populations adapt slower through common beneficial mutations of modest effect sizes (reviewed in Sniegowski and Gerrish (2010)). Interestingly, several theoretical (Lande, 1983; Orr and Coyne, 1992; Otto, 2004) and empirical (Griswold, 2007; Hague et al., 2018) studies suggest that larger mutational benefits also have heavier pleiotropic disadvantages. When we combine these two insights, a new testable hypothesis emerges: larger asexual populations should show greater specialization by adapting more specifically to their environment of selection and should also suffer heavier costs in alternative environments. To the best of our knowledge, there are no direct experimental tests of the relationships of such specializations and their underlying trade-offs with population size. To begin with, as pointed out repeatedly in the literature, experimental evolution studies of fitness trade-offs and the resulting specialization have not been conducted at variable population sizes (Bataillon, Thomas et al., 2013; Cooper, 2014; Kawecki et al., 2012; Kraemer and Boynton, 2017). Furthermore, several recent evolution experiments with microbes which have provided important insights in this regard have focused on the pleiotropic profiles of individual mutations (reviewed in Bono et al. 2017), and not on population-level properties like population size. To address this lacuna, in this study, we use experimental evolution with *Escherichia coli* populations of different sizes to test if larger populations evolve bigger fitness trade-offs and specialize more across environments.

Unfortunately, the usage of the term ‘trade-off’ itself has been quite inconsistent across evolutionary studies over the last few decades, particularly in the context of ecological specialization (Bell and Reboud, 1997; Fry, 1996, 2003; Gwynn et al., 2005; Jessup and

Bohannan, 2008; Kassen, 2002, 2014; Lang et al., 2009; Smith-Tsurkan et al., 2010). On the one hand, the term 'trade-off' has been used interchangeably with 'cost of adaptation', which represents cases where adaptation to one environment leads to maladaptation in another. Such a notion suggests that maladaptation (decrease in fitness below the ancestral level) is a pre-requisite for trade-offs (Bohannan et al., 2002; Fry, 2003; Kawecki et al., 1997; Smith-Tsurkan et al., 2010). On the other hand, some studies explicitly differentiate between trade-offs and costs of adaptation, stating that trade-offs (and thus specialization) can occur with or without such costs (Bell and Reboud, 1997; Kassen, 2014). Such discrepancy in the usage of the term trade-off can often lead to confusions while comparing the conclusions of different studies. For example, single-generation studies of trade-offs and ecological specialization routinely make conclusions about costs of adaptation while relying almost exclusively on negative correlations in fitness across environments (Fry, 2003; Gwynn et al., 2005; Jessup and Bohannan, 2008; Joshi and Thompson, 1995; Maharjan et al., 2013). However, single generation studies are not only weak in terms of detecting specialization, they can also make misleading claims regarding costs of adaptation (Kassen 2002; Fry 2003).

As shown schematically in Fig. 5.1, negative fitness correlations only imply the intersection of competing reaction norms for fitness across the two environments in question. Such intersection only means that no genotype has the highest fitness in both the environments. More importantly, reaction norms can intersect (and thus fitness correlations can be negative) even if the population ends up adapting to both the environments, albeit to different degrees (Fig. 5.1a) (Fry 1996; Kassen 2014). Thus, although negative correlations always lead to specialization across two environments, they can evolve with or without costs of adaptation (Bell and Reboud 1997; Kassen 2002, 2014) (Fig. 5.1). Thus, ecological specialization can happen even in the absence of costs of adaptation brought about by antagonistic pleiotropy. Indeed, magnitude pleiotropy (cases when the fitness effects of a mutation have the same sign but different magnitudes across environments) can lead to ecological specialization on its own (Remold 2012). Such magnitude pleiotropy has been a very common observation in recent studies of mutational fitness effects across environments (Sane et al., 2018; Schick et al., 2015).

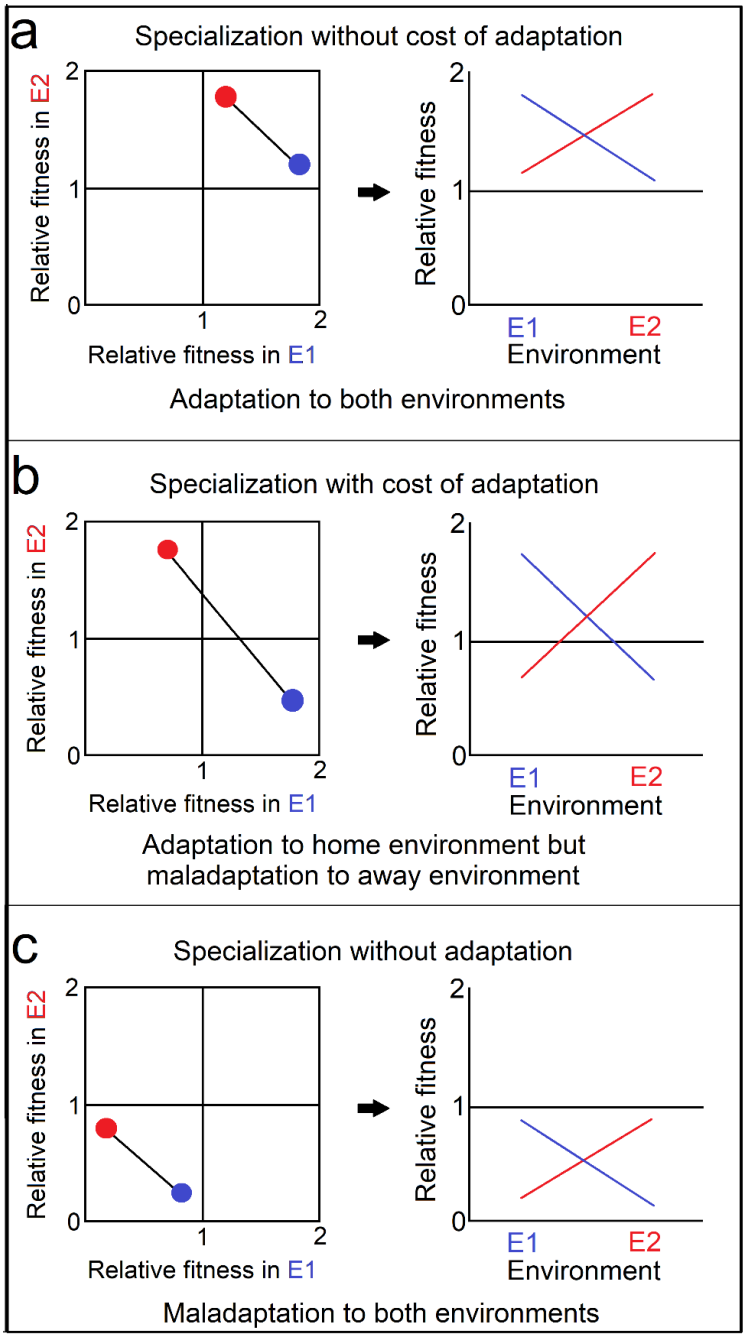

Fig. 5.1. Ecological specialization can happen with or without costs of adaptation. Specialization happens when the fittest genotype in one environment is not the fittest genotype in another environment. We consider specialization across two environments (E1 and E2) here. The blue populations evolved only in E1 while the red populations evolved only in E2. The first column across the three panels (a, b, and c) shows a negative fitness correlation in reciprocally selected lines. The second column shows the reaction norms corresponding to the correlation in the first column. Following Kassen (2014), the fitness values have been normalized by the ancestral fitness (=1) in each environment. **(a)** Specialization can happen even if reciprocally selected lines end up adapting to both the environments. **(b)** Specialization can happen if reciprocally selected lines adapt to their selective conditions but maladapt to the alternative environment. **(c)** Specialization can happen even if reciprocally selected lines end up maladapting to both the environments.

To avoid semantic ambiguity, we follow Fry (1996) and define specialization across two environments as any case where the reaction norms for fitness intersect with each other. Evolutionary experiments have conventionally studied fitness trade-offs across environments from three major perspectives: (1) as negative correlations in fitness of populations selected reciprocally in two environments (Bell and Reboud, 1997; Jessup and Bohannan, 2008; Lee et al., 2009); (2) as fitness deficits below the ancestral levels (costs of adaptation) in the alternative environment(s) that accompany adaptation to the environment in which evolution takes place (Andersson and Hughes, 2010; Bono et al., 2017; Karve et al., 2016; Lee et al., 2009); (3) as differences in fitness across environmental pairs (Kassen 2014; Schick et al. 2015). Although these perspectives can potentially be related, they are clearly not equivalent, and therefore might lead to different insights about the process of ecological specialization. Therefore, we decided to use all three perspectives to investigate how population size affects fitness trade-offs and the resulting specialization across environments.

We propagated replicate *E. coli* populations of two different sizes in two different nutritionally limiting environments (Galactose minimal medium and Thymidine minimal medium). Descending from a common ancestor, the experimental populations evolved a strong negative correlation between fitness across the two environments. We also found that the larger populations paid heavier costs of adaptation. We further assayed the fitness of the evolved populations in two more nutritionally limited environments. This enabled us to quantify the extent of specialization across six environmental pairs. Remarkably, we found that the larger populations specialized more, evolving steeper reaction norms of fitness. To the best of our knowledge, this is the first study to directly test the effects of population size on ecological specialization brought about by fitness trade-offs.

5.2. Materials and Methods

Experimental Evolution

We founded 24 populations from a single *E. coli* MG1655 colony and propagated them for ~ 480 generations at two different population sizes: Large (L) or Small (S) (defined below). For each population size, we had two different kinds of environments: Thymidine (T) or Galactose (G) as the sole carbon source in a M9-based minimal medium (for details regarding the ancestral strain and media compositions, see Appendix 8). This 2 × 2 design gave rise to four population types (TL, TS, GL, and GS) where the first letter represented the only carbon source in the selection environment, and the second letter represented the population size. We chose thymidine and galactose as the sole carbon sources in the two disparate selection environments because these two compounds have very different metabolic pathways in *E. coli* (Barupal et al., 2013; Díaz-Mejía et al., 2009; Frey, 1996; Loh et al., 2006). Each population type had six independently evolving replicate populations. We propagated all the 24 populations using the standard batch-culture technique at a volume of 300 µl in 96 well plates shaking continuously at 150 rpm in an incubator set at 37º C. The large populations (TL and GL) had a periodic bottleneck ratio of 1:10 while and the small ones (TS and GS) faced a periodic bottleneck of $1:10^4$. To ensure that the large and small populations did not spend vastly different amounts of time in the stationary-phase, we bottlenecked the large populations every 12 hrs (every 3.3 generations), and the small one every 48 hrs (every 13.3 generations). Overall, L and S corresponded approximately to 9.9×10^7 and 3.9×10^5 respectively in terms of the harmonic mean population size (Lenski et al., 1991). In terms of the measure of population size relevant for the extent of adaptation in asexual populations as reported in a recent study (Chavhan et al. 2019a), L and S corresponded approximately to 9.0×10^6 and 2.2×10^3 respectively.

Quantification of fitness and specialization across environments

At the end of our evolution experiment, we revived the cryostocks belonging to each experimental population in glucose-based M9 minimal medium and allowed it to grow for 24 hours. Next, we performed automated growth-assays on each of the 24 revived populations using a well-plate reader in multiple environments (Synergy HT, BIOTEK ® Winooski, VT, USA). Using optical density at 600 nm as the measure of population density, we obtained growth readings every 20 minutes for 24 hours. We ensured that the physical conditions during

the assays were identical to culture conditions (96 well plates shaking at 150 rpm and ambient temperature maintained at 37º C). Using a randomized complete block design (RCBD), we conducted the fitness measurements over six different days, assaying one replicate population of each type in both the environments on a given day (Milliken and Johnson, 2009). We estimated fitness as the maximum growth rate (R), which was computed as the maximum slope of the growth curve over a moving window of ten readings (Chavhan et al., 2019a, 2019b, Karve et al., 2015, 2016, 2018; Leiby and Marx, 2014).

We labelled the environment in which selection occurred as 'home' and the other (alternative) environment(s) as 'away.' The presence of the common ancestor as the reference against which fitness gains or reductions could be tested allowed us to differentiate between specialization and costs of adaptation. As mentioned earlier, we studied trade-offs and the ensuing ecological specialization from three major conventional perspectives:

(1) If fitness trade-offs exist between two environments, reciprocal selection is expected to result in strong negative correlations in fitness across them (Kassen 2014). Therefore, we determined if relative fitness in Galactose (henceforth "Gal") had a significant negative correlation with relative fitness in Thymidine (henceforth "Thy").

(2) We also determined if our experimental populations paid significant costs of adaptation. To this end, we first established whether our experimental populations had adapted significantly to their home environment (Thy for TL/TS and Gal for GL/GS). Next, we determined if the populations had maladapted significantly to their away environment (Gal for TL/TS) and Thy for GL/GS). For this, we scaled all fitness values in a given environment by the fitness of the ancestor in the corresponding environment, which is equivalent to scaling the ancestral fitness value to 1 (Kassen 2014). We then used single sample t-tests to ascertain if the fitness of a given population type differed significantly from the ancestor (i.e., 1). We corrected for the inflation of family wise error rate using the Holm-Šidák step-down procedure (Abdi, 2010). We also computed Cohen's *d* to analyse the statistical significance of these differences in terms of effect sizes (Cohen, 1988). We concluded that a population had paid a cost of adaptation only if it had adapted significantly to its home environment (i.e., scaled fitness > 1) and simultaneously maladapted significantly to its away environment (i.e., scaled fitness < 1).

We used a mixed model ANOVA with a randomized complete block design (RCBD) to analyse if population size and the identity of the home environment interacted with each other statistically to shape the fitness in home environment (henceforth $\text{Fitness}_{\text{home}}$). To this end, we

used ‘Population Size’ (two levels: L or S) and ‘Home environment’ (two levels: Gal or Thy) as fixed factors crossed with each other, and ‘Day of assay’ (six levels: 1 to 6) as the random factor. We also analysed the effect size of the main effects using partial η^2, interpreting the latter as representing small, medium, or large effect for Partial $\eta^2 < 0.06$, $0.06 <$ Partial $\eta^2 < 0.14$, $0.14 <$ Partial η^2 respectively (Cohen, 1988).

Furthermore, we analyzed if the relative extent of fitness loss in the away environment (= 1 - $Fitness_{away}$) was significantly different for the large and small populations. To this end, we used a mixed-model ANOVA (RCBD) with ‘Population Size’ (two levels: L or S) and ‘Home-Away pair’ (two levels: Gal-Thy or Thy-Gal) as fixed factors crossed with each other, and ‘Day of assay’ (six levels: 1 to 6) as the random factor.

(3) We quantified the environmental specificity of adaptation using differences in the relative fitness of experimental populations across different home-away environmental pairs. This quantity represents the difference in the degrees to which a population adapts to the two environments under consideration (Remold 2012; Schick et al. 2015). Such a difference between home- and away- relative fitness values (= $Fitness_{home} - Fitness_{away}$) can be represented graphically as slopes of reaction norms of fitness. Since the quantification of reaction norm slopes does not require reciprocal selection, we enhanced the generalizability of our study by assaying the fitness of all the 24 evolved populations (TL, TS, GL, and GS) in two more nutritionally limited environments (Maltose minimal medium (henceforth “Mal”) and Sorbitol minimal medium (henceforth “Sor”)). This allowed us to compare the reaction norm slopes of the large and small populations across six home-away environmental pairs (Thy-Gal, Thy-Mal, Thy-Sor for TL and TS; Gal-Thy, Gal-Mal, Gal-Sor for GL ad GS).

We first determined if a population type had specialized significantly across a given home-away pair. We followed Fry (1996) to identify specialization across a pair of environments as any case where the population’s reaction norm intersected with the corresponding ancestral reaction norm. This would happen whenever the unambiguous fittest type in one environment is not unambiguously the fittest type in the other environment. To identify cases of specialization, we first determined if the population type in question had significantly different fitness in its home environment as compared to the common ancestor. Next, we determined if the population type’s fitness was significantly different from that of the ancestor in the away environment. If the population type increased its fitness significantly in both its home and away environments, its reaction norm would not intersect with the ancestral norm. This would imply

lack of ecological specialization. On the other hand, if the population type's fitness increased significantly in its home environment but failed to do so in its away environment, its reaction norm would intersect with the ancestral norm, revealing significant specialization across the environmental pair in question (Fig. 5.2). We performed this procedure for each of the four population types in our study and used this information to determine if specialization had occurred across the six home-away pairs under consideration.

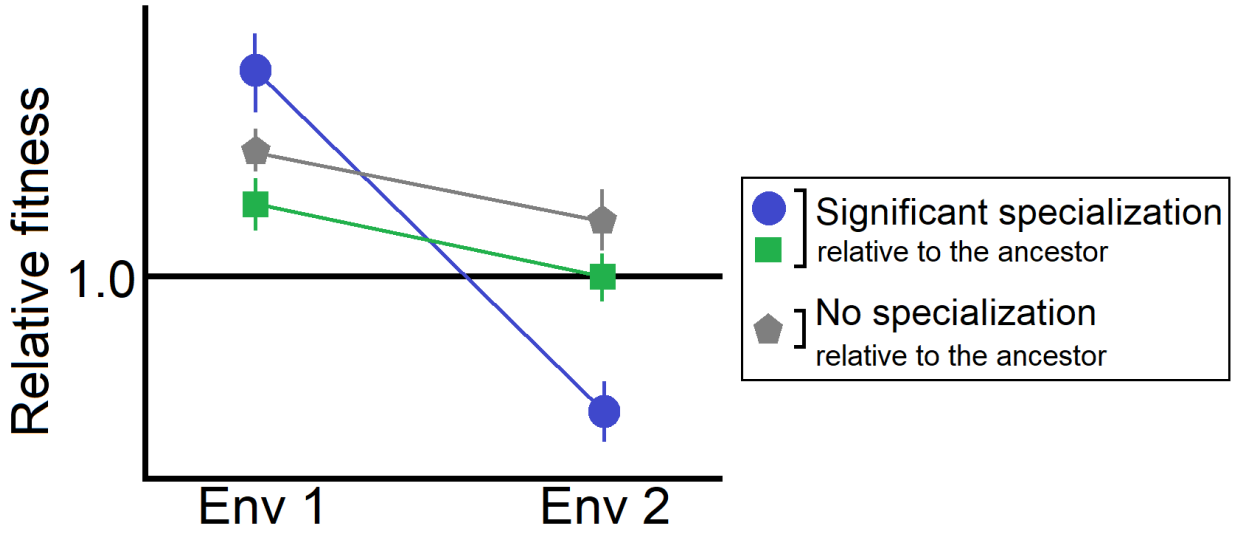

Fig. 5.2. Schematic representation of ecological specialization across two environments. All the fitness values are scaled by the ancestral value (=1 (horizontal black line)). The error bars represent 95% confidence intervals. Significant ecological specialization occurs when the reaction norms of fitness intersect (which happens when the unambiguous fittest type in one environment is not the unambiguous fittest type in the other environment).

We also compared the specificity of adaptation (reaction norm slopes) of each of the four population types with that of the ancestor across all the home-away pairs under consideration. To this end, we conducted single sample t-tests on reaction norm slopes in each pair against the ancestral level (ancestral reaction norms have zero slope), followed by correction for family-wise error rates using the Holm-Šidák procedure. We further analysed the statistical significance of these differences in terms of effect sizes using Cohen's *d*.

To further study how population size affects the specificity of adaptation, we determined if the magnitudes of reaction norm slopes were significantly different across the large and small populations. To this end, we conducted two separate mixed model ANOVAs (RCBD), one for selection in Thy and the other for selection in Gal. The design of these mixed model ANOVAs had 'Population Size' (two levels: L or S) and 'Home-Away pair' (three home-away pairs) as

fixed factors crossed with each other, and ‘Day of assay’ (six levels: 1 to 6) as the random factor.

5.3. Results

1. Fitness trade-offs as negative correlations: Fitness in Gal was negatively correlated with fitness in Thy

We found a strong negative correlation between fitness in Gal and fitness in Thy (Fig. 5.3; Spearman's ρ = -0.744; $P = 3.04 \times 10^{-5}$). This trade-off was also reflected in terms of intersecting reaction norms for fitness across these two environments (Fig. 5.4), which reveals that the fittest type in Gal was never the fittest type in Thy. As an intersection of reaction norms of fitness across is a critical pre-requisite for ecological specialization (Fry 1996), the trade-off in fitness across the two environments (Gal and Thy) implied the occurrence of ecological specialization in our experimental populations.

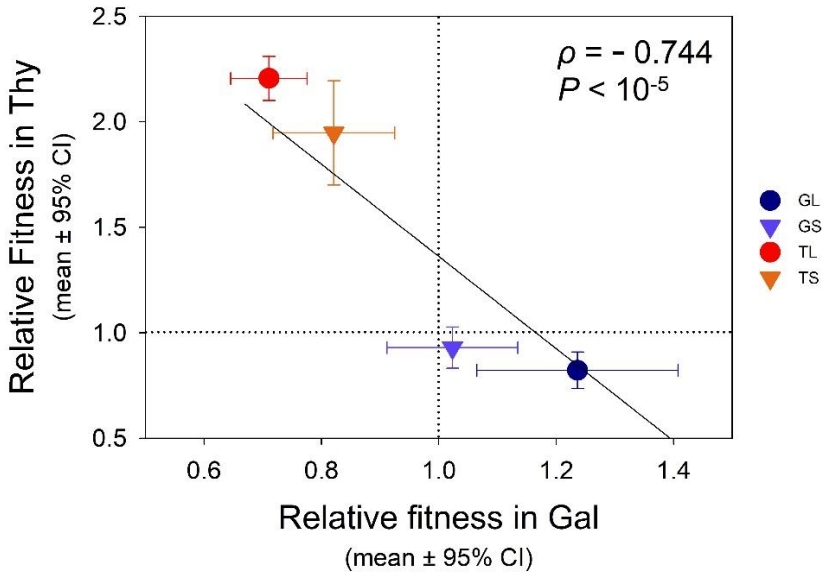

Fig. 5.3. Correlation between relative fitness values in Galactose and Thymidine minimal media after evolution in these environments at two different population sizes. The black line represents the best linear fit ($R^2 = 0.63$); the dotted lines represent ancestral levels of fitness; the bidirectional error bars represent 95% confidence intervals.

Since trade-offs based on negative fitness correlations (or equivalently, intersecting reaction norms) can potentially happen with or without costs of adaptation (Fig. 5.1; Fry 1996, Kassen 2014), we next determined if our populations had evolved significant costs of adaptation. We also examined whether the larger populations had evolved greater costs of adaptation.

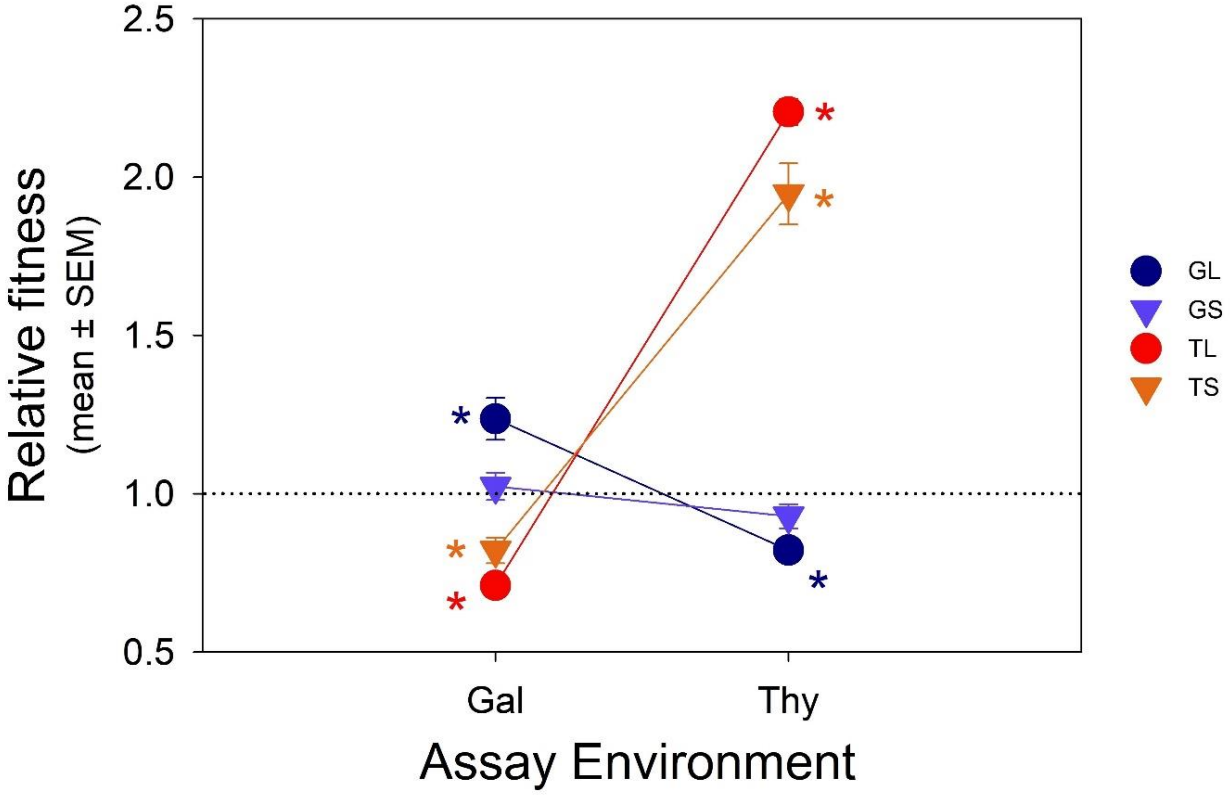

Fig. 5.4. Reaction norms of relative fitness across Gal and Thy. The dotted line represents ancestral levels of fitness. The asterisks represent significant differences with respect to the ancestor; the error bars represent SEM (N = 6; some error bars are smaller than their corresponding symbols). The crossing of reaction norms implies that the reciprocally selected population types had specialized with respect to each other across the two environments.

2. Trade-offs as costs of adaptation: Larger populations paid more costs of adaptation

Following Kassen (2014), we defined costs of adaptation as the simultaneous occurrence of fitness increase (adaptation) in the home environment and fitness decrease in the away environment (maladaptation). A comparison of Fig. 5.3 and 5.4 with Fig. 5.1 shows that the negative correlation between relative fitness values in Gal and Thy was accompanied by costs of adaptation.

We found a significant main effect of population size on adaptation to the home environment, with the large populations showing higher $Fitness_{home}$ than the small populations (mixed model ANOVA: population size ($F_{1,15}$ = 10.998, P = 0.005, η^2 = 0.423 (large effect)). We also found a significant main effect of the home environment ($F_{1,15}$ = 176.969, P = 1.044 ×10^{-9}, η^2 = 0.921 (large effect)). Importantly, we did not find a significant population size × home environment interaction ($F_{1,15}$ = 0.102, P = 0.753).

We found that evolution in Thy resulted in significant costs of adaptation in case of both large (TL) and small (TS) populations (Table 5.1), i.e., both TL and TS adapted to Thy but became maladapted to Gal (Fig. 5.4; Table 5.1). However, evolution in Gal incurred significant costs of adaptation only in case of the large populations (GL); i.e., the GL populations adapted to Gal but became maladapted to Thy (Fig. 5.4; Table 5.1). The small populations that evolved in Gal (GS) neither adapted significantly to Gal nor became significantly maladapted to Thy (Fig. 5.4; Table 5.1).

Population type	Fitness change in Gal	Fitness change in Thy	Cost of adaptation
TL	Maladaptation $\boldsymbol{P = 2.683 \times 10^{-4}}$	Adaptation $\boldsymbol{P = 3.240 \times 10^{-6}}$	Yes
TS	Maladaptation $\boldsymbol{P = 0.007}$	Adaptation $\boldsymbol{P = 7.327 \times 10^{-4}}$	Yes
GL	Adaptation $\boldsymbol{P = 0.049}$	Maladaptation $\boldsymbol{P = 0.013}$	Yes
GS	Not significant $P = 0.617$	Not significant $P = 0.122$	None

Table 5.1. Occurrence of adaptation and maladaptation events in population types selected in Gal and Thy separately. The Holm-Sidak corrected *P* values correspond to single sample t-tests against the ancestral levels of fitness (= 1). $P < 0.05$ are shown in boldface. All the cases with $P < 0.05$ were also found to have large effect sizes (See Table A9.1 (Appendix 9)).

Hence, the larger populations paid significant costs of adaptation in both the environments, but the smaller ones did so only in one of the two environments under consideration.

Next, we determined if larger populations also had a higher magnitude of loss in relative fitness below the ancestral levels in their away environments. Indeed, we found that the large populations lost significantly greater fitness than the small populations in their away environments (Fig. 5.5, mixed-model ANOVA: population size (main effect) $F_{1,15} = 9.558$, $P = 0.007$, partial $\eta^2 = 0.389$ (large effect); home environment (main effect) $F_{1,15} = 9.650$; $P = 0.007$ partial $\eta^2 = 0.391$ (large effect), population size × home environment (interaction) $F_{1,15} = 0.002$, $P = 0.963$). Interestingly, this result also implies that the effect of population size on $\text{Fitness}_{\text{away}}$ would be the opposite of its effects on $\text{Fitness}_{\text{home}}$.

Overall, larger population adapted more to their home environments while significantly paying greater costs of adaptation that made them significantly more maladapted to the away environments.

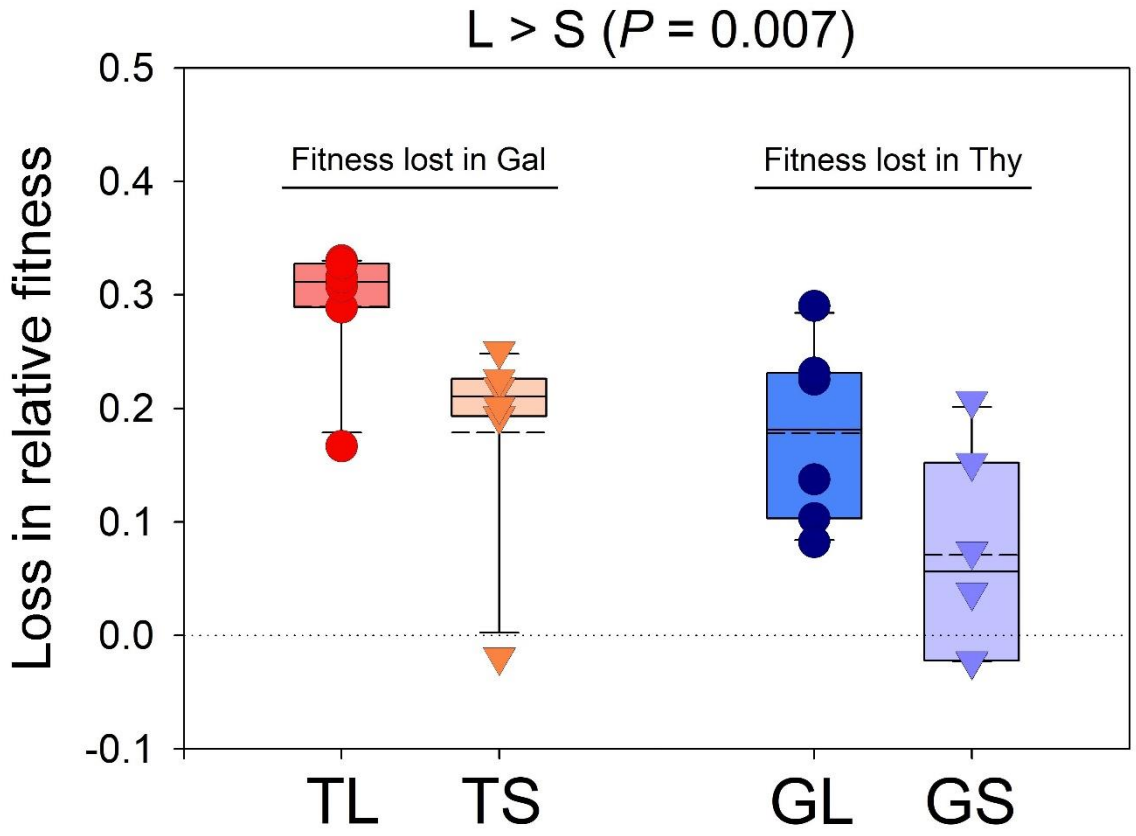

Fig. 5.5. Loss of fitness below the ancestral levels in the away environments. In each case, the loss in relative fitness was computed as the difference between the descendant population's relative fitness and the ancestor's relative fitness. L and S represent large and small populations, respectively. The solid lines in the box plots mark the 25th, 50th, and 75th percentiles while the whiskers mark the 10^{th} and 90^{th} percentiles; the dashed lines within the box plots represent means (N = 6). The dotted line represents no loss from the ancestral fitness levels. See the text for details.

Thus, as compared to the small populations, the large populations lost more fitness in the away environments. We note that the magnitudes of such fitness decline cannot reflect the full extent of ecological specialization. This is because the former only represent the fitness deficits in the

away environments whereas ecological specialization involves a difference between the extents of adaptation across the home and away environments. Thus, as the next logical step, we set out to measure the extent of specialization in our experimental populations.

3. Fitness trade-offs as extents of ecological specialization: Larger populations specialized more

We used reaction norm slopes as a measure of the specificity of adaptation, computed as the difference in the fitness values in the home and the away environments. As described in the Materials and Methods section, we assayed the fitness of our population in two more nutrient-limited environments, which allowed us to compute the reaction norm slope across six different environmental pairs (three for Gal-selected populations and three for the Thy-selected populations). Moreover, normalization of fitness in any given environment with the corresponding ancestral value ensured that the slope of the ancestral reaction norms of relative fitness is zero across all environmental pairs (Kassen 2014).

First, we determined whether our experimental populations had specialized significantly to their home environment. Following a previous study (Fry 1996), we identified the evolution of specialization as the intersection of the reaction norms of the descendant treatments with that of the ancestor.

On the one hand, the TL and TS populations had significantly greater fitness than the ancestor in their home environment (Fig. 5.4). On the other hand, the TL and TS populations had lower fitness than the ancestor in all the three away environments under consideration (See Table A9.1 (Appendix 9)). This reveals that the average reaction norms of both TL and TS populations intersected with the ancestral norms across all the three environmental pairs under consideration (T-Gal, Thy-Mal, Thy-Sor). Hence, both the TL and TS populations had specialized significantly across all the three home-away pairs.

The large populations evolved in Gal (GL) had adapted significantly to their home environments (Table 5.1; Fig. 5.4). Furthermore, the relative fitness of GL was not significantly greater than the ancestor in any of the three away environments (Table A9.1 (Appendix 9)). Combining these pieces of information, it is clear that GL populations specialized significantly (i.e., the fittest type in home environment was not the unambiguous fittest type in the away environment) across all the three home-away pairs (Gal-Thy, Gal-Mal, Gal-Sor). Interestingly,

the small populations evolved in Gal (GS) did not have significantly different fitness as compared to the ancestor in any of the four environments under consideration (Table A9.1 (Appendix 9)). This implies that the GS populations did not specialize significantly across any of the three home-away pairs under consideration.

Amongst the Thy-selected populations, we found that both the large (TL) and small (TS) populations evolved significantly greater reaction norm slopes than that of the ancestor (i.e., reaction norm slope = 0) ((Fig. 5.6, Table A9.2 (Appendix 9)) .

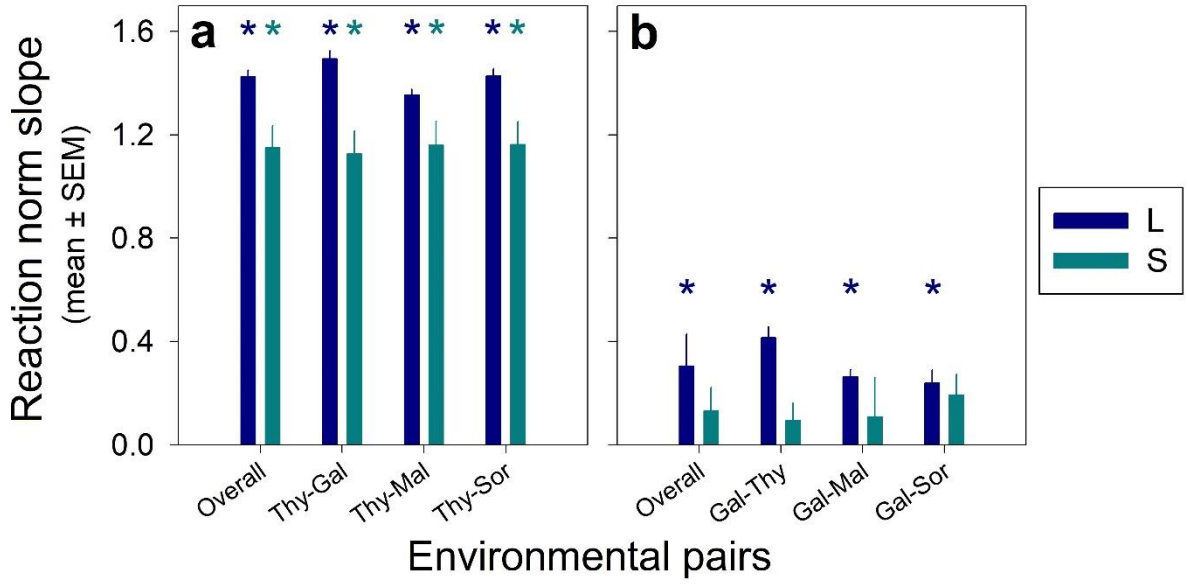

Fig. 5.6. Slopes of reaction norms of the fitness of our experimental populations across six environmental pairs. The asterisks represent significant differences (single sample t-tests ($P < 0.05$)) from the ancestral slope (= 0). See the text for details. Refer to Fig. 5.7 for the reaction norms across the six environmental pairs. **(a)** Populations evolved in Thy: reaction norm slopes ($L > S$ ($P < 10^{-6}$)). **(b)** Populations evolved in Gal: reaction norm slopes ($L > S$ ($P < 10^{-2}$)). Overall, the larger populations had steeper reaction norms.

Amongst the Gal-selected populations, we found that the GL populations had significantly steeper reaction norms than the ancestor across all the three home-away pairs under consideration (Fig. 5.6; Table A9.2 (Appendix 9)). However, the reaction norm slopes of the GS populations were not significantly different from that of the ancestor across any of the three home-away pairs (Fig. 5.6; Table A9.2 (Appendix 9)).

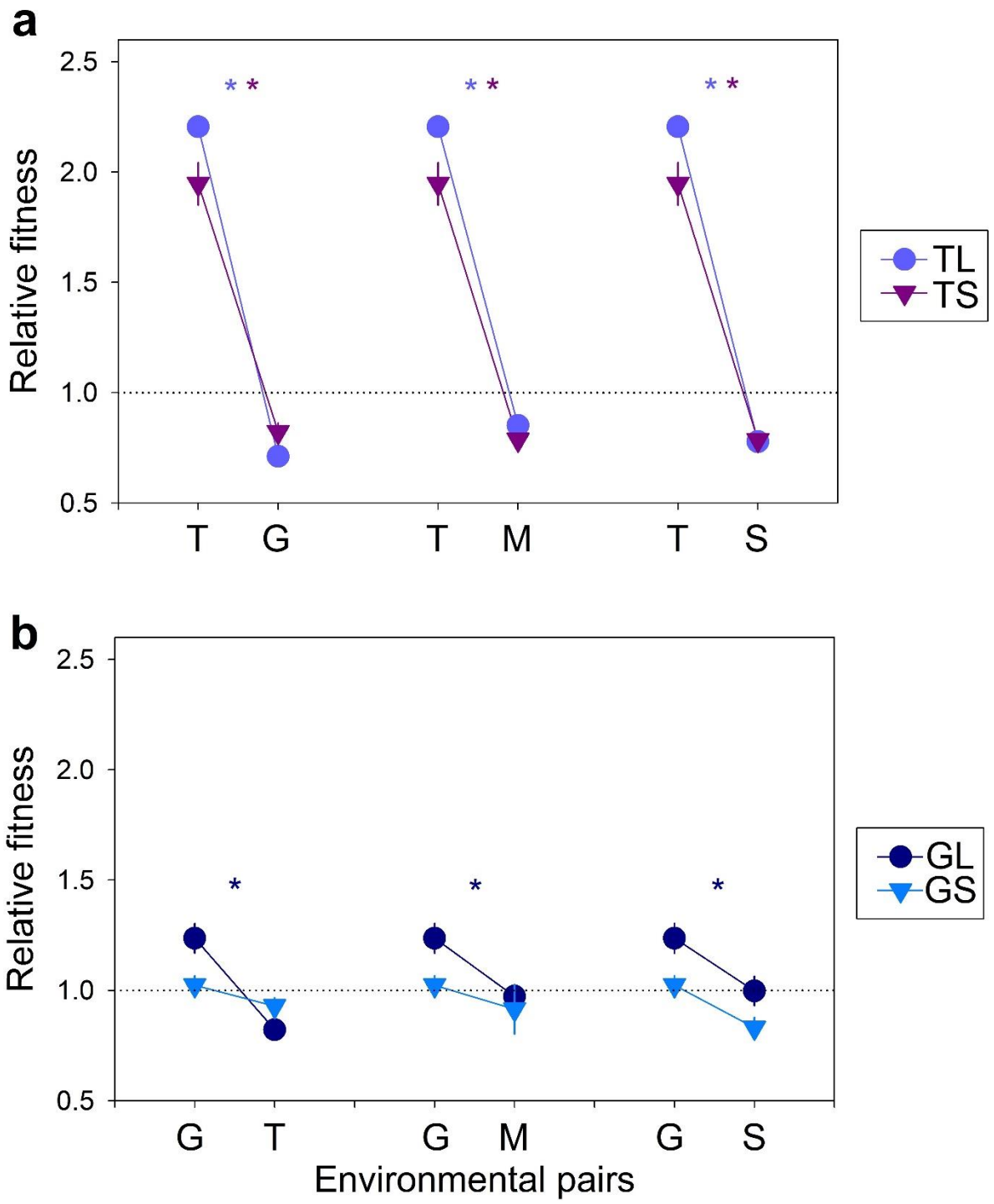

Fig. 5.7. Reaction norms of fitness across the six home-away environmental pairs used in our study. The error bars represent SEM (N = 6). The asterisks represent significantly different slope as compared to the ancestral reaction norm for the corresponding home-away pair (Holm-Šidák corrected $P < 0.05$). The dotted lines represent ancestral reaction norms **(a)** Reaction norms for populations selected in thymidine (TL (large) and TS (small). **(b)** Reaction norms for populations selected in galactose (GL (large) and GS (small).

We further determined if larger populations had steeper reaction norms than smaller populations over the six different home-away environmental pairs. Indeed, we found a significant main effect of population size, with the larger populations evolving steeper reaction norms than the smaller ones; importantly, population size and home-away pair did not show significant statistical interaction (Fig. 5.6: mixed-model ANOVA for Thy-selected lines: Population Size (main effect) $F_{1,25} = 43.664$, $P = 6.357 \times 10^{-7}$, partial $\eta^2 = 0.636$ (large effect); Home-Away pair (main effect) $F_{2,25} = 0.566$, $P = 0.575$; Population Size × Home-Away pair (interaction) $F_{2,25} = 1.471$, $P = 0.249$; mixed-model ANOVA for Gal-selected lines: Population Size (main effect) $F_{1,25} = 8.147$, $P = 0.009$, partial $\eta^2 = 0.246$ (large effect); Home-Away pair (main effect) $F_{2,25} = 0.428$, $P = 0.657$; Population Size × Home-Away pair (interaction) $F_{2,25} = 1.721$, $P = 0.199$).

Overall, the large populations not only specialized more frequently (six home-away pairs for the large populations versus three for the small ones), they also evolved higher magnitudes of environmental specificity.

5.4. Discussion

Fitness trade-offs across environments and the ensuing ecological specialization play key roles in understanding a variety of important phenomena, including the maintenance of biodiversity, local adaptation, etc. (reviewed in Futuyma and Moreno (1988); Roff and Fairbairn (2007); Agrawal et al. (2010)). However, little is known about the relationship between fitness trade-offs and population size, even in relatively simple organisms like microbes.

In this study, we conducted experimental evolution to directly test how population size influences fitness trade-offs and the resulting ecological specialization. Inconsistencies in the usage of terms like trade-offs and costs of adaptation in the evolutionary biology literature complicates comparisons across studies. Therefore, here we investigated fitness trade-offs with three different (but not necessarily independent) perspectives. Our primary result is that regardless of how we chose to visualize trade-off, larger populations suffer more fitness trade-offs and thus evolve higher extents of ecological specialization. To the best of our knowledge, this is the first study to address and experimentally demonstrate this relationship between population size and fitness trade-offs.

The theory of adaptive dynamics in asexual populations predicts that while larger populations adapt primarily via rare large effect beneficial mutations, such mutations remain largely inaccessible to small populations, which adapt via mutations of relatively smaller effect sizes (Chavhan et al., 2019a; Sniegowski and Gerrish, 2010). Moreover, a large body of studies suggests that mutational benefits of large sizes lead to heavier disadvantages due to antagonistic pleiotropy (Griswold, 2007; Hague et al., 2018; Orr and Coyne, 1992; Otto, 2004). Our result that larger population pay higher costs of adaptation can thus be explained by a combination of the above two ideas. In other words, the notion that larger populations adapt primarily via large effect beneficial mutations which, in turn, are expected to lead to higher pleiotropic disadvantages, can potentially explain why larger population paid higher costs of adaptation which resulted in steeper reaction norms (Fig. 5.6 and 5.7). Furthermore, since multiple beneficial mutations can simultaneously rise to high frequencies in large populations (Desai and Fisher 2007; Desai et al. 2007; Sniegowski and Gerrish 2010), our observations can also be explained by the pleiotropic effects of a higher number of beneficial mutations in the larger populations. We briefly note that although maladaptation to the away environments can potentially be caused by the accumulation (via drift in the home environment) of neutral mutations that are contextually deleterious in the away environments, it is unlikely to be an

explanation of our observations. There are two key reasons behind this assertion. First, a period of 480 generations is too small a time window for mutation accumulation to show its phenotypic effects in clonally derived bacterial populations with harmonic mean population sizes greater than 10^4 (which happens to be the case here) (Cooper, 2018; Kassen, 2002). Second, the effects of accumulation of conditionally neutral mutations are not expected to be different across populations of different sizes (Hall and Colegrave, 2008; Kimura, 1983).

Therefore, if the environment remains constant for long periods, adaptation in larger numbers can make populations more specialized to this environment. This relationship between population size and ecological specialization has many important implications. Foremost, owing to their higher extent of specialization, larger populations can become vulnerable to sudden changes in the environment, as predicted by a recent study (Chavhan et al., 2019b). Interestingly, if the environment abruptly shifts between two states that show fitness trade-offs with each other, then populations with a history of evolution at larger numbers would be at a greater disadvantage than historically smaller populations. Microbial populations routinely experience such abrupt shifts across environmental states that are known to show fitness trade-offs with each other. For example, costs of antibiotic resistance are expected to check the spread of resistant bacteria if the antibiotics are removed abruptly from the environments (Andersson and Hughes 2010). A similar phenomenon has recently been shown to be operating with respect to antifungal drugs other than antibiotics (Hill et al., 2015). Moreover, pathogens are also expected to experience fitness trade-offs when they migrate across different hosts (Smith-Tsurkan et al., 2010; Turner and Elena, 2000).

Our results also predict that in the face of environmental changes, larger populations may not adapt better than smaller ones. Pleiotropy has been routinely invoked to explain why evolution should mostly proceed via small effect mutations in nature (where the environment is rarely constant, both spatially and temporally) (Dillon et al., 2016; Lande, 1983; Orr and Coyne, 1992; Tenaillon, 2014). Our results are in accord with this long-held assumption and lead to the prediction that environmental fluctuations across states that show fitness trade-offs can potentially explain why small populations can be successful in nature. We note that the evolution of ecological specialization may sometimes require thousands of generations (Kassen 2002, 2014; Cooper 2014). It would therefore be particularly interesting to study how specialization evolves in populations of different sizes if the environment fluctuates at much smaller timescales (tens of generations). Our results can act as stepping-stones for more

complex investigations of the links between population size and trade-offs, particularly in fluctuating environments.

Chapter 6

An interaction of environmental heterogeneity and population size explains the rarity of detectable fitness costs

Highlights

- We conducted experimental evolution with *Escherichia coli* populations of two different sizes in heterogenous and homogenous environments and studied the evolution of fitness costs.
- To the best of our knowledge, this is the first experimental evolution study carried out in heterogenous environments at multiple population sizes.
- We demonstrate a previously unreported interplay of population size and environmental heterogeneity that determines the evolutionary emergence (or avoidance) of fitness costs. We show that population size has opposite relationships with fitness costs in homogenous versus heterogenous environments.
- Large population size and environmental heterogeneity led to fitness cost avoidance when present together but not on their own. This provides a novel explanation for the rarity of detectable fitness costs in evolutionary and ecological studies.
- Interestingly, fitness costs can be avoided even when most mutations show antagonistic pleiotropy.
- Heterogenous environment can make larger populations avoid fitness costs despite giving rise to steeper reaction norms of fitness.

‘**Chavhan, Y.D.**, Malusare, S., and Dey, S. (2019). An interaction of environmental heterogeneity and population size explains the rarity of detectable fitness costs (Manuscript under preparation).’

6.1. Introduction

Costs of adaptation, also known as 'fitness costs' and 'true trade-offs,' entail that fitness increase in one environment leads to fitness decline (maladaptation) in another, and are a fundamental assumption of a large number of models of evolution (Futuyma and Moreno, 1988; Levins, 1962, 1968; Lynch and Gabriel, 1987; Stearns, 1989). Such fitness costs are the basis of ecological specialization, which explain why a given environment favors the survival and reproduction of some particular species over others (Fry, 1996; Futuyma and Moreno, 1988; Kassen, 2002). Such significance of fitness costs in theoretical models and evolutionary ecological explanations notwithstanding, a rather large number of evolutionary studies spanning diverse taxa have failed to detect them (Coustau et al., 2000; Friman and Buckling, 2013; Futuyma and Philippi, 1987; Neve et al., 2009; Nidelet and Kaltz, 2007; Rausher, 1984; Vasilakis et al., 2009; Via, 1984; Vila-Aiub et al., 2009). Explaining this rarity of detectable fitness costs has been a major challenge for evolutionary studies over the last two decades (Agrawal et al., 2010; Fry, 1996; Joshi and Thompson, 1995; Remold, 2012).

Here we investigate the evolutionary emergence and avoidance of fitness costs in asexual microbial populations, which have proven to be convenient model systems for experimental evolution studies of fitness costs over several hundred generations (Bataillon et al., 2013; Bono et al., 2017; Kassen, 2002, 2014; Kawecki et al., 2012). Moreover, unlike multicellular organisms, the physiological and molecular bases of fitness costs are fairly well understood in asexual microbes (Ferenci, 2016). Numerous microbial experimental evolution studies have reported the absence of detectable fitness costs altogether (Bennett and Lenski, 1999; Bono et al., 2013; Buckling et al., 2000; Friman and Buckling, 2013; Kassen and Bell, 1998; Sacristán et al., 2005; Vasilakis et al., 2009). Moreover, several studies have found such costs in some experimental populations but not in others (Bennett and Lenski, 2007; Bono et al., 2015, 2017; Buckling et al., 2007; Ciota et al., 2014; Duffy et al., 2006, 2007; Hughes et al., 2007; Jasmin and Kassen, 2007a, 2007b; Jasmin and Zeyl, 2013; Ketola and Saarinen, 2015; Lee and Marx, 2012; Nidelet and Kaltz, 2007; Ostrowski et al., 2005; Roemhild et al., 2015; Satterwhite and Cooper, 2015; Velicer and Lenski, 1999; Wenger et al., 2011). An important but trivial explanation for the failure to find fitness costs is the absence of any real costs altogether (Coustau et al., 2000). Indeed, some recent investigations of single-step mutations in *Escherichia coli* have found mutational pleiotropy to be largely positive (and not antagonistic) (Dillon et al., 2016; Sane et al., 2018). Importantly, the extant literature offers three conventional explanations as to why fitness costs may exist but remain undetected in empirical

studies (Coustau et al., 2000; Velicer and Lenski, 1999): (1) Fitness costs can be detected only under certain environmental conditions which the experimental setup fails to provide (Agrawal et al., 2010; Coustau et al., 2000; Jaenike, 1990; Kassen, 2014; Strauss et al., 2002). (2) Detecting antagonistic pleiotropy (the very foundation of fitness costs) is statistically demanding. This is because the significance of adaptive and maladaptive events needs to be established simultaneously, and the experiment does not have enough statistical power to do so (Ågren et al., 2013; Anderson et al., 2013; Bono et al., 2017; Coustau et al., 2000). (3) The emergence of fitness costs is expected to require a threshold amount of time; such costs may appear only after several thousand generations of microbial evolution has taken place (Jasmin and Kassen, 2007a; Jasmin and Zeyl, 2013; Satterwhite and Cooper, 2015; Schick et al., 2015; Velicer and Lenski, 1999).

A recent meta-analysis of microbial experimental evolution studies provides a new explanation for the emergence of fitness costs based on environmental heterogeneity, suggesting that environments imposing a single (homogenous) selection pressure frequently lead to fitness costs that can be avoided in heterogeneous environments (which fluctuate across multiple individual selection pressures) (Bono et al., 2017). These observations can be explained by the blindness of selection to fitness changes in environments that are not encountered during evolution (Bono et al., 2017; Kassen, 2002, 2014). Antagonistic pleiotropy (wherein a mutation that is beneficial in one environment is deleterious in others) can evolve freely if the environment does not allow the ensuing costs of adaptation to be expressed. Since selection would be blind to the antagonistic pleiotropic effects if the environment does not change, fitness costs are more likely to appear in homogeneous environments with a single selection pressure as compared to heterogenous environments which fluctuate across multiple selection pressures.

However, in contrast to the above expectation, numerous microbial experimental evolution studies have failed to find a significant decrease in the emergence of fitness costs owing to the multiplicity of selection pressures in heterogeneous environments (Deardorff et al., 2011; Friman and Buckling, 2013; Jasmin and Kassen, 2007b, 2007b; Ketola and Saarinen, 2015; Presloid et al., 2008). This suggests that factors other than environmental heterogeneity may be important in shaping the evolution of fitness costs.

In a previous study, we had demonstrated that bacterial population size is an important factor that influences how fitness costs evolve in homogeneous environments. Specifically, we had

found that larger populations evolving in a homogenous environment containing a single carbon source suffer greater fitness costs in alternative carbon sources (Chapter 5). In another study, we had demonstrated that when evolution happens in an unchanging environment for a few hundred generations, adapting in larger numbers can make bacterial populations more susceptible to environmental changes (Chavhan et al., 2019b). The results of these studies could be explained with a combination of two notions. First, adaptation in very large populations is primarily driven by beneficial mutations of large effect sizes (Chavhan et al., 2019a; Desai and Fisher, 2007; Sniegowski and Gerrish, 2010). Second, larger beneficial mutations are expected to carry heavier disadvantages in alternative environments (Lande, 1983; Orr and Coyne, 1992).

Thus, the extant literature suggests that environmental heterogeneity and population size are two important factors that can potentially shape the evolution of fitness costs. However, it is unknown how these two factors interact with each other to influence the evolution of fitness costs. Interestingly, this interaction of environmental heterogeneity and population size is expected to act in contrasting ways: (1) If mutational pleiotropy across environmental components is largely antagonistic and no mutations can be beneficial in multiple components, larger populations would not have a major evolutionary advantage in a heterogeneous environment despite having access to relatively more variation. (2) If a large majority of mutations show antagonistic pleiotropy but some extremely rare mutations can be simultaneously beneficial in multiple environmental components, then larger populations (which can access such rare mutations) would adapt better to the heterogenous environment than the smaller ones (where such rare mutations would remain inaccessible). To the best of our knowledge, no studies in the existing literature have tested these contrasting expectations empirically.

To investigate how environmental heterogeneity interacts with population size, we carried out experimental evolution with clonally derived *Escherichia coli* populations in both heterogeneous and homogenous environments at two different population sizes for ~480 generations (Fig. 6.1). We investigated if population size has similar effects on fitness costs in homogeneous and heterogenous environments. We also tested if evolving in a heterogenous environment can lead to evolutionary avoidance of fitness costs, regardless of the population size. Based on these investigations, we propose a new explanation for the rarity of fitness costs evolutionary and ecological studies, which can account for several contrasting observations made in the last two decades of microbial experimental evolution.

6.2. Materials and Methods

Experimental evolution

We carried out experimental evolution with clonally derived *Escherichia coli* populations in five different nutrient limited environmental conditions at two different population sizes (L (large) and S (small) (defined below)) for ~480 generations (Fig. 6.1). This gave rise to ten different evolutionary lines. Our experimental design had six replicates for each of these ten lines—making it a total of 60 independently evolving populations. In our principal environmental treatment (heterogenous environment), the sole carbon source fluctuated randomly across four different states (thymidine, galactose, sorbitol, and maltose) approximately every 13.3 generations. Our study also involved four different homogeneous environmental controls, each with an unchanging supply of one of the above four carbon sources.

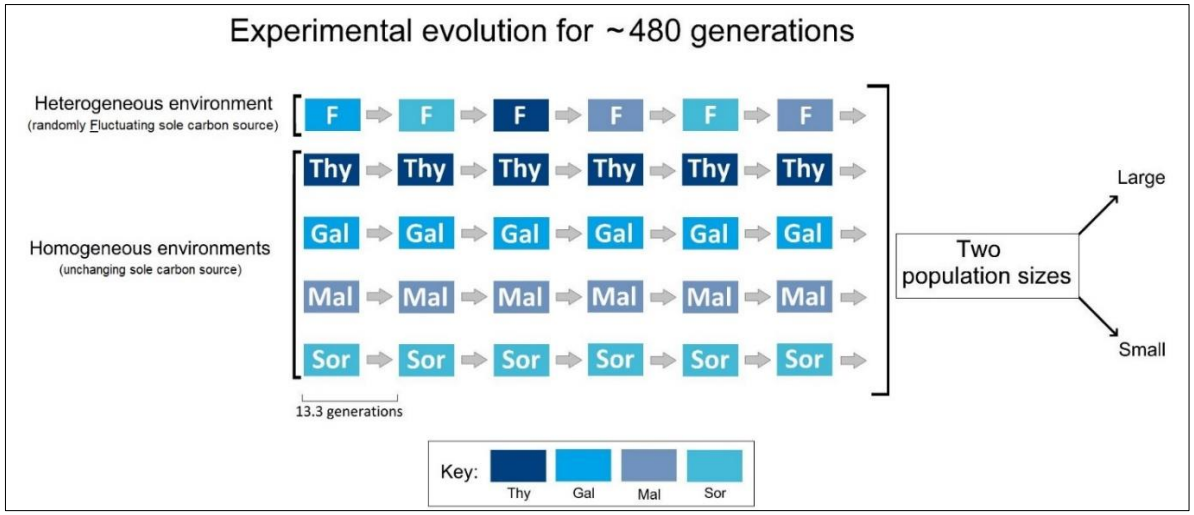

Fig. 6.1. A schematic representation of the evolution experiment used in this study. The text on each coloured box denotes the identity of the sole carbon source in its selection environment (F denotes fluctuating carbon source (heterogeneous environment).

Using the standard batch culture technique, we let all the 60 populations propagate as continuously shaken cultures (150 rpm) in 96 well plates maintained at 37º C. In all the 60 populations, the culture volume was fixed at 300 µl. The larger populations faced a periodic bottleneck ratio of 1:10 while the smaller ones had a periodic bottleneck of $1:10^4$. To ensure that populations of different sizes did not remain in the stationary phase for significantly different time periods, we bottlenecked the larger populations every 12 hrs (~3.3 generations), and the smaller ones every 48 hrs (~13.3 generations). The harmonic mean population size was

~9.9×10^7 for the larger (L) populations and 3.9×10^5 for the smaller ones (S) (Lenski et al. 1991). L and S corresponded approximately to 9.0×10^6 and 2.2×10^3 respectively in terms population size relevant for the extent of adaptation in periodically bottlenecked asexual systems (Chavhan et al. 2019a) (see Chapter 2).

Quantification of fitness

We conducted fitness measurements for all the 60 independently evolving populations in all four carbon sources (T, G, M, and S) at the end of the evolution experiment (~480 generations). To this end, we revived the cryostocks belonging to each of the 60 experimental populations in a common nutrient limited environment that was not encountered by any population during the ~480 generations of our experiment (glucose based M9 minimal medium) and allowed them to grow for 24 hours. Using a well-plate reader (Synergy HT, BIOTEK ® Winooski, VT, USA), we then performed automated growth measurements on each of the 60 revived populations in all four different minimal media, each based on one of T, G, M, or S. Ensuring that the physical conditions during the fitness measurements were the same as the culture conditions (96 well plates shaking at 150 rpm and ambient temperature maintained at 37º C), we obtained growth readings every 20 minutes for 24 hours. We used optical density at 600 nm as the measure of population density. We note that the data pertaining to selection in G and T homogenous environments has been reported in a previous study (See Chapter 5).

Since the total number of growth curves (= 240) was much larger than number of wells in the assay plate (= 96), we used a randomized complete block design (RCBD) for growth measurements (Milliken and Johnson, 2009). Specifically, we assayed one replicate population of each of the ten different evolutionary lines in all four environments on a given day. Since there were six replicates for each evolutionary line, we conducted growth measurements over six different days. We used the maximum growth rate (R) as the measure of fitness. We computed R as the maximum slope of the growth curve over a dynamic window of ten OD 600 readings (Chavhan et al., 2019a, 2019b, Karve et al., 2015, 2016, 2018; Leiby and Marx, 2014).

Costs of adaptation

We identified cases of costs of adaptation as cases that showed significant adaptation to one environment and simultaneous maladaptation to another. To this end, we carried out single

sample t-tests with the ancestral fitness level (scaled to 1) as the reference value. We corrected for family-wise error rates using the Holm-Sidak procedure (Abdi, 2010). Cases with fitness > 1 (corrected $P < 0.05$) were identified as adaptations; analogously, cases with fitness < 1 (corrected $P < 0.05$) were identified as maladaptations.

At the end of the experiment, we also computed the geometric mean fitness across each of the four carbon sources for all the sixty independently evolving populations. We used a mixed model ANOVA to compare the geometric mean fitness across the populations evolved in the heterogenous environment (FL and FS). In this analysis, the population size (two levels: large (L) and small (S)) was taken as the fixed factor and the day of assay as the random factor, with each day corresponding to one biological replicate. We also determined the effect size of the difference between FL and FS using partial η^2, interpreting the latter as showing small, medium, or large effect for Partial $\eta^2 < 0.06$, $0.06 <$ Partial $\eta^2 < 0.14$, $0.14 <$ Partial η^2 respectively (Cohen 1988).

We also conducted single sample t-tests for comparing the geometric mean fitness of all the ten evolutionary lines with that of the ancestor, correcting for family-wise errors using the Holm-Sidak procedure.

We tested if the treatment populations evolved in the heterogenous environment (FL and FS) had evolved significantly different geometric mean fitness (over T,G, M, and S) as compared to the control populations evolved in homogenous environments. To this end, we conducted a mixed model ANOVA with evolutionary line (ten levels) as the fixed factor and day of assay (six levels) as the random factor. The we used two post-hoc tests (Dunnett's procedure), one with reference to FL and the other with reference to FS to make pairwise comparisons.

Environmental specificity of adaptation

We quantified the specificity of adaptation our treatment populations (FL and FS) across the four different components of the heterogenous environment. We used the slope of pairwise reaction norms as the measure of the environmental specificity of adaptation, with steeper reaction norms corresponding to greater difference in adaptedness across environmental components, and thus, greater specificity of adaptation. We compared the reaction norm slopes of the FL and FS populations across all the six possible environmental pairs. To this end, we used a mixed model ANOVA with population size (two levels: large (L) and small (S)) and

environmental pair (six levels: T-G, T-M, T-S, G-M, G-S, M-S) as fixed factors crossed with each other, and ‘day of measurement’ as the random factor. We also computed partial η^2 to determine the effect size of the difference between FL and FS.

6.3. Results and Discussion

Large population size and environmental heterogeneity led to fitness cost avoidance when present together but not on their own

We compared the fitness values of ten different evolutionary treatments with the ancestral fitness in four distinct environments (single sample t-tests, N = 6). Therefore, there were forty possible cases of adaptation or maladaptation. After correcting for family-wise error rates using the Holm-Sidak procedure (Abdi, 2010), we found that twenty-one out of these forty possible cases showed significant fitness changes as compared to the common ancestor (corrected $P < 0.05$; Table A10.1). As described earlier, the simultaneous occurrence of adaptation to one carbon source and maladaptation to another was a pre-requisite for the cost of adaptation across an environmental pair to be significant. We used this information to analyse the effects of two factors that are expected to be important in shaping the evolution of fitness costs in bacterial populations, namely population size and environmental heterogeneity. We found that population size had opposite effects on costs of adaptation during evolution in homogeneous versus heterogeneous environments.

We found that the large populations that evolved in the heterogeneous environment (FL) completely avoided costs of adaptation across all the six environmental pairs under consideration (Fig. 6.2; Table 6.1). These populations adapted simultaneously to both thymidine and galactose and did not show a change in fitness (vis-à-vis the common ancestor) in sorbitol and maltose (Fig. 6.2; Table A10.1 (Appendix 10)). On the other hand, the small (FS) populations suffered costs of adaptation across three out of the six environmental pairs while showing simultaneous maladaptation in the other three (Fig. 6.2; Table 6.1). The FS populations adapted only to thymidine, becoming maladapted to the other three carbon sources (galactose, sorbitol, and maltose) (Fig. 6.2). To summarize, when evolved in the heterogeneous (randomly fluctuating) environment, the large populations avoided costs of adaptation in all cases, but the small populations failed to do so.

Interestingly, the above pattern of costs of adaptation reversed completely when we investigated evolution in homogeneous (single carbon source) environments. Specifically, in homogeneous environments, the large populations paid heavier costs of adaptation than the small populations (Table 6.2; the results pertaining to selection in homogeneous thymidine and galactose environments have been previously reported in Chapter 5).

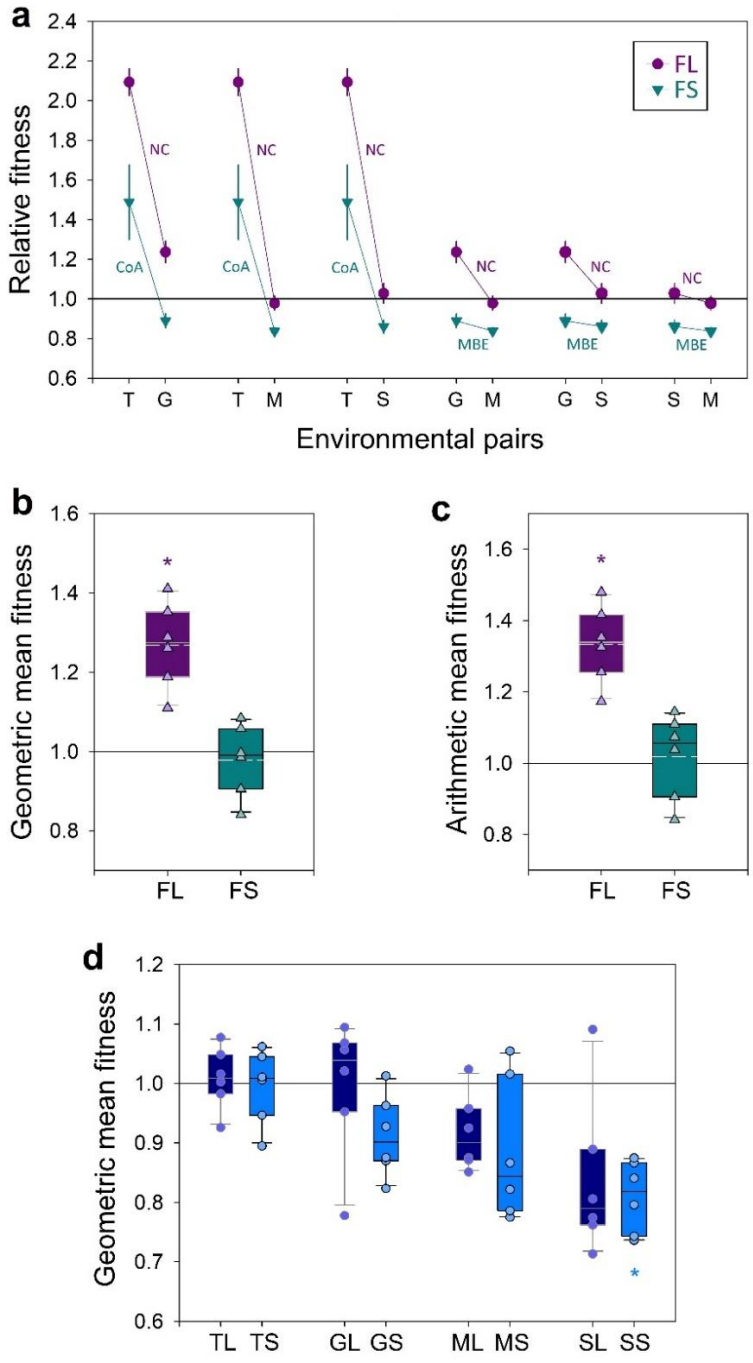

Fig. 6.2. The effects of population size (L (large) versus S (small)) on evolution in heterogeneous and homogeneous environments. T, G, M, and S represent thymidine, galactose, maltose, and sorbitol, respectively; NC = no costs; CoA = costs of adaptation; MBE = maladaptation to both environments. The solid line represents the ancestral level of the ordinate in each graph. The asterisks represent $P < 0.05$ (single-sample t-tests against the ancestral level). (a) Reaction norms of fitness of large (FL) and small (FS) populations evolved in heterogeneous environments across the environmental states faced during evolution. The error bars represent SEM. (b) Geometric mean fitness (across the four carbon sources (T, G, M, and S)) of populations evolved in the heterogeneous environment. FL > FS ($P < 0.01$) (c) Arithmetic mean fitness (across T, G, M, and S) of populations evolved in the heterogeneous environment. FL > FS ($P < 0.05$) (d) Geometric mean fitness (across T, G, M, and S) of populations evolved in homogeneous environments. See the text for details.

Evolution in heterogeneous environment			
Case(s) with costs of adaptation		Case(s) with simultaneous adaptation	
Population size	Environmental pair(s)	Population size	Environmental pair(s)
Small	T (adaptation) - G (maladaptation)	Large	T (adaptation) – G (adaptation)
Small	T (adaptation) - S (maladaptation)		
Small	T (adaptation) - M (maladaptation)		

Table 6.1. The evolutionary emergence or avoidance of costs of adaptation in populations evolved in the heterogeneous environment. T, G, M, and S represent thymidine, galactose, maltose, and sorbitol, respectively. In the heterogeneous environment, the larger (FL) populations avoided all the costs suffered by the smaller ones (FS). Only the FL populations could adapt simultaneously to multiple carbon sources. See the text and Table A10.1 for details.

Evolution in homogeneous (single carbon source) environments			
Case(s) with costs of adaptation			Case(s) with simultaneous adaptation
Selection environment	Population size	Environmental pair(s)	None
G	Large	G (adaptation) - T (maladaptation)	
T	Large	T (adaptation) - G (maladaptation)†	
T	Large	T (adaptation) - S (maladaptation)†	
T	Large	T (adaptation) - M (maladaptation)†	
T	Small	T (adaptation) - G (maladaptation)	
T	Small	T (adaptation) - Sor (maladaptation)	
T	Small	T (adaptation) - M (maladaptation)	

Table 6.2. The evolutionary emergence or avoidance of costs of adaptation in populations evolved in the homogeneous environments. T, G, M, and Sor represent thymidine, galactose, maltose, and sorbitol, respectively. In homogeneous environments, the larger populations suffered greater costs than the smaller ones. See the text and Table A10.1 for details. †Across the T - M, T - G, and T - S pairs, the costs suffered by the larger populations were greater than those suffered by the smaller populations. None of the populations evolved in homogeneous environments could adapt simultaneously to multiple carbon sources, regardless of their population size. See the text and Table A10.1 for details.

It has been previously reported that when evolved in homogeneous (single carbon source) environments, adaptation to thymidine was accompanied by maladaptation to galactose, and vice-versa (Chapter 5). Here we find that when populations faced the heterogeneous environment with fluctuating carbon sources, the small populations (FS) indeed suffered from the thymidine-galactose costs (adapting to thymidine but maladapting to galactose) (Fig. 6.2), similar to the populations evolved in homogeneous environments containing either galactose or thymidine. Surprisingly, the large populations evolved in the heterogeneous environment (FL) completely bypassed the thymidine-galactose trade-off, adapting simultaneously to both the carbon sources, thereby avoiding the costs of adaptation across this environmental pair (Fig. 6.2a, Table 6.1).

Thus, neither population size nor environmental heterogeneity could sufficiently explain the emergence (or avoidance) of costs of adaptation on their own (Tables 6.1 and 6.2). Instead, an interplay of these two factors shaped how fitness costs evolved in our experiment. Overall, costs of adaptation were avoided only when populations evolved in heterogeneous environments at large population size (the FL populations (Table 6.1; Fig. 6.2)).

Interestingly, the evolutionary success in fluctuating environments is reflected by the geometric mean fitness across the states about which the environment fluctuates (and not necessarily the arithmetic mean fitness) (Kassen, 2014; Orr, 2007). Therefore, we compared the geometric mean fitness of the FL and FS populations using a mixed model ANOVA (see Materials and Methods). We found that the FL populations had significantly higher geometric mean fitness than the FS populations (Fig. 6.2b; mixed-model ANOVA: population size (main effect): $F_{1, 5} = 18.002$; $P = 0.008$; partial $\eta^2 = 0.783$ (large effect)). Thus, the large (FL) populations adapted better than the small (FS) populations to the heterogeneous environment.

We also found that among the populations evolved in the heterogeneous environment (FL and FS), only the large populations (FL) could significantly enhance their geometric mean fitness with respect to the common ancestor (Fig. 6.2b; Table A10.2 (Appendix 2)). Despite showing significant fitness changes in thymidine (adaptation) and galactose (maladaptation), the FS populations did not have significantly different geometric mean fitness as compared to the common ancestor (Fig. 6.2b; Table A10.2). Interestingly, we also found that the FL populations had higher arithmetic mean fitness than the FS populations across the four environments (Fig. 6.2c). This observation can be explained by the avoidance of fitness costs by the FL populations. Adaptation to homogeneous environments is not expected to entail increased

geometric mean fitness over several (unexposed) environments. Indeed, we found that the geometric mean fitness over the four carbon sources did not increase significantly as compared to the ancestral level in any of the homogeneous environment treatments, regardless of the population size (Fig. 6.2d; Table A10.2).

Although the FL populations had a much larger geometric mean fitness than all the homogeneous environment treatments (Table A10.3 (Appendix 10)), surprisingly, the FS populations did not have significantly different geometric mean fitness as compared to seven out of the eight homogeneous environment treatments (Table A10.3). This shows that, despite evolving in the heterogeneous environment for several hundred generations, the FS populations did not become fitter in the fluctuating environmental as compared to most of our homogeneous environment treatments. This highlights the role played by population size in shaping fitness relationships across the components of heterogeneous environments. The mere presence of multiple selective pressures in a heterogeneous environment was not enough to prevent costs of adaptation. which ultimately precluded increase in geometric mean fitness in small populations.

Conventional explanations fail to account for the avoidance of fitness costs in our experiments

None of the conventional explanations for the rarity of detectable fitness costs could account for our observations (Coustau et al., 2000; Velicer and Lenski, 1999). Specifically, the inability of our experimental setup to provide the relevant conditions for costs to be expressed (Coustau et al., 2000), substantial statistical demands of establishing antagonistic pleiotropy (Ågren et al., 2013; Anderson et al., 2013; Bono et al., 2017; Coustau et al., 2000), and the inadequacy of the duration of our evolution experiment (Jasmin and Kassen, 2007a; Satterwhite and Cooper, 2015; Schick et al., 2015; Velicer and Lenski, 1999) could not explain why the FL populations avoided fitness costs suffered by the other evolutionary lines. Foremost, several environmental pairs in our study showed real costs of adaptation and we could detect them despite the substantial statistical demands imposed by antagonistic pleiotropy. Moreover, apart from the intentional differences in terms of population size and carbon source composition (which were integral to our experimental design), our experimental treatments had faced identical physical conditions, both during the ~ 480 generations of evolution and during the fitness assays. Lastly, ~ 480 generations were enough for large fitness costs to emerge in our

experiments, even in heterogeneous environments. Therefore, none of the above conventional explanations can account for the observation that the FL populations avoided all the costs that were detected in the other experimental treatments in our study (Table 6.1).

Fitness costs can be avoided even when most mutations show antagonistic pleiotropy

Our experimental design allowed us to analyse how population size and environmental heterogeneity interacted with each other to influence the emergence (or avoidance) of fitness costs. The positive relationship between population size and fitness costs observed in homogeneous environments can be readily explained using antagonistic pleiotropy (Cohan et al., 1994; Cooper, 2014; Cooper and Lenski, 2000; Holt, 1996; Rose and Charlesworth, 1980). Since these populations faced only one carbon source throughout the experiment, their evolution was blind to fitness changes in other carbon sources. The pleiotropic disadvantages of beneficial mutations are generally expected to be correlated with their direct effects (Chavhan et al., 2019b; Lande, 1983; Orr and Coyne, 1992; Otto, 2004). Since the latter are generally greater in larger asexual populations (Chavhan et al., 2019a, 2019b; Desai and Fisher, 2007; Desai et al., 2007; Sniegowski and Gerrish, 2010), adapting to single carbon source environments in larger numbers should lead to greater costs of adaptation, as observed in our study (Table 6.2).

We now provide a putative explanation to why the FL populations avoided costs of adaptation, but the FS populations could not do so. Although the environment of the F populations contained only a single carbon source at any given point of time, the identity of this carbon source fluctuated randomly over four states every 13.3 generations. Therefore, selection was not blind to the pleiotropic fitness effects of mutations across the four constituent carbon sources for the F populations. Imagine a pair of environments (A and B) that show a negative correlation in terms of the fitness effects of mutations, as shown schematically in Fig. 6.3. Here A and B can be taken as proxies for galactose and thymidine, respectively, because fitness values in these carbon sources are known to be negatively correlated (Chapter 5). Our explanation here is based on two environments for the sake of simplicity and can easily be extended to multiple environments.

Fig. 6.3 shows two different scenarios where the distributions of fitness effects (DFEs) have identical shapes but different locations on the fitness plane. Both DFEs agree with common empirical observations that deleterious mutations are much more frequent than beneficial ones

and beneficial mutations with greater effect sizes are rarer (Eyre-Walker and Keightley, 2007; Kassen and Bataillon, 2006; Neher, 2013). If populations of different sizes adapt to a homogeneous environment with a single selection pressure (say environment B), both DFEs would lead to greater fitness costs in the larger populations, thereby explaining our observations for single carbon source populations (Table 6.1). However, if populations experience selection in both the environments (A and B) during evolution, the DFE of Fig. 6.3a would never lead to simultaneous adaptation in both the environments. This is because adaptation along any one environmental axis would necessarily lead to maladaptation along the other one, and there are no mutations in Fig. 6.3a that can simultaneously increase the fitness along both axes. Thus, the DFE of Fig. 6.3a can explain our results pertaining to homogeneous environments in our study but not of the F populations which evolved in the heterogeneous (fluctuating) environment.

Contrastingly, the DFE of Fig. 6.3b allows a small fraction of mutations to be beneficial in both the environments. Since mutations that are beneficial in a single environment are known to be rare, mutations that are simultaneously beneficial in two environments are expected to be even rarer. Small populations are unlikely to stumble upon such rare doubly beneficial mutations. However, very large populations can readily do so. Thus, the DFE of Fig. 6.3b predicts that very large populations are likely to adapt simultaneously to both environments, but very small populations are unlikely to do so. This can explain why the FL populations could adapt simultaneously to both thymidine and galactose, but the FS populations could not. Note that the schematic DFEs of Fig. 6.3 are asymmetric with respect to the two environmental axes (many more beneficial mutations are available in environment B than in environment A). In other words, the scope of adaptation in environment B is greater than that in environment A. This asymmetry is in close agreement with our observation that the scope of adaptation in thymidine was much greater than that in galactose (Table A10.1; also see Chapter 5). Indeed, we found that the GS populations could not adapt significantly to galactose, but the TS populations increased their fitness in thymidine by more than 1.5-fold (Table A10.1). This shows that the size of our small-population treatments was sufficient to adapt via beneficial mutations with respect to thymidine. This can explain why the FS populations adapted to thymidine even if they were not large enough to adapt via doubly beneficial mutations (Fig. 6.2, Table 6.1). Such singly beneficial mutations in thymidine are expected to lead to fitness costs

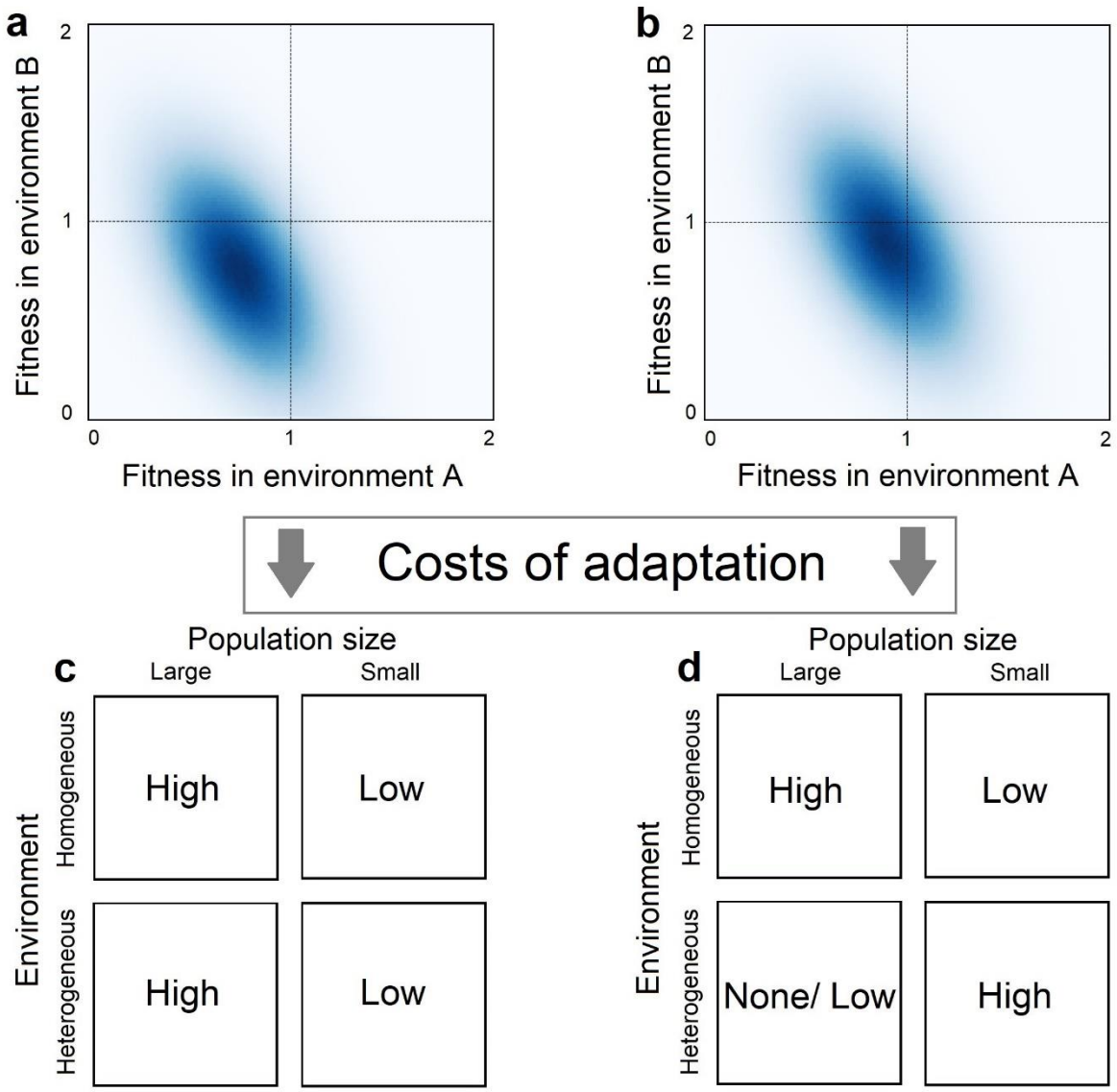

Fig. 6.3. A schematic representation of hypothetical bivariate frequency distributions of fitness effects (DFE) of mutations across two different environments. The color intensity correlates with mutational frequency. (a) A DFE where fitness in environment A is negatively correlated with fitness in environment B and no mutations are beneficial in both the environments. (b) A DFE where fitness values in environments A and B are negatively correlated, but some mutations are beneficial in both the environments. (c) and (d) Predictions regarding the effects of population size on costs of adaptation in homogeneous and heterogeneous environments based on the DFEs of (a) and (b), respectively.

in galactose (see Chapter 5). In line with this expectation, the FS populations became significantly maladapted to galactose despite the latter being one of the four component selection pressures faced by these populations (Fig. 6.2; Table 6.1).

Thus, even when most (but not all) mutations exhibit antagonistic pleiotropy across two environments, large asexual populations can adapt simultaneously to them and avoid fitness costs.

Larger populations evolved steeper reaction norms of fitness even when they avoided fitness costs

It has been reported earlier that in homogeneous environments with a single carbon source, larger *E. coli* populations showed more specificity of adaptation across environments (where the latter was measured as the steepness of reaction norms across environmental pairs) (Chapter 5). This result derives from the expectation that adaptation of larger populations to homogeneous environments occurs primarily via mutations that not only carry greater direct benefits but also show stronger pleiotropic effects (Chavhan et al., 2019b; Lande, 1983; Orr and Coyne, 1992; Otto, 2004). However, this does not imply that larger populations evolving in heterogeneous environments would also show steeper reaction norms across the environmental components. Since selection is not blind to the pleiotropic disadvantages across the components of a heterogeneous environment, the latter is expected to favor generalist mutations (Kassen, 2002, 2014). As described above, larger populations suffered greater fitness costs than smaller populations when evolved in homogeneous environments. However, in heterogeneous environments, smaller populations evolved substantial fitness costs while the larger populations avoided them altogether. Along similar lines, the steepness of reaction norms of fitness can also have opposite relationships with population size in homogeneous and heterogeneous environments, with larger populations evolving steeper norms in homogeneous environments but flatter ones in heterogeneous environments. We tested this hypothesis by measuring the slopes of reaction norms of the populations evolved in fluctuating environments (FL and FS) across all the six possible environmental pairs and determined if the large populations (FL) had significantly different slopes than the small populations (FS). Surprisingly, we found that FL populations evolved steeper reaction norms than the FS populations (Fig. 6.4, mixed model ANOVA: population size (main effect): $F_{1,55} = 22.959$; $P = 1.295 \times 10^{-5}$; partial $\eta^2 = 0.294$ (large effect)). We also found a significant main effect of environmental pairs, which is evident from Fig. 6.2 ($F_{1,55} = 35.119$; $P = 5.55 \times 10^{-16}$; partial $\eta^2 = 0.761$ (large effect)). However, the interaction of the two main effects (population size and environmental pairs) was not significant ($P = 0.195$). Combining this observation with

previously reported results (Chapter 5), we find that the relationship between population size and reaction norm slopes applies was robust to changes in environmental heterogeneity (Table 6.3). This contrasts with the relationship of population size with costs of adaptation, which is not only dependent in the environmental heterogeneity, but also shows opposite trends in homogeneous and heterogeneous environments.

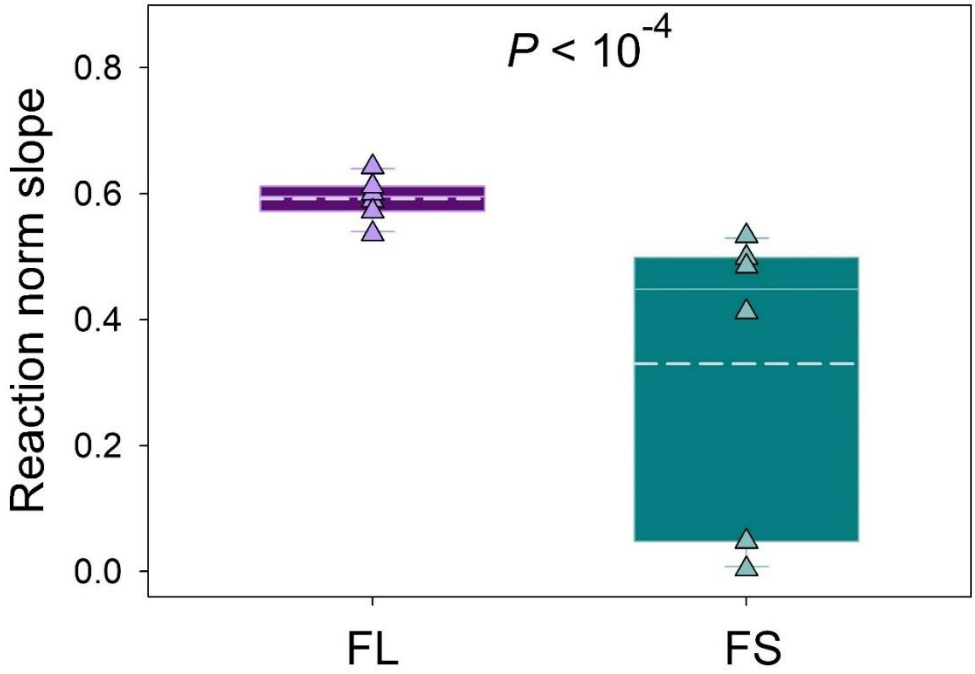

Fig. 6.4. The specificity of adaptation across environmental pairs as reflected by the slopes of pairwise reaction norms belonging to large (FL) and small (FS) populations evolved in the heterogeneous environment. The solid lines in the box plots mark the 25th, 50th, and 75th percentiles while the whiskers mark the 10^{th} and 90^{th} percentiles; the dashed lines within the box plots represent means (N = 6). The FL populations had steeper reaction norms than the FS populations.

Interestingly, the effects of population size on the specificity of adaptation (measured as reaction norm slopes) are in stark contrast with contrast with its effects on costs of adaptation. As depicted in Table 6.3, the (FS) populations suffered heavier costs of adaptation as compared to the large populations which faced fluctuating environment (FL). Surprisingly, the FL populations escaped the costs of adaptation across all the six environmental pairs despite consistently evolving steeper reaction norms across them (Table 6.3). On the one hand, higher specificity of adaptation accompanies greater costs of adaptation in homogeneous environments. On the other hand, when bacterial populations adapt in a heterogeneous

environment, higher specificity of adaptation can accompany simultaneous adaptation to multiple environmental components, thereby preventing costs of adaptation.

	Heterogeneous environment		Homogeneous environment	
	Large populations	Small populations	Large populations	Small populations
Reaction norm slope	High	Low	High	Low
Costs of adaptation	None	High	High	Low

Table 6.3. A summary of our experimental observations depicting two key results: First, population size has opposite relationship with costs of adaptation in heterogeneous versus homogeneous environments. Second, population size has similar relationship with reaction norm slopes in heterogeneous versus homogeneous environments.

Implications

In this study, we offer a novel explanation for an important evolutionary conundrum, the rarity of detectable fitness costs in empirical studies. Specifically, we demonstrate a previously unreported interaction of population size and environmental heterogeneity that determines the evolutionary emergence (or avoidance) of fitness costs (Table 6.1 and 6.2). These results can potentially explain how evolving populations can escape fitness costs despite substantial antagonistic pleiotropy across environmental states. Our study shows that a combination of two conditions, namely large population size and heterogeneous environment, can avoid all fitness costs that can potentially evolve when these conditions are not present simultaneously.

The environments of most natural populations of asexual microbes are known to be heterogeneous (Angel et al., 2010; Freedman and Zak, 2015; Green and Bohannan, 2006; Morin and McGrady-Steed, 2004; Muscarella et al., 2019; Schaum et al., 2016; Yan et al., 2017). Moreover, such natural asexual populations are also known to have extremely large sizes (Tenaillon et al., 2010; Torsvik et al., 2002; Walter and Ley, 2011; Whitman et al., 1998). Our results suggest that if the asexual population under consideration has a history of evolving in heterogeneous environments in large numbers, it is expected to have reached its current state

after having avoided fitness costs during its past evolution. Therefore, if a sample from such a population is now employed to analyse fitness correlations in a single-generation study, such correlations may not be negative. This explains why single-generation studies (which are based on standing genetic variation) can reveal non-negative fitness correlations even if most *de novo* mutations exhibit antagonistic pleiotropy across environments.

Our observations also suggest that a combination of large population size and homogeneous environment is highly likely to give rise to fitness costs. This can explain why a considerable number of microbial experimental evolution studies that make clonally derived asexual populations evolve in homogeneous environments at effective sizes exceeding tens of millions of individuals have successfully revealed fitness costs (Bedhomme et al., 2012; Cooper and Lenski, 2000; Cooper et al., 2001; Ensminger et al., 2012; Hall and Colegrave, 2008; Kassen and Bell, 1998; Kubinak and Potts, 2013; Leiby and Marx, 2014; Nilsson et al., 2004; Philippe et al., 2009; Presloid et al., 2008; Vasilakis et al., 2009).

The results of our study have important practical implications for understanding phenomena where the environment fluctuates randomly across states that exhibit fitness costs. We emphasize that our experiment can be treated as a case-study of how population size and environmental fluctuations interact with each other to shape fitness costs in asexual microbes. This is particularly true because the heterogenous environment used in our study fluctuated across two states (galactose and thymidine) that are known to show reciprocal fitness trade-offs with each other when they are present as the sole source of carbon in a homogenous environment (Chapter 5; Table 6.2). Furthermore, although the environments used in our experimental setup were nutritionally challenging minimal media, the explanation of our observations applies to the general notion of fitness costs across multiple environments in asexual microbial populations.

Our results can have particularly important implications for understanding the rampant evolution and spread of antibiotic resistance, which has direct practical value. Mutations that confer resistance to antibiotics have been routinely shown to bear fitness costs in drug-free conditions (Andersson and Hughes, 2010; Angst and Hall, 2013; Gifford and MacLean, 2013; MacLean et al., 2010; Paulander et al., 2009; Song et al., 2014; Vogwill and MacLean, 2015). Interestingly, resistant microbes mostly evolve in a heterogeneous environment that fluctuates randomly across antibiotic-laden and antibiotic-free conditions (Baquero et al., 1998). Our results predict that small populations evolving in heterogeneous environments suffer heavy

fitness costs while large populations are likely to avoid them altogether (Fig. 6.2). Thus, even if most antibiotic resistance mutations carry a cost in drug-free conditions, large microbial population sizes stemming from lack of sanitary conditions and proper medical waste-disposal (Cantón et al., 2013; Kümmerer, 2003; Okeke et al., 1999) could themselves lead to vigorous spread of cost-free resistance.

Chapter 7

Conclusions, implications, and future avenues

Evolutionary theory predicts that larger populations typically adapt faster. Experimental evolution with asexual microbes is an important approach for testing such theoretical predictions by studying the process of evolution as it unfolds under a set of carefully regulated conditions (Bataillon et al., 2013; Cvijović et al., 2018; Kassen, 2002, 2014; Kawecki et al., 2012). In most of these studies, the population size does not remain constant over successive generations (reviewed in Cvijović et al., 2018; Kawecki et al., 2012). Instead, most experimental populations of asexual microbes are cultivated under resource limited conditions, and their growth is punctuated by severe reductions in the number of individuals (bottlenecks), which happen periodically every few generations (LeClair and Wahl, 2018; Lenski et al., 1991; Wahl and Gerrish, 2001; Wahl et al., 2002). Almost all experimental evolution studies dealing with such periodically bottlenecked asexual populations investigate the changes in average population-wide fitness assuming that for any given number of individuals in the periodic bottleneck (N_0) and number of generations between successive bottlenecks (g), the harmonic mean size ($HM = N_0 g$) should predict the population's adaptive dynamics (de Visser and Rozen, 2005; Desai et al., 2007; Lachapelle et al., 2015; Lenski et al., 1991; Samani and Bell, 2010; Vogwill et al., 2016). Unfortunately, the validity of this widespread assumption had never been subjected to theoretical or empirical tests.

In Chapter 2, we used a combination of evolution experiments with *Escherichia coli* populations and individual-based simulations to test the above (widely assumed) relationship between average fitness and the conventional measure of population size (*HM*). We found that *HM* fails to predict or explain the trajectories of average fitness in asexual populations. We also showed that our results are valid over the timescales employed in almost all evolution experiments till date. Our observations call for a re-evaluation of the effects of population size in explaining the observations of a large number of empirical studies and can potentially account for the concerns regarding the adaptive relevance of *HM* (Raynes et al., 2014).

We further investigated why *HM* fails to predict average fitness and found that while the latter varies positively with N_0, it varies negatively with g. Therefore, any measure of population size that is an increasing function of both N_0 and g would fail to predict the average fitness. Our study also established that periodic bottlenecks play a dual role in terms of influencing variation in asexual populations. On the one hand, for a given size of the periodic bottleneck (N_0), harsher periodic bottleneck ratios (higher g) lead to increased variation, thereby allowing populations to explore rare large-effect beneficial mutations. On the other hand, higher g also reduces the efficacy with which selection enriches beneficial mutations and removes

deleterious ones. Overall, higher g impedes adaptation. We show that the inability of the conventional measure to incorporate this dual aspect of periodic bottleneck leads to an overestimation of the rate of production of variation that can successfully survive sampling. Thus, Chapter 2 calls for an updated view of the effects of periodic population bottlenecks on adaptation.

We used the above insights to show that not only does N_0/g predict average fitness much better than N_0g, but populations with identical N_0/g also have similar fitness trajectories. Thus, N_0g can be a potential measure of population size that can predict average fitness in asexual systems. This measure should aid not only in designing better evolution experiments, but also in comparisons of adaptive dynamics across disparate studies in similar environmental contexts.

We emphasize that N_0/g is an empirically (and not analytically) derived quantity that is congruent with our finding that the average fitness varies positively with N_0 but negatively with g. It is possible that other theoretical expressions that also capture these relationships are better than N_0/g at predicting the trajectories of average fitness. A detailed theoretical study that derives the appropriate measure of population size incorporating the above findings is an important future direction in this context.

We also note that the conventional measure of population size ($HM = N_0g$) can still explain the trends of average fitness in several empirical studies where the final size just prior to the bottleneck (N_f) is held constant (Desai et al., 2007; Raynes et al., 2014; Vogwill et al., 2016). This is because both N_0g and N_0/g would predict the same trends for average fitness over the empirically relevant ranges of N_0 and g, if the populations being compared have similar N_f. Importantly, this congruence will break down and HM would fail to predict average fitness if N_f varies across experimental treatments.

Our results also show that carrying capacity (K) can evolve rapidly in a few hundred generations, which calls for a re-evaluation of theoretical models that treat N_f as a constant. This could be particularly important for understanding adaptive dynamics because an increased K can itself accelerate adaptation in such systems via a feedback loop. Specifically, an increase in K leads to increased N_f, which itself leads to increased N_0 (as g typically remains constant within a given experiment). Since the average fitness varies positively with N_0, an increase in the latter would also lead to an increase in the former. Finally, since our results apply generically to periodically bottlenecked asexual systems, they are also relevant for

understanding practically important phenomena like the evolution of drug-resistance in pathogens (whose population growth within a single host is punctuated by bottlenecks during host-to-host transfers).

In Chapter 3, we followed up on the evolution experiment of Chapter 2 to investigate the effects of historical population size on the vulnerability of asexual populations to sudden changes in the environment. We found that when the selection environment consisted of sub-lethal concentrations of three antibiotics, the efflux activity decayed in the larger populations but enhanced in the smaller ones. It should be noted here that the generic ability to actively efflux xenobiotic chemicals is not only expected to be a major determinant of fitness in the presence of antibiotics (Kumar & Schweizer 2005, but is also a generic mechanism for combating stressful chemicals like heavy metals (Nies, 2003; Poole, 2005), bile salts (Thanassi *et al.*, 1997), organic solvents (Fernandes *et al.*, 2003), intercalating mutagens (Ma *et al.*, 1993; Nishino *et al.*, 2009), etc. We further found that the efflux activity was not only negatively correlated with population size, it also showed a strong negative correlation with the speed of adaptation. Our experimental design allowed us to attribute the differences in the evolved efflux activities to the population sizes of the treatments. We further established that the evolution of efflux activity was driven largely by pleiotropic response to selection and not by random accumulation of conditionally neutral mutations via genetic drift. The decay of efflux activity in the larger populations is expected to render them less fit in a variety of stressful environments that had not been encountered earlier. This was confirmed by the fitness trends in four different alternative stressful environments.

Chapter 3 presents the first study to empirically demonstrate a link between historic population size and the vulnerability to sudden environmental changes. We found that efficient natural selection in an unchanging environment could make large populations undergo highly focused adaptation that can, in turn, render them vulnerable to sudden changes in their environments. This counterintuitive insight finds an analogue in a recent study documenting the rise and fall of passenger pigeon populations (Murray et al., 2017). Using DNA sequence data, it was shown that the passenger pigeon populations in North America were so large that natural selection resulted in highly reduced diversity at important loci. Such reduction in diversity might have made these populations highly vulnerable to the sudden environmental change brought upon by the industrial revolution, which could have ultimately contributed to their extinction. While this study pertains to a completely different model system as compared to our experiments, the congruence between its observations and our experimental results lend credence to the idea

that adapting in larger numbers can potentially increase the susceptibility to environmental changes.

Chapter 3 also uncovers a novel trade-off between maximizing the speed of adaptation and decreasing vulnerability to environmental changes, which should be pertinent to asexual populations of microbes that face periodic bottlenecks (e.g., host-to-host transfer of gut microbiota or pathogens), particularly if their environment changes across states that show fitness trade-offs (e.g., costs of drug resistance in drug-free conditions (Andersson and Hughes, 2010)). Specifically, larger asexual populations can face severe fitness declines if their environment changes across such states.

The results of Chapter 3 also have important implications for understanding the evolutionary fates of putatively important biological characters. Conventionally, the decay or enhancement of a biological character has been attributed to the environment in which evolution occurs. For example, the evolutionary decay of eyes in multiple cave-dwelling animals has been attributed to dark environments (Jeffery, 2005; Protas et al., 2011). Our study presents the first demonstration that selection can make a biological character decay or enhance rapidly over evolutionary time in the same environment. This result calls for a re-evaluation of the importance conventionally given to the environmental conditions while making *a posteriori* claims with respect to character divergence across disparate populations. Specifically, our study demonstrates that differences in environmental conditions (and hence the direction of selection) need not always be invoked to explain such character divergence; instead differences in population size can themselves lead to the divergence of a putatively important biological character in the same selection environment. To investigate this phenomenon further, we used Wright Fisher simulations of evolutionary dynamics under different population sizes.

In Chapter 4, we present a generalizable simulation framework to study how differences in the sizes of asexual populations adapting to the same environment can translate into divergent fates of an important fitness-affecting character. Our simulations reveal that sign epistasis and differential mutational supply across fitness-affecting loci can lead to such divergence in character fates when present together, but not on their own. Most importantly, our study demonstrates that a simple two-locus three-allele landscape with sign epistasis and differential supply of mutations is sufficient for translating quantitative differences in population sizes to qualitative differences (decay versus enhancement) in character fates in the same environment. Chapter 4 thus provides a putative population genetic explanation for divergent character

evolution observed in Chapter 3. Sign epistasis is expected to be a very common feature of natural fitness landscapes (Szendro et al., 2013a). Moreover, a potential source of differential mutational supply loci is the substantial variation in gene length (Eyre-Walker, 1996; Moriyama and Powell, 1998; Wright, 1990). Thus, the conditions required for divergent fates of a fitness-affecting character are expected to be fairly widespread in asexual populations. Chapter 4 thus provides a parsimonious explanation for rapid microevolutionary character divergence that does not require the presence of contrasting environmental conditions.

While Chapter 3 dealt with the phenotypic aspects of divergent character (efflux) evolution in the same environment, Chapter 4 provided a simple simulation framework that could reproduce this phenomenon in a generalizable manner. A logical next step is to study the molecular details of the divergent efflux evolution in the antibiotic cocktail to verify these explanations using whole-genome whole-population sequencing. We have already initiated this line of work and expect to gain new insights from the resulting data. The latter would also aid in testing some key predictions made in Chapter 2. Specifically, these data can be compared with the prediction made in terms of the changes in genotypic distributions within populations of different sizes.

A significant gap in the existing understanding of the population genetics of fitness trade-offs concerns the lack of clarity regarding the effects of population size on fitness trade-offs. An important contributor to the lack of understanding of the population genetics of fitness trade-offs concerns the inconsistencies across different studies in the perspectives for studying fitness trade-offs across environments. Chapter 5 presents an evolution experiment with *E. coli* populations of two different sizes in multiple nutritionally limited homogenous environments. In this study, we looked at trade-offs from three different perspectives and found that regardless of the choice of perspectives, larger populations evolved greater fitness trade-offs. Importantly, our results were robust to changes in the identity of the environmental pair across which trade-offs were studied. This is the first study to propose and demonstrate a simple relationship between population size and fitness trade-offs and should add to the current understanding of the population genetics of ecological specialization.

We note that the results of Chapter 5 corroborate the results of Chapter 3, which revealed that adapting in larger populations could be disadvantageous to fitness in other environments. On the one hand, the selection environment in Chapter 3 contained a cocktail of three antibiotics while the alternate environments offered diverse challenges to bacterial fitness. On the other hand, the experiment of Chapter 5 had multiple selection environments based on different

individual carbon sources. Moreover, while the experiment of Chapter 3 did not involve reciprocal selection and fitness measurements in multiple environments, the experiment of Chapter 5 did so. Overall, the congruence between the observations of Chapter 3 and Chapter 5 shows that adapting in larger numbers can make asexual populations suffer greater fitness trade-offs. This result can be particularly relevant in scenarios where the environment abruptly shifts between two states that exhibit fitness trade-offs with each other. Such sudden environmental shifts are routinely encountered by bacteria. For example, pathogens have been shown to experience fitness trade-offs when they migrate across different hosts (Smith-Tsurkan et al., 2010; Turner and Elena, 2000). Our results predict that larger populations would suffer heavier fitness trade-offs across environments and evolve greater extent of ecological specialization. Thus, our results show that adapting to a single host in larger numbers for several hundred generations should lead to specialist pathogens with narrow host ranges. Thus, our results can have practical implications for scenarios where fitness trade-offs play an important role.

Following up on the observations of Chapter 5, Chapter 6 offers a novel explanation for the scarcity of detectable fitness costs in evolutionary and ecological studies. Here we conducted an evolution experiment with *E. coli* populations of two different sizes in a heterogeneous environment (randomly fluctuating across four different sole carbon sources) and four distinct homogenous environments, each with one of the above four sole carbon sources. To the best of our knowledge, this is the only experimental evolution study carried out in heterogenous environments at multiple population sizes. We found that population size has opposite relationships with fitness costs in homogenous and heterogenous environments. Specifically, in heterogenous environments, smaller populations suffered more fitness costs than the larger ones. Interestingly, the larger populations evolved in the heterogeneous environments not only avoided all fitness costs under consideration, but also showed simultaneous adaptation to a pair of environments known to show strong fitness trade-offs with each other. On the contrary, in homogenous environments, the larger populations suffered greater fitness costs than the smaller ones. Previous studies suggest that the magnitudes of fitness costs should be lower in heterogenous environments as compared to homogenous ones (Bono et al., 2017), predicting that multiple selection pressures faced in a heterogenous environment should lead to cost avoidance. However, we found that cost avoidance depends on an interplay between environmental heterogeneity and population size. The simultaneous presence of large population size and heterogenous environment led to the avoidance of fitness costs, but neither

of these two factors could lead to cost avoidance on their own. Importantly, we explain why large populations can avoid fitness costs while evolving in a heterogenous environment even when most mutations show antagonistic pleiotropy across different environmental components.

The observations of Chapter 6 cannot be accounted for by any of the conventional explanations for the lack of detectable fitness costs, which include the experimental setup's failure to provide the relevant conditions to express costs (Coustau et al., 2000), high statistical demands of detecting antagonistic pleiotropy (Ågren et al., 2013; Anderson et al., 2013; Bono et al., 2017; Coustau et al., 2000), and inadequate experimental durations (Jasmin and Kassen, 2007a; Satterwhite and Cooper, 2015; Schick et al., 2015; Velicer and Lenski, 1999). Instead, our experiments suggest that asexual populations that have a history of evolving in unstable environments in large numbers can harbour mutations that are simultaneously beneficial in multiple environmental components. Importantly, the explanation of our results pertains to the generic concept of fitness costs across different environments in asexual microbial populations. The natural environments of human microbial pathogens routinely fluctuate between drug-containing and drug-free conditions. If our prediction regarding the evolution of fitness costs in fluctuating environments also applies to costs of antibiotic resistance, lack of proper sanitary conditions (which would result in large microbial population sizes) itself should result in the evolution and vigorous spread of cost-free resistance, even if most mutations that provide antibiotic resistance carry a cost in drug-free conditions. Therefore, a clear future extension of Chapter 6 is to directly study the evolution of antibiotic resistance and its costs in an experiment with multiple population sizes where the environment fluctuates between antibiotic-containing and antibiotic-free states.

We note that this thesis deals exclusively with asexual systems. One of the principal messages of Chapter 2 is that the conventional measure of population size (*HM*) fails to explain the fitness trajectories of asexual populations with fluctuating sizes. As discussed earlier, this is partly due to wastage of several beneficial mutations due to clonal interference. Interestingly, *HM* is also the conventional measure of population size in sexual populations (Charlesworth, 2009; Kosheleva and Desai, 2018; McDonald et al., 2016; Rice, 2004). Since clonal interference is not as relevant in sexual populations due to recombination, it is possible that *HM* succeeds in explaining the trajectories of fitness in sexual populations. There are no empirical or theoretical tests of this idea, and due caution should be observed while extrapolating our results to sexual populations.

Finally, the phenomenon of horizontal gene transfer (HGT) across microbial taxa is a significant source of adaptive variation in microbial communities (Niehus et al., 2015; Springael and Top, 2004; Wiedenbeck and Cohan, 2011). The variation provided by HGT transcends single-species microbial populations, and when such variation becomes adaptively relevant, the concept of single-species microbial populations needs to be re-evaluated (Joyce et al., 2002). We note that the observations of this thesis do not apply to systems where HGT plays a significant role and testing how HGT alters our predictions, particularly in the context of microbial communities, can be of considerable interest.

Appendices

Appendix 1

Culture environment for experimental evolution:

24 independent bacterial populations *Escherichia coli* K-12 MG1655 were grown in Nutrient Broth with a fixed concentration of an antibiotic cocktail containing a mixture of three antibiotics at the following sub-lethal concentrations:

1. Norfloxacin (0.015 μg/ml)
2. Rifampicin (6 μg/ml)
3. Streptomycin (0.1 μg/ml)

Composition of Nutrient Broth (Himedia Laboratories Pvt. Ltd.):

- Peptic digest of animal tissue (5 g/l)
- Sodium chloride (5 g/l)
- Beef extract (1.50 g/l)
- Yeast extract (1.50 g/l)

Appendix 2

Algorithm for the individual based model used in this study:

Our model simulates the growth of individual bacteria in density-dependent resource-limited conditions. Each bacterium is represented by an array which has the following components:

1. Determinant of efficiency (K_eff): determines how much food can be assimilated per unit time
2. Determinant for threshold (thres): how much food needs to be assimilated in order to divide
3. Bodymass: how big is the bacterium (where is it along its cell cycle) at any given time

At the beginning of the simulation, two global scaling quantities, Food_Proxy and Body_Proxy are declared for the whole population. As the names suggest, Food_Proxy acts as a proxy for the amount of available resources initially, while Body_Proxy (=250) is a proxy for bodymass of the ancestor. Each simulation run is started with 100 individuals and each individual is allotted K_eff_i value given as Eff_i * Food_Proxy. Here, Eff_i is a random number picked from a uniform distribution U(0.95-1.05) and K_eff_i determines how much food would be consumed in a density–dependent manner and when its food consumption would stop (as per the conditions given below). Similarly, the parameter for the threshold for each of the 100 starting individuals is assigned as a random number picked from a uniform distribution between 0.95*(Body_Proxy) and 1.05*(Body_Proxy).

Each bacterium has the same initial biomass (an arbitrarily small quantity, 10 units in this case).

Time is implicitly defined in our code and each iteration signifies one unit of time.

In each iteration, each bacterium "grows and divides" according to the following rules:

If for bacterium i, (population size/ K_eff_i) ≥ 1, it doesn't eat anything: its bodymass remains the same as the earlier iteration.

If for bacterium i (population size/ K_eff_i) < 1, it eats 10(1 - (population size/ K_eff_i)) units of food in this iteration: its bodymass increases by 10*(1- (population size/ K_eff_i) units).*

If at the end of this iteration, $bodymass_i$ > $thres_i$, bacterium i divides into two equal parts. A small thermodynamic cost (constant for all individuals) is deducted so that the sum of

the bodymass of the daughter cells is exactly 1 unit less than the bodymass of the mother cell at the time of division.

If the bacterium divides, there is a 1 in 100 chance for each of the daughter cells that it mutates. If a mutation occurs, the new parameter for efficiency is drawn from an already defined normal distribution that is used throughout the simulation. The same applies to threshold. (Threshold and efficiency mutate independently in each bacterium.)

The total size of the population is saved at the end of every iteration. The total amount of food consumed during each iteration is also computed.

The above description (italics) represents all the processes that happen within an iteration.

The process is repeated (and the population grows) until the following conditions are fulfilled:

1. The number of iterations is greater than 2000.
2. The amount of food consumed during each iteration < 0.08* Food_Proxy

If the above conditions are met simultaneously, food consumption is stopped, a defined fraction of individuals are sampled randomly and the whole process is started with this sample population (this represents bottlenecking). The above process is continued for *q* bottlenecks. The bottleneck ratio and the number *q* are predefined, depending upon the type of population being studied. This gives rise to *q* sigmoidal growth curves. Two quantities are extracted from each sigmoidal curve:

1. Carrying capacity (*K*, the maximum size of the population in each growth phase)
2. Maximum linear growth rate (*R*, the maximum slope of population growth over 100 iterations). Straight lines were fit on overlapping moving windows of 100 iterations on the entire time-series of population size values within each growth-phase. The maximum value of the slope observed within the entire time-series of population size values (sigmoidal curve) was taken to be the maximum growth rate (*R*).

Time series of carrying capacities and maximum growth rates are computed using the series of *q* sigmoidal curves.

In each simulation used in this study, the carrying capacity of the first growth phase was ≈ 1.8*Food_Proxy. The value of Food_Proxy was adjusted in such a way that it gave rise to the desired value of the carrying capacity of the first growth phase in a simulation. The carrying capacities corresponding to the subsequent growth phases was an emergent result of Darwinian

evolution in the simulations. A summary of the key differences between our model and previous theoretical studies is given below in Table A2.1.

Property	Wahl and Gerrish (2001)	Desai and Fisher (2007)	Campos and Wahl (2009)	Campos and Wahl (2010)	Wahl and Zhu (2015)	Our study
Direct analysis of bottlenecks	Yes	No	Yes	Yes	Yes	**Yes**
Direct predictions about *EoA*	No	Yes	No	No	No	**Yes**
Deleterious mutations present	No	No	Yes	Yes	Yes	**Yes (Majority)**
Clonal interference allowed	No	Yes	Yes	Yes	No	**Yes**
Size(s) of distinct beneficial mutations	Not applicable	Constant	Variable	Constant*	Not applicable	**Variable**
Variable N_f across populations being compared	No	Not applicable	No	No	No	**Yes**
Evolution in resource limited conditions	No	No	No	No	Yes	**Yes**
Overlapping generations	Yes	No	No	No	Yes	**Yes**

Table A2.1. A summary of the key differences between our model and previous theoretical studies.

*While assuming that distinct beneficial mutations can have different effects, Campos and Wahl (2010) arrive at an expression of N_e that itself depends upon the size of such effects (s_b). They then state that "Clearly an effective population size that depends on s_b is unsatisfactory." Then they assume that "all beneficial mutations have the same effect" to arrive at a much simpler expression.

Appendix 3

Parameter and distribution settings for the individual based simulations in Chapter 2

Table A3.1. The simulation settings used in the individual-based simulations

Population type	Food_proxy	Bottleneck ratio	Number of bottlenecks (400 generations)
XX’	$2*10^4$	1/10	120
SS’	10^5	$1/10^2$	60
SL’	$5*10^6$	$1/10^4$	30
BN1	$5*10^3$	1/10	120
BN2	$5*10^4$	$1/10^2$	60
BN3	$5*10^5$	$1/10^3$	40
BN4	$5*10^6$	$1/10^4$	30
SM1	$5*10^3$	1/10	120
SM4	$5*10^6$	$1/10^4$	30
HB	$5*10^6$	$1/10^4$	30
LB	$2*10^4$	1/10	120
LBbar	$1.25*10^3$	1/10	120
MBbar	$2.5*10^4$	$1/10^2$	60
SNFBN1	$5*10^4$	1/10	120
SNFBN2	$5*10^4$	$1/10^2$	60
SNFBN4	$5*10^4$	$1/10^4$	30
Fig. 2.10a (small bottleneck size)	$5*10^4$	1/10	120
Fig. 2.10a (large bottleneck size)	$5*10^6$	1/10	120
Fig. 2.10b (small bottleneck size)	$5*10^4$	$1/10^3$	40
Fig. 2.10b (large bottleneck size)	$5*10^6$	$1/10^3$	40
Fig. 2.10c (small bottleneck size)	$5*10^5$	$1/10^4$	30
Fig. 2.10c (large bottleneck size)	$5*10^6$	$1/10^4$	30
Fig. 2.11a (lenient bottleneck)	$5*10^2$	1/10	120
Fig. 2.11a (harsh bottleneck)	$5*10^6$	$1/10^5$	24
Fig. 2.11b (lenient bottleneck)	$5*10^4$	1/10	120
Fig. 2.11b (harsh bottleneck)	$5*10^6$	$1/10^3$	40
Fig. 2.11c (lenient bottleneck)	$5*10^5$	1/10	120
Fig. 2.11c (harsh bottleneck)	$5*10^7$	$1/10^3$	40
Fig. 2.11d (lenient bottleneck)	$5*10^6$	1/10	120
Fig. 2.11d (harsh bottleneck)	$5*10^7$	$1/10^2$	60

Table A3.2. Distributions for parameters used in simulations

Distribution used for	**Distribution for efficiency parameter**	**Distribution for threshold parameter**
Starting the simulation	Uniform random (0.95,1.05)	Uniform random (237.5,262.5)
Mutation	Normal random (0.9,0.22)	Normal random (1.1,0.22)

Appendix 4

Statistical analysis of the empirical results of Chapter 2

Generation	ANOVA $F_{(2,21)}$	ANOVA *P*	Holm-Šidàk corrected *P*	Tukey *P* LL-SL	Tukey *P* LL-SS	Tukey *P* SL-SS
40	36.75	1.4E-7	1E-6	0.0001	0.0001	0.0001
80	13.319	0.0002	0.0011	0.0001	0.0001	0.7942
120	14.365	0.0001	0.0008	0.0001	0.0001	0.0001
160	17.282	3.7E-5	0.0003	0.0001	0.0001	0.0001
200	2.894	0.0776	-	-	-	-
240	9.359	0.0012	0.0050	0.0001	0.0001	0.0001
280	12.110	0.0003	0.0016	0.0001	0.0001	0.0001
320	6.769	0.0054	0.0161	0.0001	0.0001	0.0001
~ 390	3.33	0.0556	-	-	-	-

Table A4.1. A summary of statistical analysis of carrying capacity (*K*) measurements in empirical populations. The values in red represent statistically significant difference ($P < 0.05$). The *P*-values corresponding to nine independent ANOVAs (corresponding to nine different time points) were subjected to Holm-Šidàk correction. Post-hoc (Tukey) comparisons were done only in cases where the ANOVA *P*-values were less than 0.05 after Holm-Šidàk correction. These post–hoc comparisons were done for three pairwise differences (LL-SL, LL-SS, and SL-SS) at each time point. Holm-Šidàk correction was not done on Tukey *P*-values. The *P*-values are reported to four decimal places.

Generation	ANOVA $F_{(2,21)}$	ANOVA *P*	Holm-Šidàk corrected *P*	Tukey *P* LL-SL	Tukey *P* LL-SS	Tukey *P* SL-SS
40	56.631	3.5E-9	3.1E-8	0.0001	0.0001	0.0001
80	5.996	0.0087	0.0344	0.0001	0.0001	0.3229
120	12.027	0.0003	0.0023	0.0001	0.0001	0.0001
160	12.287	0.0003	0.0023	0.0001	0.0001	0.0001
200	3.093	0.0665	0.1286	-	-	-
240	8.757	0.0017	0.0103	0.0001	0.0001	0.0001
280	7.785	0.0030	0.0147	0.0001	0.0001	0.0001
320	4.096	0.0315	0.0915	-	-	-
~ 390	0.925	0.4122	-	-	-	-

Table A4.2. A summary of statistical analysis of maximum growth rate (*R*) measurements in empirical populations. The values in red represent statistically significant difference ($P < 0.05$). The values in red represent statistically significant difference ($P < 0.05$). The *P*-values corresponding to nine independent ANOVAs (corresponding to nine different time points) were subjected to Holm-Šidàk correction. Post-hoc (Tukey) comparisons were done only in cases where the ANOVA *P*-value was less than 0.05 after Holm-Šidàk correction. These post–hoc comparisons were done for three pairwise differences (LL-SL, LL-SS, and SL-SS) at each time point. Holm-Šidàk correction was not done on Tukey *P*-values. The *P*-values are reported to four decimal places.

Appendix 5

Details of the protocol used for measuring energy-dependent efflux activity:

We used a previously-established fluorescence-based assay for measuring energy-dependent efflux activity (EA) in Gram negative bacteria (Karve et al., 2015; Webber and Coldham, 2010). We used a small molecule (bis-benzimide) that enters bacterial cells and fluoresces after intercalating with DNA (bis-benzimide excites at 355 nm and emits at 465 nm). The details of the protocol are as follows:

- Cryostocks belonging to each of the 25 populations (24 descendants and 1 ancestor) were revived in Nutrient Broth (NB) for 18 hours.
- The revived populations were brought to similar sizes by dilutions with (NB) so that the OD at 600 nm was between 0.03 and 0.06 when measured using Nanodrop (Thermo Scientific, 2000).
- The above cultures (with similar sizes) were centrifuged at 13,400 rpm for 2 minutes. The supernatant was discarded, and PBS buffer (at pH 7.4) was used to resuspend the pellet.
- A small aliquot of the ancestral culture was heated at 60 ^{O}C to set the upper range of fluorescence gain.
- 8 µl of glucose solution (1% w/v) was also added because the aim was to measure energy-dependent efflux activity.
- 20 µl of bis-benzimide was added to each well containing 168 µl live culture. The same volume of bis-benzimide was also added to the control well with dead cells.
- An automated plate reader (Tecan Infinite M200 Pro) was used to measure fluorescence.

Initially, we measured fluorescence for 40 min. All the fluorescence curves had reached a steady state by 35 min.

- After 40 minutes, the plate was taken out and 4 µl of a non-specific inhibitor of active efflux was added to all the wells. The inhibitor of efflux used in our study was CCCP (Carbonyl Cyanide m-Chlorophenylhydrazone; C2759 Sigma).
- The measurement of fluorescence was resumed and continued for a further 30 minutes so that a new steady state could be reached by all the fluorescent curves.
- Efflux activity was measured using the following formula:

  (Fluorescence at 70 min – Fluorescence at 35 min) / (Fluorescence at 35 min)

Appendix 6

Statistical details of ANOVAs done separately in each alternative environment (Chapter 3):

Alternative environment	ANOVA *P*	Benjamini-Hochberg critical value	$F_{2,21}$	Partial η^2	Pairwise comparisons					
					LL-SL		LL-SS		SL-SS	
					Tukey *P* (post-hoc)	Cohen's d	Tukey *P* (post-hoc)	Cohen's d	Tukey *P* (post-hoc)	Cohen's d
Ampicillin	0.000005	0.0375	23.34	0.689729 (large effect)	0.000126	2.463 (large effect)	0.000126	2.641 (large effect)	0.641707	-
Sorbitol	0.006558	0.075	6.448	0.380455 (large effect)	0.000126	1.493 (large effect)	0.000126	1.665 (large effect)	0.471524	-
Urea	0.018380	0.1125	4.864	0.316563 (large effect)	0.000126	1.332 (large effect)	0.000126	1.226 (large effect)	0.853422	-
Copper	0.037171	0.15	3.867	0.269149 (large effect)	0.000126	1.571 (large effect)	0.000126	0.699 (medium effect)	0.000126	0.613 (medium effect)

Table A6.1. Summary of the statistical analysis done separately for each of the four alternative environments in terms of K. Statistically significant *P* values are shown in red. The False Discover Rate (FDR) used here is 0.15 (McDonald, 2009). Partial η^2 was interpreted as: Partial $\eta^2 < 0.06$ (small effect); $0.06 <$ Partial $\eta^2 < 0.14$ (medium effect); $0.14 <$ Partial η^2 (large effect). Cohen's d was interpreted as: $0.2 < d < 0.5$ (small effect), $0.5 < d < 0.8$ (medium effect); $d > 0.8$ (large effect). Tukey's post-hoc test was done only when the ANOVA results were significant after the Benjamini Hochberg procedure. Cohen's d was interpreted only when the pairwise differences (revealed by Tukey's post-hoc test) were significant.

Alternative environment	ANOVA *P*	Benjamini-Hochberg critical value	$F_{2,21}$	Partial η^2	Pairwise comparisons					
					LL-SL		LL-SS		SL-SS	
					Tukey *P* (post-hoc)	Cohen's d	Tukey *P* (post-hoc)	Cohen's d	Tukey *P* (post-hoc)	Cohen's d
Sorbitol	0.036469	0.0375	3.893	0.270475 (large effect)	0.000128	1.208 (large effect)	0.000126	1.388 (large effect)	0.634558	-
Ampicillin	0.051636	0.075	3.424	0.245908 (large effect)	0.000126	0.901 (large effect)	0.000126	1.059 (large effect)	0.467068	-
Urea	0.277341	0.1125	1.364	0.114979 (medium effect)	-	-	-	-	-	-
Copper	0.438481	0.15	0.858	0.075515 (medium effect)	-	-	-	-	-	-

Table A6.2. Summary of the statistical analysis done separately for each of the four alternative environments in terms of R. Statistically significant *P* values are shown in red. The False Discover Rate (FDR) used here is 0.15 (McDonald, 2009). Partial η^2 , Tukey's post-hoc test, and Cohen's d were interpreted as described above for Table A6.1.

Appendix 7

Ancestral fitness values in the alternative environments (Chapter 3):

Alternative environment	K	R
Sorbitol	0.652	0.054
Ampicillin	0.836	0.118
Urea	0.427	0.049
Copper	0.924	0.170

Table A7.1. Fitness values of the common ancestor in the four alternative environments in terms of K and R.

Appendix 8

Details of the ancestral strain and media compositions in Chapter 5 and Chapter 6:

Ancestral strain: *Escherichia coli* MG1655 lacY::kan (resistant to kanamycin).

Composition of the minimal media: Our experiment involved four different M9-based minimal media, each containing one of the following as the only source of carbon:

1. Thymidine
2. Galactose
3. Maltose
4. Sorbitol

1 litre of each minimal medium contained the following:

- 12.8 g $Na_2HPO_4.7H_2O$
- 3.0 g KH_2PO_4
- 0.5 g NaCl
- 1.0 g NH_4Cl
- 240.6 mg $MgSO_4$
- 11.1 mg $CaCl_2$
- 4g of the pre-decided carbon source
- 50 mg Kanamycin sulphate

Appendix 9

Analysis using single-sample t-tests in Chapter 5:

Population type	Assay environment	*P* value	Corrected *P* value	Cohen's *d* (Effect size)
TL	Thy	8.100×10^{-7}	3.240×10^{-6}	12.129 (large)
TL	Gal	8.943×10^{-5}	2.683×10^{-4}	- 4.670 (large)
TL	Mal	5.553×10^{-4}	5.553×10^{-4}	- 3.187 (large)
TL	Sor	1.135×10^{-4}	2.270×10^{-4}	- 4.445 (large)
TS	Thy	1.832×10^{-4}	7.327×10^{-4}	4.024 (large)
TS	Gal	6.839×10^{-3}	6.839×10^{-3}	- 1.807 (large)
TS	Mal	4.576×10^{-4}	1.372×10^{-3}	- 3.318 (large)
TS	Sor	6.190×10^{-4}	1.238×10^{-3}	- 3.111 (large)
GL	Thy	0.003	0.013	- 2.158 (large)
GL	Gal	0.016	0.049	1.448 (large)
GL	Mal	0.633	0.633	-
GL	Sor	0.973	0.973	-
GS	Thy	0.122	0.122	-
GS	Gal	0.617	0.617	-
GS	Mal	0.483	0.483	-
GS	Sor	0.016	0.061	-

Table A9.1. Single-sample t-tests against the ancestral fitness values (N = 6). The fourth column shows Holm-Šidák corrected *P* values. Effect sizes were interpreted as the following: $0.2 < d < 0.5$ (small effect), $0.5 < d < 0.8$ (medium effect); $d > 0.8$ (large effect). Effect sizes were not interpreted for cases where Holm-Šidák corrected $P > 0.05$.

Population type	Home-Away pair	*P* value	Corrected *P* value	Cohen's d (Effect size)
TL	T-G	6.481×10^{-8}	6.481×10^{-8}	20.131 (large)
TL	T-M	2.467×10^{-8}	7.402×10^{-8}	24.427 (large)
TL	T-S	5.935×10^{-8}	1.187×10^{-7}	20.490 (large)
TS	T-G	5.633×10^{-5}	5.633×10^{-5}	5.136 (large)
TS	T-M	5.490×10^{-5}	1.098×10^{-4}	5.163 (large)
TS	T-S	4.714×10^{-5}	1.414×10^{-4}	5.328 (large)
GL	G-T	1.830×10^{-4}	5.488×10^{-4}	4.025 (large)
GL	G-M	2.217×10^{-4}	4.434×10^{-4}	3.866 (large)
GL	G-S	5.910×10^{-3}	5.910×10^{-3}	1.873 (large)
GS	G-T	0.232	0.232	-
GS	G-M	0.504	0.504	-
GS	G-S	0.061	0.061	-

Table A9.2. Single-sample t-tests against the ancestral reaction norm slopes (N = 6). The fourth column shows Holm-Šidák corrected *P* values. Effect sizes were interpreted as the following: $0.2 < d < 0.5$ (small effect), $0.5 < d < 0.8$ (medium effect); $d > 0.8$ (large effect). Effect sizes were not interpreted for cases where Holm-Šidák corrected $P > 0.05$.

Appendix 10

Statistical summary for the analyses carried out in Chapter 6:

Selection environment	Population type	Assay environment	*P* value	Corrected *P* value	Inference
Heterogeneous	FL	Thy	1.58×10^{-5}	6.33×10^{-5}	Adaptation
Heterogeneous	FL	Gal	7.025×10^{-3}	0.021	Adaptation
Heterogeneous	FL	Mal	0.564	-	-
Heterogeneous	FL	Sor	0.612	-	-
Heterogeneous	FS	Thy	0.049	0.049	Adaptation
Heterogeneous	FS	Gal	0.025	0.051	Maladaptation
Heterogeneous	FS	Mal	2.5×10^{-4}	0.001	Maladaptation
Heterogeneous	FS	Sor	0.009	0.02635	Maladaptation
Homogeneous	TL	Thy	8.1×10^{-7}	3.24×10^{-6}	Adaptation
Homogeneous	TL	Gal	8.94×10^{-5}	2.68×10^{-4}	Maladaptation
Homogeneous	TL	Mal	5.53×10^{-4}	5.53×10^{-4}	Maladaptation
Homogeneous	TL	Sor	1.13×10^{-4}	2.27×10^{-4}	Maladaptation
Homogeneous	TS	Thy	1.83×10^{-4}	7.33×10^{-4}	Adaptation
Homogeneous	TS	Gal	6.839×10^{-3}	6.839×10^{-3}	Maladaptation
Homogeneous	TS	Mal	4.58×10^{-4}	1.372×10^{-3}	Maladaptation
Homogeneous	TS	Sor	6.19×10^{-4}	1.238×10^{-3}	Maladaptation
Homogeneous	GL	Thy	0.003	0.013	Maladaptation
Homogeneous	GL	Gal	0.016	0.049	Adaptation
Homogeneous	GL	Mal	0.633	0.633	-
Homogeneous	GL	Sor	0.973	0.973	-
Homogeneous	GS	Thy	0.122	0.122	-
Homogeneous	GS	Gal	0.617	0.617	-
Homogeneous	GS	Mal	0.483	0.483	-
Homogeneous	GS	Sor	0.016	0.061	-

continued

continued

Selection environment	Population type	Assay environment	*P* value	Corrected *P* value	Inference
Homogeneous	ML	Thy	0.252	-	-
Homogeneous	ML	Gal	0.036	0.134661	-
Homogeneous	ML	Mal	0.090	-	-
Homogeneous	ML	Sor	0.762	-	-
Homogeneous	MS	Thy	0.134	-	-
Homogeneous	MS	Gal	0.164	-	-
Homogeneous	MS	Mal	0.066	-	-
Homogeneous	MS	Sor	0.069	-	-
Homogeneous	SL	Thy	3.14×10^{-4}	1.257×10^{-3}	Maladaptation
Homogeneous	SL	Gal	7.59×10^{-3}	0.023	Maladaptation
Homogeneous	SL	Mal	0.088	-	-
Homogeneous	SL	Sor	0.690	-	-
Homogeneous	SS	Thy	5.5×10^{-3}	0.011	Maladaptation
Homogeneous	SS	Gal	6.4×10^{-5}	2.56×10^{-4}	Maladaptation
Homogeneous	SS	Mal	0.001	0.003	Maladaptation
Homogeneous	SS	Sor	0.366	-	-

Table A10.1. Analysis of adaptation and maladaptation events in all ten evolutionary lines using single-sample t tests (N = 6) with reference to the ancestral fitness in each carbon source (scaled to 1) followed by Holm-Sidak corrections.

Selection environment	Population type	*P* value	Inference
Heterogeneous	FL	0.002	GM enhanced
Heterogeneous	FS	0.584	-
Homogeneous	TL	0.703	-
Homogeneous	TS	0.826	-
Homogeneous	GL	0.922	-
Homogeneous	GS	0.026	GM reduced
Homogeneous	ML	0.027	GM reduced
Homogeneous	MS	0.069	-
Homogeneous	SL	0.034	GM reduced
Homogeneous	SS	5.96×10^{-4}	GM reduced

Table A10.2. Summary of single-sample t-tests (N = 6) of differences in the geometric mean fitness (calculated over the four carbon sources) of the ten evolutionary lines with the corresponding ancestral value (= 1).

Selection environment	Population type	*P* value (Dunnett (reference: FL))	*P* value (Dunnett (reference: FS))
Heterogeneous	FL	-	8.508×10^{-6}
Heterogeneous	FS	8.508×10^{-6}	-
Homogeneous	TL	2.214×10^{-5}	0.992
Homogeneous	TS	1.222×10^{-5}	0.999
Homogeneous	GL	1.260×10^{-5}	0.999
Homogeneous	GS	6.865×10^{-6}	0.652
Homogeneous	ML	6.872×10^{-6}	0.737
Homogeneous	MS	6.855×10^{-6}	0.302
Homogeneous	SL	6.853×10^{-6}	0.036
Homogeneous	SS	6.853×10^{-6}	0.007

Table A10.3. Summary of Dunnett post-hoc tests (N = 6) with respect to FL and FS done after an analysing the geometric mean fitness differences across the ten evolutionary lines using a mixed model ANOVA (see section 6.2 (Chapter 6)). This analysis revealed a significant main effect of the identity of the evolutionary line: $F_{9,45} = 14.566$, $P = 1.129 \times 10^{-6}$.

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List of publications and manuscripts under preparation

Publications:

- **Chavhan, Y.D.**, Ali, S.I., and Dey, S. (2019). Larger Numbers Can Impede Adaptation in Asexual Populations despite Entailing Greater Genetic Variation. Evol. Biol. *46*, 1–13.

- **Chavhan, Y.**, Karve, S., and Dey, S. (2019). Adapting in larger numbers can increase the vulnerability of *Escherichia coli* populations to environmental changes. Evolution *73*, 836–846.

Manuscript under review:

- **Chavhan, Y.**, Malusare, S., and Dey, S. (2019). Larger *Escherichia coli* populations suffer greater fitness trade-offs and undergo more ecological specialization. (Under review)

Manuscripts under preparation:

- **Chavhan, Y.**, Shah, S., and Dey, S. (2019). Minimal requirements for divergent character fates in populations adapting to the same environment at different sizes.

- **Chavhan, Y.**, Malusare, S., and Dey, S. (2019). An interaction of environmental heterogeneity and population size explains the rarity of detectable fitness costs.

Apart from the research articles mentioned above, I have also been associated with the following publication, which is not a part of this thesis:

- Karve, S.M., Daniel, S., **Chavhan, Y.D.**, Anand, A., Kharola, S.S., and Dey, S. (2015). *Escherichia coli* populations in unpredictably fluctuating environments evolve to face novel stresses through enhanced efflux activity. J. Evol. Biol. *28*, 1131–1143.