The influence of fluctuating antibiotic exposures and population sizes on the evolution of multidrug resistance

A Thesis

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by

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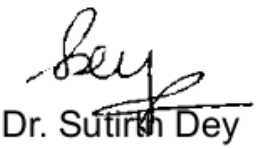
Certificate

This is to certify that this dissertation entitled “The influence of fluctuating antibiotic exposures and population sizes on the evolution of multidrug resistance” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Kasturi Lele at Indian Institute of Science Education and Research under the supervision of Dr. Sutirth Dey, Professor, Department of Biology, during the academic year 2020-2021.



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Declaration

I hereby declare that the matter embodied in the report entitled “The influence of fluctuating antibiotic exposures and population sizes on the evolution of multidrug resistance” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sutirth Dey and the same has not been submitted elsewhere for any other degree.

Kasturi Lele

Date: 13/05/2021

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Abstract

Experimental evolution is an important tool used to study the long-term effects of multidrug resistance in bacterial populations. Two major factors that can potentially affect the evolution of resistance are fluctuations in exposures to antibiotics and differences in population sizes. To investigate this, replicate populations of *Escherichia coli* were subjected to either a single antibiotic or fluctuations between a pair of antibiotics. After these selections, the increase in antibiotic resistance was measured as an increase in MIC, growth rate and carrying capacity compared to the ancestral populations. Contrary to prior expectations, we found that large populations did not show an increase in antibiotic resistance compared to small populations when selected under increasing concentrations of a single antibiotic. Also, large populations showed a lack of costs when exposed to novel antibiotics. When exposed to fluctuations between a pair of antibiotics, large populations were able to evolve a higher resistance than small populations, but again showed a lack of costs in novel antibiotics. These results indicate that there might be a few factors with different levels of influence on large and small populations, such as the presence of resistant subpopulations, upregulation of efflux genes and differences in the distribution of fitness effects. Further experiments are required to clarify the effect of these individual factors.

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Introduction

Since the development of multiple classes of antibiotics to target pathogens, it has also become abundantly clear that bacteria are capable of evolving multidrug resistance. Hence, a large body of research has been devoted to tackle the problem of slowing down or reversing resistance in bacteria that have been exposed to multiple antibiotics (reviewed in Baym et. al., 2016 and Roemhild and Schulenberg, 2019). Using the tool of experimental evolution to understand the long-term effects of antibiotic resistance (Baym et. al., 2016) several factors that influence the development of multidrug resistance have been identified. For instance, it has been shown that alternating exposures between a pair of antibiotics have the potential to slow down the evolution of antibiotic resistance (Imamovic and Sommer, 2013; Kim et. al., 2014). This is due to a phenomenon known as collateral sensitivity, where an increase in resistance to one antibiotic can lead to a subsequent decrease in resistance to another antibiotic (Imamovic and Sommer, 2013). The conditions under which fluctuating drug exposure can successfully limit bacterial growth have been extensively investigated. The primary aim of such studies has been to identify the fitness costs in novel antibiotics for specific drug mutations which give rise to antibiotic resistance (Pál et. al., 2015, Roemhild and Schulenberg, 2019) or studying the interactions between antibiotics (Rodriguez de Evgrafov et. al., 2015; Dean et. al., 2019; Barbosa et. al., 2019). However, a factor that has not been adequately addressed is the role of population dynamics in the development of antibiotic resistance.

The exploration of the role of population size in the development of antibiotic resistance has mostly centered on single-exposure experiments focusing on the initial inoculum size. It has been shown that an increase in the initial number of bacteria can lead to an increase in the minimum inhibitory concentration (MIC), i.e. the minimum concentration at which there is no visible growth (Brook, 1989). This has led to further single-exposure studies that focus on the relationship between the molecular basis of drug action and the number of bacteria present in the population (Udekwu et. al, 2009; Martinez et. al.,

2012). Additionally, recent studies have shown that population bottlenecks might play an important role in shaping the trajectories of antibiotic resistance evolution (Garoff et. al., 2020; Windels et. al., 2021).

However, a large body of literature has gradually amassed in the past few years to demonstrate the crucial role of population dynamics in shaping the evolution of bacterial populations. The extent of mutation supply can differ between populations of different size, which can in turn influence the extent of adaptation (Desai and Fisher, 2007, Chavhan et. al, 2019a), evolutionary trajectories (Lachapelle et. al., 2015) and efficiency of natural selection (Chavhan et. al., 2019a). Thus, large populations evolve mainly via beneficial mutations of large effects. Conversely, small populations are often unable to access beneficial mutations of large effect, and hence are limited to relatively common mutations of smaller beneficial effects (Sniegowski and Gerrish, 2010). However, beneficial mutations of large effect can have higher fitness costs in novel environments and thus populations evolved at a larger population size can do poorly when exposed to novel environments (Chavhan et. al., 2019b; Chavhan et. al., 2020).

Similarly, populations of different sizes might follow different evolutionary trajectories in fluctuating environments, as fitness costs that are imposed in constant environments might be avoided in fluctuating environments (Bono et. al., 2017). This is because while it is possible to increase fitness in constant environments through antagonistically pleiotropic mutations, when adapting in a temporally fluctuating environment it is necessary to avoid a decline in fitness in both environments (Boyer et. al., 2021). Since large populations have access to a greater number of mutations, they are more likely to access mutations that might be beneficial in both environments. This access to rare beneficial mutations might make it easier for larger populations to grow successfully under exposure to fluctuations between two antibiotics. Indeed, multidrug resistant mutants have been shown to have a higher likelihood of appearing in large populations (Jiao et. al., 2016; Mahrt, 2020). Also, large populations were able to avoid fitness costs when evolving under fluctuations between two carbon sources (Chavhan et. al., 2021).

It remains to be seen whether this would hold for populations evolving under fluctuating antibiotic exposure and how this could influence networks of collateral resistance.

Since population size has been shown to influence evolutionary trajectories and fitness in novel environments for bacterial populations, this study aimed to understand the effect of population size on the evolution of multidrug resistance and collateral networks. To study this, two different selection experiments were conducted using replicate populations of *Escherichia coli*. First, bacterial populations were exposed to increasing concentrations of the same antibiotic at two different effective population sizes for ~200 generations. Second, bacterial populations were exposed to fluctuations between two different antibiotics for ~200 generations in each antibiotic (a total of ~400 generations). After the selection, the minimum inhibitory concentration (MIC) was determined for the antibiotics used for the selection as well as for novel antibiotics. Also, the growth rate and carrying capacity were measured in sublethal concentrations of these antibiotics.

We found that the effect of population size on the evolution of antibiotic resistance was different from prior expectations. After exposure to increasing concentrations of a single antibiotic, large populations did not evolve a higher resistance than small populations and also failed to show higher costs when assayed in novel antibiotics. However, when exposed to fluctuations between two antibiotics, large populations were able to avoid fitness costs while small populations were not able to do so. Taken together, these results indicate the presence of other influencing factors, and further experiments are needed to better understand the effect of these factors.

Materials and Methods

2.1. Selection protocol in the presence of a single antibiotic.

A common ancestor derived from the *E. coli* strain MG 1655 was used to initiate 64 replicate populations (henceforth referred to as SAS populations). 16 populations were subjected to increasing concentrations of each of the four chosen antibiotics in nutrient broth (see table 2.1 for antibiotics and corresponding population codes). These antibiotics were chosen as they have different targets inside the cell as well as different modes of action. Ampicillin disrupts cell wall formation, Chloramphenicol inhibits the 50S subunit of the ribosome, Kanamycin inhibits the 30S subunit of the ribosome, and Nitrofurantoin interrupts the synthesis of folic acid (Kohanski et. al., 2010). Of the 16 populations of each treatment, 8 populations were subcultured with a bottleneck ratio of 1/10 (henceforth referred to as the L populations). For the other 8, a bottleneck ratio of 1/10000 was used (henceforth referred to as the S populations).

As all the populations were grown in 96-well plates, the final population size (N_f) was constrained by the culture volume (200 µl). However, for a population that was propagated under batch culturing, the evolutionarily relevant size has been shown to be the effective pop size $N_e = N_0 * g$ (Lenski et. al., 1991; Lachapelle et. al., 2015). Hence, the difference in effective population size was realized by using two different bottleneck ratios (1/10 and 1/10000) at subculture. A bottleneck ratio of 1/10 (L populations) resulted in ~3.3 generations between subcultures whereas a ratio of 1/10000 (S populations) resulted in ~13.3 generations ($\log_2$(bottleneck ratio)). Hence, the effective population size for L populations was approximately 10^8 and for S populations was approximately 10^5.

The L populations reached their carrying capacity much earlier (within ~12 hours) than the S population (within ~24 hours). Therefore, to ensure that all populations experience a given antibiotic concentration for ~13.3 generations, the L populations were subcultured 4 times, every 12 hours, in the same antibiotic concentration while the S populations were subcultured only once, every 24 hours. Between subcultures, all

populations were incubated at 37℃ and shaken at 150 RPM. The S populations, subjected to harsher bottlenecks, were likely more prone to extinctions when subjected to the next higher concentration of antibiotics. To circumvent this risk, the S populations were grown at multiple concentrations of the antibiotic (0.9x, 1x, 1.05x, 1.1x), where 1x is the previous concentration at which they were able to successfully grow. The highest concentration at which the OD_{600} of all replicates was greater than 0.3 was considered the concentration of successful growth for the S populations. To ensure that the L and S populations face the same environmental conditions, the L populations were grown in the same antibiotic concentration where S populations showed successful growth.

Both L and S populations were subjected to 15 rounds of antibiotic exposures, which resulted in ~ 200 generations of evolution. At the end of the selection, all evolved populations were frozen as 15% glycerol stocks.

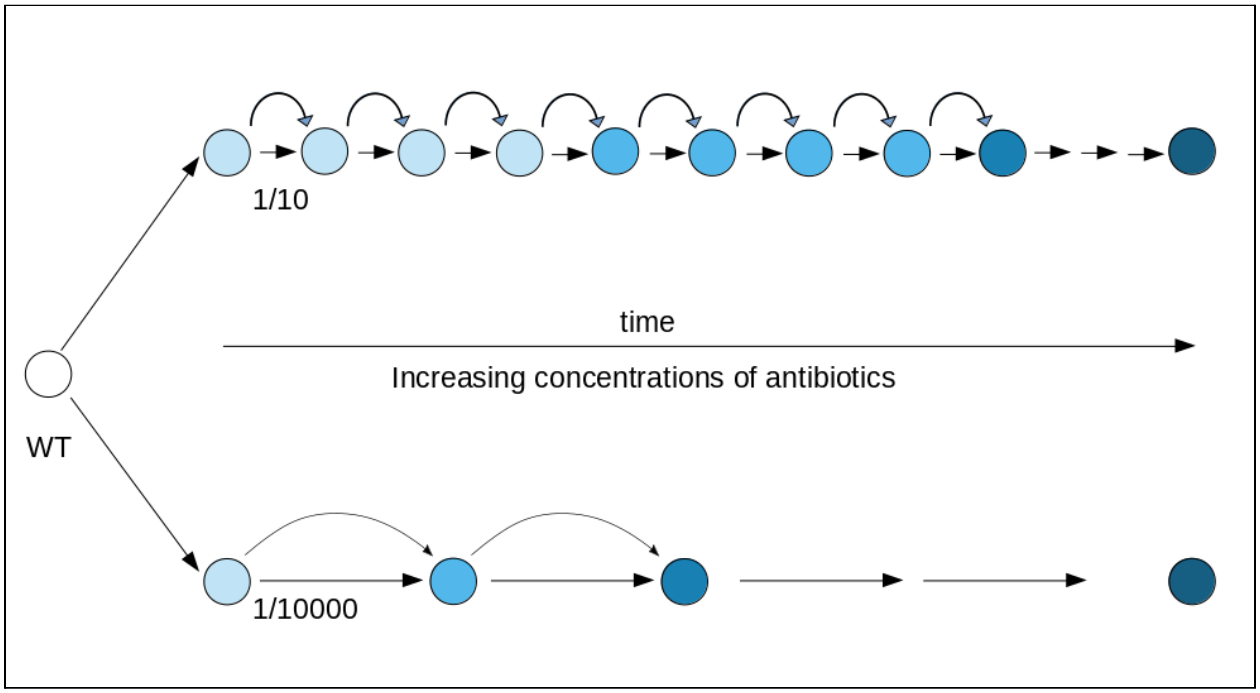


Figure 2.1. Schematic for single antibiotic selection. For L populations (1/10), the antibiotic concentration will be increased every four subcultures. For S populations (1/10000), the antibiotic concentration will be increased at each subculture.

No.	Antibiotics used in the selection	Population code
1	Ampicillin	A
2	Chloramphenicol	C
3	Kanamycin	K
4	Nitrofurantoin	N

Table 2.1. Information regarding all populations that were a part of the single antibiotic selection. The second column gives the antibiotics used for that particular population. The third column denotes the code used to refer to the population in this study.

2.2 Selection protocol for the fluctuating selection.

96 replicate populations were initiated from the same common ancestor as above and subjected to a fluctuating selection (henceforth referred to as FS populations). 16 populations were subjected to increasing concentrations of each of the six antibiotic pairs that were part of the selection regime (see Table 2.2 for the antibiotic pairs and populations codes). Out of these 16, 8 populations were subcultured with a bottleneck ratio of 1/10 (L populations). For the other 8, a bottleneck ratio of 1/10000 was used (S populations). Similar to SAS selection, populations were subjected to 15 rounds of antibiotic exposure for each antibiotic in a given pair. Hence, both L and S populations were subjected to 30 rounds of antibiotic exposure. The culture conditions and the pattern of increasing antibiotic concentrations were the same as for the SAS populations. At the end of 30 antibiotic exposures or ~400 generations of evolution, all evolved populations were frozen as 15% glycerol stocks.

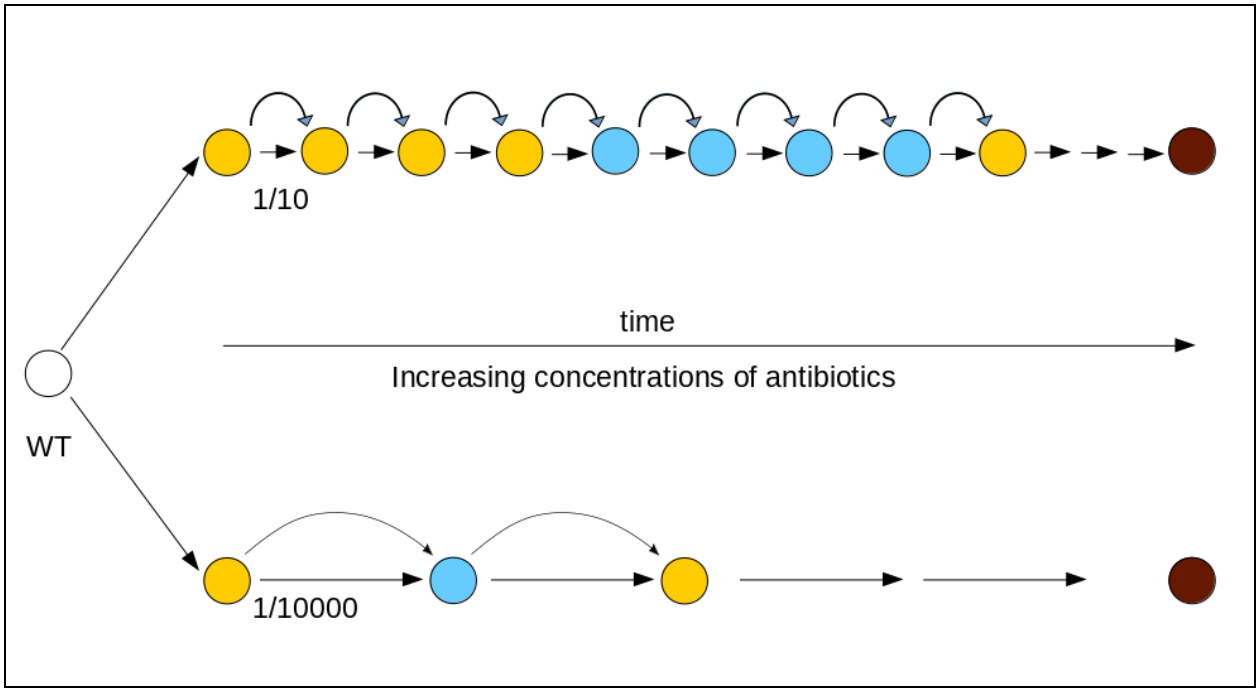


Figure 2.2. Schematic for fluctuating selection. For L populations (1/10), the antibiotic was changed every four subcultures. For S populations (1/10000), the antibiotic was changed at each subculture. After one set of exposures to each antibiotic, the antibiotic concentration was increased.

No.	Antibiotics used in the selection	Population code
1	Ampicillin and Chloramphenicol	AC
2	Ampicillin and Kanamycin	AK
3	Ampicillin and Nitrofurantoin	NA
4	Chloramphenicol and Kanamycin	CK
5	Chloramphenicol and Nitrofurantoin	CN
6	Kanamycin and Nitrofurantoin	KN

Table 2.2. Information regarding all populations that were a part of the fluctuating selection. The second column gives the antibiotics used for that particular population. The third column denotes the code used to refer to the population in this study.

2.3. Assays on the selected populations

After selection for increased resistance to either a single antibiotic or a pair of antibiotics, the extent of resistance evolution was evaluated in these populations. Resistance was measured in the antibiotic(s) used for selection as well as novel antibiotics. Resistance was measured in two ways: change in minimum inhibitory concentration (MIC) and growth parameters at sub-lethal concentrations.

2.3.1 Determination of minimum inhibitory concentration (MIC) for antibiotics

The Minimum inhibitory concentration (MIC) of the selected populations was measured in the four antibiotics used in the selection (Ampicillin, Chloramphenicol, Kanamycin, and Nitrofurantoin) as well as two new antibiotics, Nalidixic acid and Rifampicin. Before the assay, the selected populations were revived from glycerol stocks and incubated at 37°C, 150 RPM for 16-22 hours. The assay was done in a 96-well microplate, where the last row of wells had the highest concentration of the antibiotic used in the assay and the subsequent wells had half the previous concentration. The range of concentrations used in this assay is given in Table 2.3a. The revived bacterial cultures were added at a dilution of 1/1000. Then, the plates were incubated with shaking at 37℃ and 150 RPM for 48 hours. The plates were visually scored for lack of bacterial growth, and the lowest concentration of antibiotic which failed to show growth was taken as the MIC. In this manner, MIC was measured for three measurement replicates for each selected population in every antibiotic that was a part of the assay, and the MIC for two out of the three measurement replicates was taken as the final value of MIC.

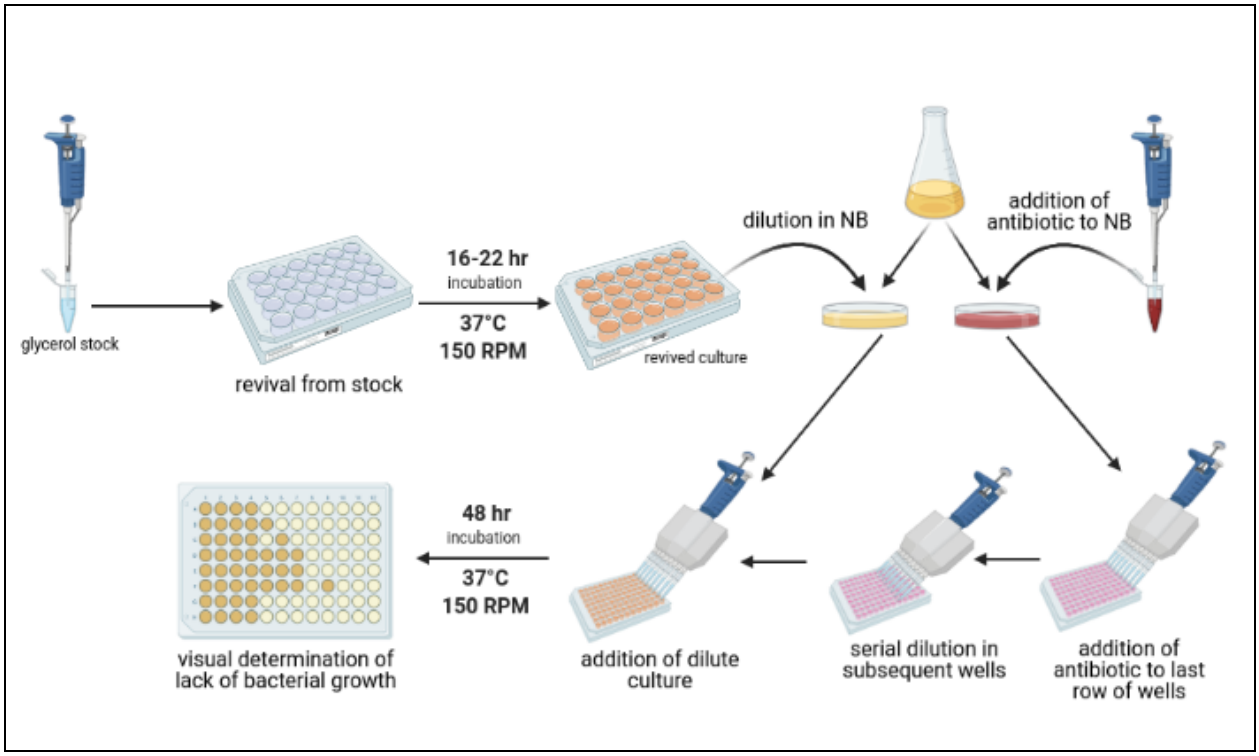


Figure 2.3. Schematic of the assay for determination of MIC for antibiotics. Created in Biorender.com

2.3.2. Determination of growth rate and carrying capacity in sub-lethal concentrations of antibiotics

The growth rate and carrying capacity of the selected populations were estimated at sub-lethal concentrations of all antibiotics as well as in Nutrient Broth as a control environment. The concentration of antibiotic that resulted in a 40% -70% reduction in growth rate/carrying capacity of the ancestor was used as the sub-lethal concentration. The evolved populations were revived as before. The revived cultures were diluted to 1/1000 and added to a 96-well microplate containing NB mixed with antibiotic at the sub-lethal concentration (see Table 2.3b for the antibiotic concentrations used). The plate was incubated at 37°C with constant shaking for 48 hours in a plate reader (Synergy HT BioTek, Winooski, VT, USA), during which OD_{600} was measured every 20 minutes (Figure 2.2). The OD_{600} of the populations over 48 hours was used to determine the growth rate and carrying capacity in the antibiotic used in the assay. A custom python script was used to determine the growth rate and carrying capacity from the

OD_{600} readings. The growth rate was calculated by finding the maximum slope over 10 consecutive readings, and the carrying capacity was the highest OD_{600} value (Karve et. al., 2015).

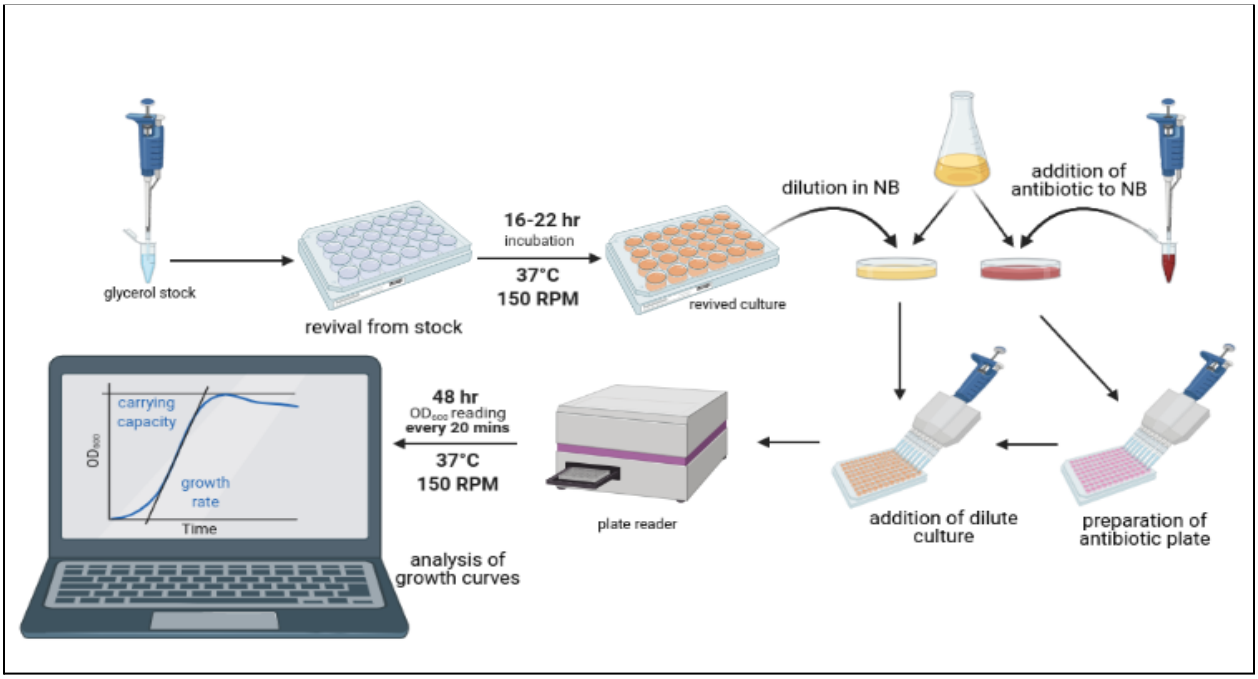


Figure 2.4. Schematic of the assay for determining growth rate and carrying capacity at sub-lethal concentrations of antibiotics. Created in Biorender.com.

a) Concentrations of antibiotics used in the MIC assay			
Antibiotic	Range of concentrations	Antibiotic	Range of concentrations
Ampicillin	1 - 2048 µg/ml	Nitrofurantoin	1 - 512 µg/ml
Chloramphenicol	1 - 1024 µg/ml	Nalidixic acid	1 - 128 µg/ml
Kanamycin	1 - 128 µg/ml	Rifampicin	1 - 256 µg/ml
b) Sublethal concentrations of antibiotics used to determine growth rate and carrying capacity			
Antibiotic	Concentration	Antibiotic	Concentration
Ampicillin	28 µg/ml	Nitrofurantoin	7 µg/ml
Chloramphenicol	3 µg/ml	Nalidixic acid	3 µg/ml

Kanamycin	1.5 µg/ml	Rifampicin	8 µg/ml

Table 2.3. All the antibiotic concentrations used in the two assays - a) range of concentrations used in the MIC assay, b) the concentration at which growth rate and carrying capacity was measured.

2.4. Analysis

2.4.1 Comparison with the ancestor

The data from all the assays was first scaled by the ancestor values in the corresponding assay environments. The MIC data was scaled by the ancestor MIC and log-transformed before performing statistical tests on it. This was because MICs for antibiotics have been shown to follow a log-normal distribution (Turnidge et. al., 2006). The growth rate and carrying capacity data were each scaled by the growth rate and carrying capacity for the ancestor in the corresponding environments. A value greater than 1 represents a gain in fitness and a value less than 1 represents a loss in fitness. Then, to determine whether there was an increase or decrease in fitness compared to the ancestor, the data from all assays was compared separately to the ancestor value using two-tailed t-tests.

2.4.2. Analysis of MIC, growth rate and carrying capacity in the selection environments

The scaled values of MIC, growth rate and carrying capacity in the selection environments were analyzed using two-way ANOVAs. Separate ANOVAs were conducted for MIC, growth rate and carrying capacity, and for the single antibiotic selection and the fluctuating selection, resulting in six independent ANOVAs. For the single antibiotic selection, the fixed factors were bottleneck ratio (2 levels, L and S) and selection environment (4 levels for the four different antibiotics used for the selection). For the fluctuating selection, the geometric mean of the MIC, growth rate and carrying capacities in the two selection environments was taken before scaling with the ancestor values. The fixed factors were bottleneck ratio (2 levels, L and S) and selection environment (6 levels for the six different antibiotic pairs used for the selection). When

the main effects or interaction was significant, pairwise comparisons between L and S populations were performed as two-tailed t-tests, and further corrected with a Holm-Šídák correction (Abdi, 2010). The t-tests and the Holm-Šídák correction on the t-tests were performed in LibreOffice Calc (The Document Foundation), and the ANOVAs were performed in STATISTICA 8.0 (Statsoft, Inc.).

2.4.3. Analysis of MIC, growth rate and carrying capacity in novel environments

The scaled values of MIC, growth rate and carrying capacity in novel environments were analyzed separately for all the selected populations. Separate ANOVAs were conducted for MIC, growth rate and carrying capacity, and for the single antibiotic selection and the fluctuating selection, resulting in 30 independent ANOVAs. For the single antibiotic selected populations, the fixed factors were bottleneck ratio (2 levels, L and S) and assay environment (5 levels for the five antibiotics other than the selection antibiotic). For the fluctuating selection, the fixed factors were Population size (2 levels, L and S) and assay environment (4 levels for the four antibiotics other than the selection antibiotics). For the growth rate and carrying capacity, Nutrient Broth was also an assay environment, and hence the assay environment factor had 6 levels for the SAS populations and 5 levels for the FS populations. When the main effects or interaction was significant, pairwise comparisons between L and S populations were performed as two-tailed t-tests, and further corrected with a Holm-Šídák correction (Abdi, 2010). The t-tests and the Holm-Šídák correction on the t-tests were performed in LibreOffice Calc (The Document Foundation), and the ANOVAs were performed in STATISTICA 8.0 (Statsoft, Inc.).

Results

3. 1. Large populations did not always evolve larger fitness than small populations in single antibiotic environments.

The first set of populations were those selected under exposure to a single antibiotic (henceforth referred to as SAS populations). We looked at the change in resistance to the antibiotics used for selection. This was examined by performing two-tailed t-tests with the scaled ancestor values of MIC, growth rate and carrying capacity. There were four antibiotics used in the selection – Ampicillin (Amp), Chloramphenicol (Chl), Kanamycin (Kan) and Nitrofurantoin (Nit). For both the bottleneck ratios, all populations show an increase in MIC compared to the ancestor (L - Amp – $p < 1E-07$, Chl – $p = 5.97E-07$, Kan – $p = 9.58E-05$, Nit – $p = 0.00312$; S – Amp – $p = 3.71E-06$, Chl – $p = 7.34E-05$, Kan – $p = 0.033146$, Nit – $p = 7.75E-05$). (Fig. 3.1. and 3.4a.) This uniform increase was not seen for the growth rate (r) and carrying capacity (K) at sublethal concentrations of the selection antibiotic. Populations that faced large bottlenecks (L populations) showed a significant increase in growth rate when selected in Chl ($p = 2.4E-06$) and Kan ($p = 0.003746$) and an increase in carrying capacity when selected in Amp, Chl and Kan (Amp - $p = 1E-04$, Chl - $p = 7.13E-07$, Kan - $p = 4.93E-06$). For populations that faced small bottlenecks (S populations), we saw an increase in growth rate when selected in Amp ($p = 0.002375$) and Chl (3.15E-09) and an increase in carrying capacity when selected in Amp ($p = 1.48E-08$), Chl ($p = 7.47E-08$), Kan ($p = 0.000584$) and Nit ($p = 0.001402$). There was no decrease in growth rate and carrying capacity for any of the SAS populations. (Figures 3.2, 3.3, 3.4b and 3.4c)

MIC													
	L							S					
	Amp	Chl	Kan	Nit	Nal	Rif		Amp	Chl	Kan	Nit	Nal	Rif
A	↑	≈	≈	≈	≈	≈		↑	↑	↓	↓	↓	↓
C	↑	↑	↓	≈	↑	≈		↑	↑	↓	≈	↑	↓
K	≈	≈	↑	≈	≈	≈		↑	≈	↑	≈	↑	≈
N	≈	≈	≈	↑	≈	≈		≈	↑	↑	↑	≈	↑
AC	↑	↑	↓	↑	↑	↑		≈	↑	↓	≈	↑	↑
AK	↑	≈	↑	↓	≈	≈		↑	≈	↑	≈	≈	≈
NA	↑	↑	↓	↑	≈	≈		↑	↑	≈	↑	↑	≈
CK	↑	↑	↑	↑	↑	↑		↑	↑	≈	≈	↑	≈
CN	↑	↑	↓	↑	↑	↑		↑	↑	≈	↑	↑	↑
KN	↑	≈	↑	↑	≈	↑		≈	↑	≈	↑	≈	≈

Figure 3.1. Visual representation of changes in MIC with respect to the ancestor for all the selected populations. The rows denote the antibiotic used for selection (Ampicillin (A), Chloramphenicol (C), Kanamycin (K), Nitrofurantoin (N), Ampicillin and Chloramphenicol (AC), Ampicillin and Kanamycin (AK), Ampicillin and Nitrofurantoin (NA), Chloramphenicol and Kanamycin (CK), Chloramphenicol and Nitrofurantoin (CN) and Kanamycin and Nitrofurantoin (KN)). The columns denote the antibiotic for which the MIC was assayed (Amp for Ampicillin, Chl for Chloramphenicol, Kan for Kanamycin, Nit for Nitrofurantoin, Nal for Nalidixic acid, and Rif for Rifampicin). L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively.

growth rate														
	L							S						
	Amp	Chl	Kan	Nit	Nal	Rif	NB	Amp	Chl	Kan	Nit	Nal	Rif	NB
A	≈	↑	↓	↓	≈	≈	≈	↑	↑	≈	↓	↓	≈	↓
C	≈	↑	↓	↓	≈	≈	↓	↓	↑	≈	↓	↓	≈	↓
K	≈	↓	↑	↓	↓	≈	≈	≈	≈	≈	≈	↓	≈	≈
N	↓	≈	≈	≈	≈	≈	≈	↓	↑	≈	≈	≈	≈	↓
AC	≈	↑	↓	↑	≈	≈	≈	≈	↑	↓	≈	≈	≈	≈
AK	≈	≈	↑	↓	≈	≈	≈	≈	≈	≈	↓	↓	≈	↓
NA	≈	≈	≈	≈	≈	≈	≈	≈	↑	≈	↓	≈	≈	≈
CK	≈	↑	↑	↓	≈	≈	↓	≈	↑	≈	↓	≈	≈	↓
CN	≈	↑	↓	≈	↑	≈	↓	≈	↑	≈	↓	↑	↓	↓
KN	≈	≈	↑	≈	≈	≈	≈	≈	↑	↓	≈	≈	≈	≈

Figure 3.2. Visual representation of changes in growth rate with respect to the ancestor for all the selected populations. The rows denote the antibiotic used for selection (Ampicillin (A), Chloramphenicol (C), Kanamycin (K), Nitrofurantoin (N), Ampicillin and

Chloramphenicol (AC), Ampicillin and Kanamycin (AK), Ampicillin and Nitrofurantoin (NA), Chloramphenicol and Kanamycin (CK), Chloramphenicol and Nitrofurantoin (CN) and Kanamycin and Nitrofurantoin (KN)). The columns denote the antibiotic for which the growth rate was assayed (Amp for Ampicillin, Chl for Chloramphenicol, Kan for Kanamycin, Nit for Nitrofurantoin, Nal for Nalidixic acid, and Rif for Rifampicin). L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively.

yield														
	L							S						
	Amp	Chl	Kan	Nit	Nal	Rif	NB	Amp	Chl	Kan	Nit	Nal	Rif	NB
A	↑	↑	↓	↓	≈	≈	↓	↑	↑	≈	↓	≈	↑	≈
C	≈	↑	↓	↓	≈	↓	↓	≈	↑	≈	↓	≈	↑	↓
K	≈	↓	↑	↓	↓	≈	↓	≈	≈	↑	≈	≈	≈	↓
N	↓	≈	≈	≈	↓	≈	↓	↓	↑	≈	↑	≈	≈	↓
AC	↑	↑	≈	≈	↓	↓	↓	↑	↑	↓	≈	≈	↑	↓
AK	≈	≈	↑	↓	≈	≈	↓	↑	≈	↑	↓	↓	≈	↓
NA	≈	≈	≈	≈	↓	≈	≈	↑	↑	≈	↓	≈	≈	↓
CK	↑	↑	↑	≈	≈	≈	↓	↑	↑	↑	≈	↑	≈	↓
CN	↑	↑	↓	↓	↓	↓	↓	↑	↑	≈	≈	↑	↓	↓
KN	≈	↓	↑	≈	≈	↓	↓	≈	↑	≈	≈	≈	≈	≈

Figure 3.3. Visual representation of changes in carrying capacity with respect to the ancestor for all the selected populations. The rows denote the antibiotic used for selection (Ampicillin (A), Chloramphenicol (C), Kanamycin (K), Nitrofurantoin (N), Ampicillin and Chloramphenicol (AC), Ampicillin and Kanamycin (AK), Ampicillin and Nitrofurantoin (NA), Chloramphenicol and Kanamycin (CK), Chloramphenicol and Nitrofurantoin (CN) and Kanamycin and Nitrofurantoin (KN)). The columns denote the antibiotic for which the carrying capacity was assayed (Amp for Ampicillin, Chl for Chloramphenicol, Kan for Kanamycin, Nit for Nitrofurantoin, Nal for Nalidixic acid, and Rif for Rifampicin). L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively.

Next, we examined whether the pattern of L > S holds for all populations selected under exposure to a single antibiotic. When ancestor-scaled MICs for L and S populations in their selection environments were assayed together, the interaction of bottleneck ratio and assay environment was significant (Figure 3.4a; $F_{3,42}$ = 14.4468, p = 1E-06). Indeed, for populations selected under Chl and Kan exposure, populations subjected to large bottlenecks (L) had a significantly higher MIC than populations subjected to small bottlenecks (S) (Chl – p = 2.47E-05, carrying capacity– p = 0.004374) (Fig. 3.4a). However, S populations had a higher MIC in Amp than L populations (p = 0.004688) after selection in Amp, and for Nit-selected populations, there was no difference between MIC for L and S populations (p = 0.584319)(Fig. 3.4a).

This lack of a consistent pattern was seen for growth rate and carrying capacity at sublethal concentrations of the selection antibiotic as well. Two-way ANOVAs were conducted to look at the relationship between L and S populations. For both growth rate and carrying capacity, the interaction between bottleneck ratio and carrying capacity was significant (growth rate – Figure 3.4b; $F_{3,42}$ = 6.729, p = 0.000825; carrying capacity - Figure 3.4c; $F_{3,42}$ = 16.658, p < 1E-07). For populations selected under exposure to Amp, S populations had a higher growth rate and carrying capacity than L populations (growth rate – p = 0.00792, carrying capacity – p = 0.026371) (Fig. 3.4b and c). There was no difference in growth rate between L and S populations for any of the other antibiotics used in the selection (Chl – p = 0.099054, Kan – 0.119014, Nit = 0.468706). For the Kan-selected populations, L populations had a higher carrying capacity than S populations (p = 0.013771), but for the populations selected under Chl (p = 0.001967) and Nit (p = 0.014785), S populations had a higher carrying capacity than L populations (Fig. 3.4b and c).

In summary, For Amp selected populations, we see a consistent trend in the other direction, where S > L for MIC, growth rate and K. However, for populations selected under exposure to Chl, the MICs show the expected pattern of L > S, but not the growth rates and carrying capacities. Only the populations selected under exposure to Kanamycin have the large populations consistently reaching a greater fitness in their

selection environments. Taken together, these results show that the expected pattern of large populations being able to reach a greater fitness in the selection environment is not upheld consistently for all the antibiotics used in the selection.

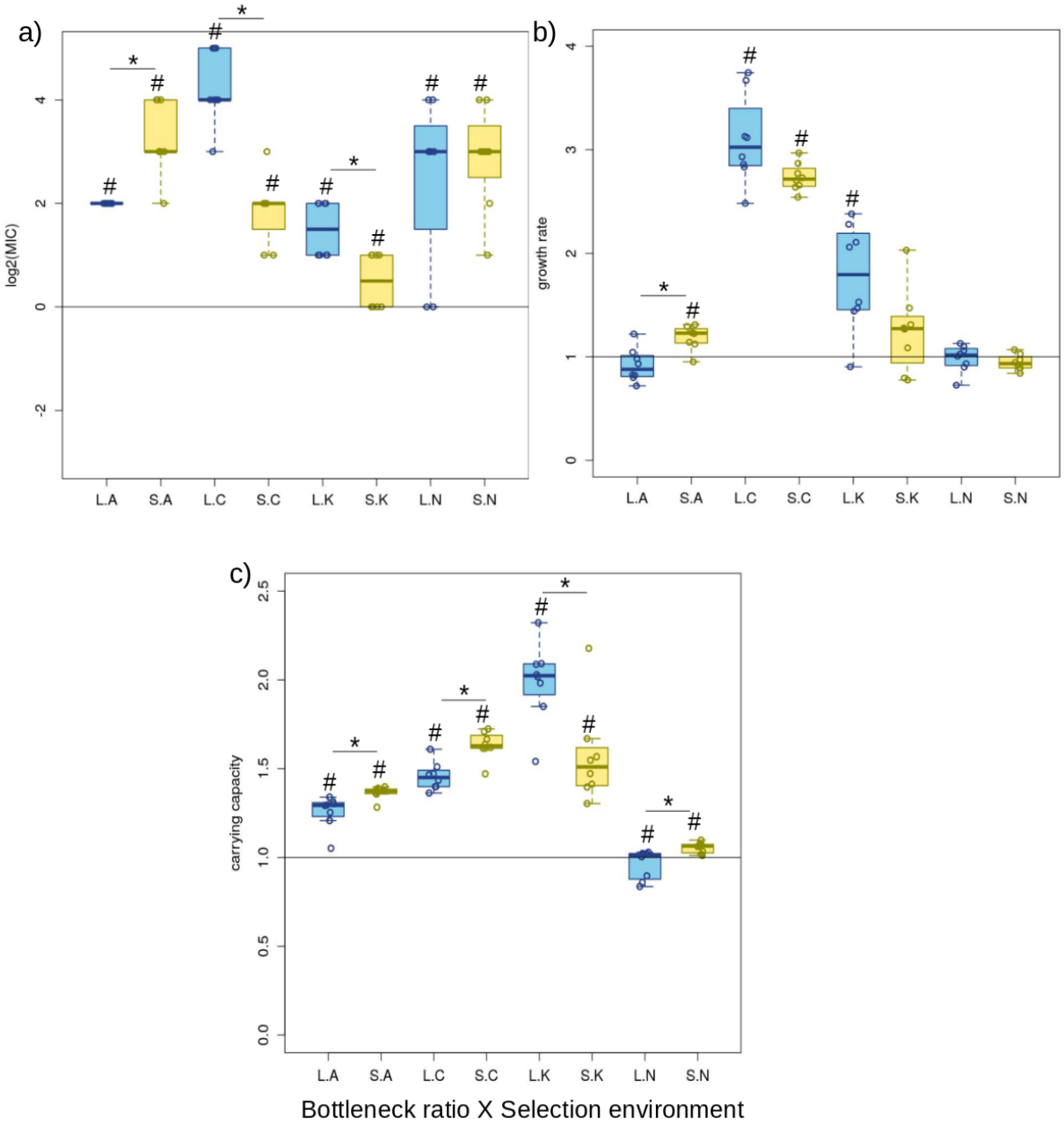


Figure 3.4. a) log_2(MIC)s, b) growth rates and c) carrying capacities for single antibiotic selected populations in their selection environments. The labels on the x-axis denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box

*plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes p < 0.05 in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant (p < 0.05) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

3.2. Large populations do not consistently face higher costs in novel environments after being selected under exposure to a single antibiotic.

First, we looked at the change in resistance to novel antibiotics for populations selected under exposure to single antibiotics. Similar to the previous analysis, this was examined by performing two-tailed t-tests with the ancestor values of MIC, growth rate and carrying capacity. There were four antibiotics used in the selection – Ampicillin (Amp), Chloramphenicol (Chl), Kanamycin (Kan), and Nitrofurantoin (Nit). MIC, growth rate and carrying capacity was measured for all four of these antibiotics, with the addition of two more antibiotics – Nalidixic acid (Nal) and Rifampicin (Rif).

In the populations selected under exposure to Amp, we saw that populations that faced large bottlenecks (L) showed no change in MIC for any novel antibiotic that was part of the assay (Chl – p = 0.350617, Kan – p = 1, Nit – p = 0.079602, Nal – p = 0.170471, Rif – p = 0.1707471). However, populations that faced small bottlenecks (S) showed an increase in resistance to Chl (p = 4.65E-05), but a significant decrease in resistance to Kan (p = 0.000122), Nit (p = 0.033146), Nal (p = 0.000212), and Rif (p = 0.000212) (Fig. 3.1 and 3.5a). This was reflected in the comparison between L and S populations, where the interaction between bottleneck ratio and assay environment was significant in the two-way ANOVA ($F_{4,56}$ = 24.78125, p < 1E-07). Thus, further comparison between L and S populations in each assay environment separately showed that S populations had a higher resistance than L populations in Chl, but the reverse was true in Kan, Nal and Rif (Holm-Šídák corrected p-values: Chl – p = 8.545E-05, Kan – p = 0.00062, Nal – p = 0.018925, Rif – p = 0.000424, Nit – p = 0.641987)(Fig. 3.5a).

For populations selected under exposure to Chl, for both bottleneck ratios there was an increase in resistance to Amp (L – p = 5.97E-07, S – p = 0.000136) and Nal (L – p = 0.000136, S – p = 0.011201), and a decrease in resistance to Kan (L – p < 1E-07, S – p = 0.011201). S populations additionally showed a decrease in resistance to Rif (p = 0.002536), which was not seen for L populations (p = 0.350617)(Fig. 3.1 and 3.5b). Both populations showed no change in resistance to Nit (L – p = 1, S – p = 1). In the two-way ANOVA carried out for comparison between L and S populations, the interaction between bottleneck ratio and assay environment was significant ($F_{4,56}$ = 5.16822, p = 0.001292). Further comparison between L and S populations for individual assay environments showed that L populations had a higher resistance than S populations for Amp as well as Nal, and no difference was seen between L and S populations for the other novel environments (Holm-Šídák corrected p-values: Amp – p = 0.025799, Nal – p = 0.045915, Kan – p = 0.220301, Nit – p = 1, Rif – p = 0.225537) (Fig. 3.5b).

L populations did not show any change in resistance, with respect to the ancestral MIC, to any novel antibiotic when selected under exposure to Kan (Amp – p = 1, Chl – p = 1, Nit – p = 0.103552, Nal – p = 0.350617, Rif – p = 0.64912). The corresponding S populations, however, showed a significant increase in resistance to Amp (p = 0.002536) and Nal (0.0033146). There was no change in resistance to the remaining novel antibiotics (Chl – p = 0.350617, Nit – p = 0.170471, Rif – p = 0.227453)(Fig. 3.1 and 3.5c.). For further comparison between L and S populations selected under exposure to these antibiotics, two-way ANOVAs were carried out. For populations selected under exposure to Kan, the interaction between bottleneck ratio and assay environment was not significant ($F_{4,56}$ = 0.9791, p = 0.426386), but the main effect of bottleneck ratio was significant ($F_{1,56}$ = 8.699029, p = 0.010558), with S populations having an overall higher MIC than L populations. (Fig. 3.5c)

For populations selected under exposure to Nit, L populations showed no change in resistance to any novel environment (Amp – p = 0.731788, Chl – p = 0.170471, Kan – p = 0.079602, Nal – p = 0.598331, Rif – p = 0.110778). (Fig. 3.1 and 3.5d.) S populations

showed a significant increase in resistance to Chl ($p = 0.001216$), Kan ($p = 7.75E-05$) and Rif (0.002536), and no change in resistance to Nit ($p = 0.731788$) The interaction between bottleneck ratio and assay environment was significant ($F_{4,56} = 3.39363$, $p = 0.014828$) in the two-way ANOVA carried out for further comparison. In the individual assay environments, S populations had a significantly higher MIC than L populations in Chl and Kan, with no difference between the two bottleneck ratios in other novel environments. (Holm-Šídák corrected p-values: Chl – $p = 0.0011153$, Kan – $p = 0.0334481$, Amp – $p = 0.429902$, Nal – $p = 1$, Rif – $p = 0.964244$)(Fig. 3.5d).

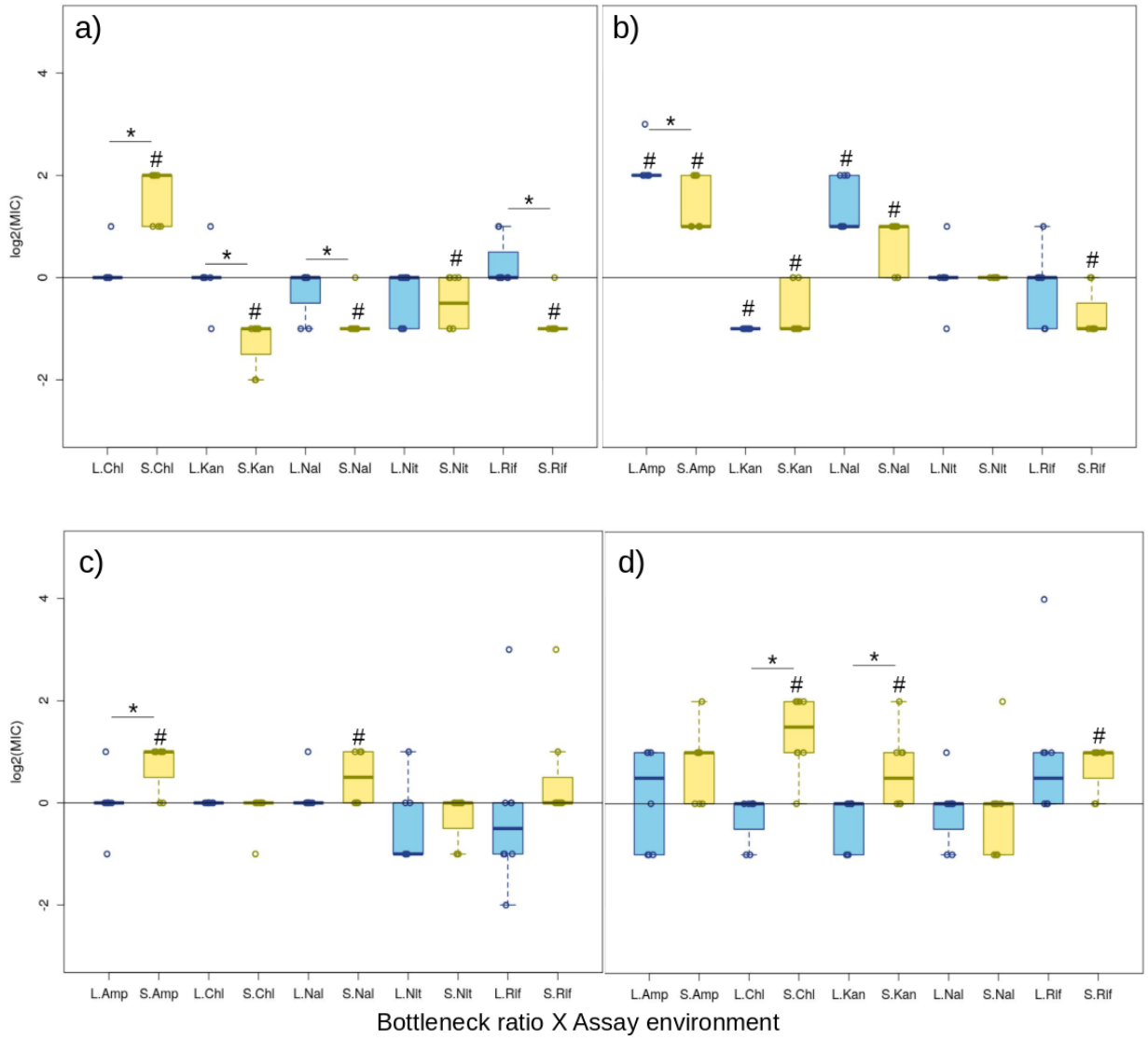


*Figure 3.5. log_2(MIC)s for a) Ampicillin-selected b) Chloramphenicol-selected c) Kanamycin-selected and d) Nitrofurantoin-selected single antibiotic selected populations in their novel environments. The labels on the x-axis denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes p < 0.05 in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant (p < 0.05) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

None of the selected populations show a significant increase as compared to the ancestral values of growth rate and carrying capacity in nutrient broth (NB). Populations subjected to both bottleneck ratios under exposure to Chl show a significant decrease in growth rate (L – p = 0.020941, S – p = 1.37E-05), while only S populations selected in Amp (p = 0.002828) and Nit (p = 0.001455) show a significant decrease in growth rate. There was no difference compared to the ancestor growth rate for any other population. However, all the L populations showed a significant decrease in carrying capacity (Amp – p = 0.000411, Chl – p = 0.000284, Kan – p = 0.000174, Nit – p = 0.000198), while Amp-selected S populations were the only ones not to show a significant decrease in carrying capacity in nutrient broth (Amp – p = 0.11623, Chl – p = 0.00067, Kan – p = 0.026902, Nit – p = 0.049708) (Fig. 3.6 and 3.7).

Among the other novel antibiotics, Amp-selected populations subjected to both bottleneck ratios showed a significant increase in growth rate in Chl (L – p = 0.003862, S – p = 9.5E-05) and a significant decrease in growth rate in Nit (L – p = 0.002204, S – p = 0.005201). Additionally, L populations showed a significant decrease in growth rate in Kan (p = 0.01361), and S populations showed a significant decrease in growth rate in Nal (p = 0.002004). There was no change in growth rate for L populations in Nal (p = 0.25658) and Rif (p = 0.537917), and S populations in Kan (p = 0.936483) and Rif (p = 0.220804). (Fig. 2 and 3.6a) Despite these differences, a two-way ANOVA showed no significant interaction between bottleneck ratio and assay environment ($F_{5,77}$ =1.6716, p = 0.152873). Additionally, even the main effect of bottleneck ratio was not significant ($F_{1,77}$ = 0.5377, p = 0.475485) (Fig. 3.6a).

In populations selected in Chl, L populations showed a significant decrease in growth rate in Kan (p = 0.002919) and Nit (p = 0.018969) and S populations showed a significant decrease in growth rate in Amp (p = 0.016941), Nit (p =0.029718) and Nal (p = 0.013661). There was no change in growth rate for L populations in Amp (p = 0.449635), Nal (p = 0.666721) and Rif (p = 0.166942), and S populations in Kan (p = 0.648261) and Rif (p = 0.345611). (Fig. 2 and 3.6b) A two-way ANOVA to further see

the differences between L and S populations showed that the interaction between bottleneck ratio and assay environment was significant ($F_{5,77}$ = 4.6206, p = 0.001061). However, there was no significant difference between L and S populations after a Holm-Šídák correction. (Holm-Šídák corrected p-values: Amp – p = 0.340771, Kan – p = 0.312401, Nit – p = 0.309947, Nal – p = 0.279432, Rif – p = 0.314881) (Fig. 3.6b).

L populations selected in Kan showed a significant decrease in growth rate in Chl (p = 0.007347), Nit (p = 9.5E-06) and Nal (0.000634), while S populations showed a significant decrease only in Nal (p = 0.034077). There was no change in growth rate for L populations in Amp (p = 0.151021) and Rif (p = 0.174403), and S populations in Amp (p = 0.199029), Chl (p = 0.865696), Nit (p = 0.155049) and Rif (p = 0.534149). (Fig. 2 and 3.6c) These differences were further elucidated with the help of a two-way ANOVA, in which the interaction between bottleneck ratio and assay environment was significant ($F_{5,77}$ = 2.8637, p = 0.020723). S populations had a significantly higher growth rate than L populations in Chl and Nit, and for the other novel environments, there was no difference between L and S populations (Holm-Šídák corrected p-values: Amp – p = 0.194277, Chl – p = 0.034591, Nit – p = 0.94341, Nal – p = 0.380408, Rif – p = 0.320289) (Fig. 3.6c).

For Nit-selected populations, both L and S populations showed a significant decrease in growth rate in Amp (L – p = 2.6E-06, S – p =), and S populations additionally showed a significant increase in growth rate in Chl (p = 0.018691). There was no change in growth rate for L populations in Chl (p = 0.232159), Kan (p = 0.063214), Nal (p = 0.268554) and Rif (p = 0.931097), and S populations in Kan (p = 0.089911), Nal (p = 0.39214) and Rif (p = 0.737725). (Fig. 2 and 3.6d) This difference between the growth rate in Chl was seen in the results of a two-way ANOVA, where the interaction between bottleneck ratio and assay environment was significant ($F_{5,77}$ =4.9792), p = 0.000586. In Chl, S populations had a higher growth rate than L populations, and there was no difference in growth rate in any other novel antibiotic (Holm-Šídák corrected p-values: Amp – p = 0.99853, Chl – p = 0.040598, Kan – p = 0.987132, Nal – p = 0.085206, Rif – p = 0.999078) (Fig. 3.6d).

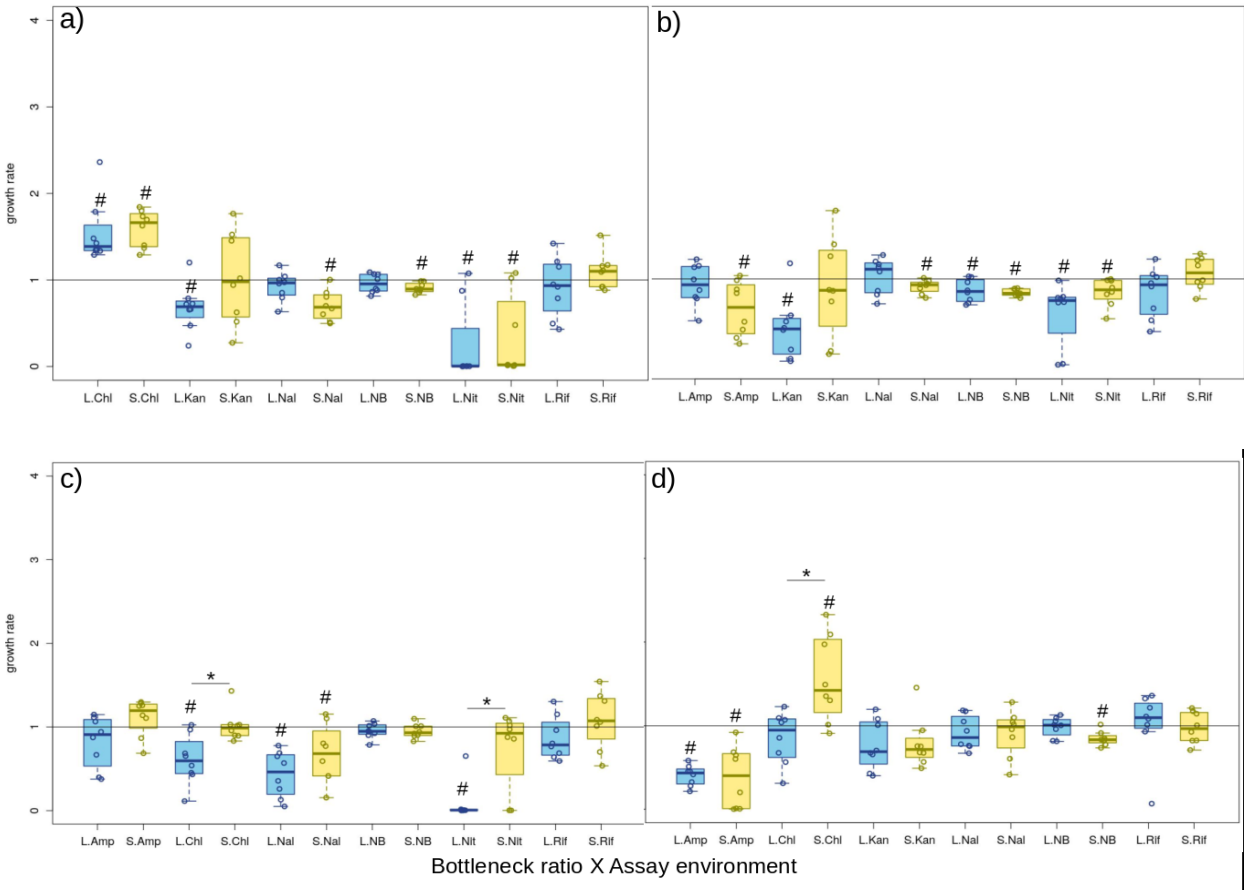


*Figure 3.6. Growth rates for a) Ampicillin-selected b) Chloramphenicol-selected c) Kanamycin-selected and d) Nitrofurantoin-selected single antibiotic selected populations in their novel environments. The labels on the x-axis denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes p < 0.05 in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant (p < 0.05) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

While looking at the carrying capacities, Amp-selected populations subjected to both bottleneck ratios showed a significant increase in carrying capacity in Chl (L - p = 4.75E-05, S – p = 1.3E-05) and a significant decrease in carrying capacity in Nit (L – p =

0.002108, S – p = 0.010508), when compared to ancestral carrying capacity values. Additionally, L populations showed a significant decrease in carrying capacity in Kan (p = 0.010081), and S populations showed a significant increase in carrying capacity in Rif (p = 0.030428).There was no change in carrying capacity for L populations in Nal (p = 0.710572) and Rif (p = 0.769135), and S populations in Kan (p = 0.365656) and Nal (p = 0.054515)(Fig. 3 and 3.7a). To further understand the differences between populations bottlenecked at the two different ratios, a two-way ANOVA was carried out. The interaction between bottleneck ratio and assay environment was not significant ($F_{5,77}$ = 1.77, p = 0.129086), but the main effect of bottleneck ratio was significant ($F_{1,77}$ = 4.941, p = 0.029154), with S populations having a higher carrying capacity than L populations(Fig. 3.7a).

In populations selected in Chl, L populations showed a significant decrease in carrying capacity in Kan (p = 0.003459), Nit (p = 0.03614) and Rif (p = 0.040509). S populations, however, showed a significant decrease in carrying capacity in Nit (p = 0.032016) but a significant increase in carrying capacity in Rif (p = 0.008668). There was no change in carrying capacity for L populations in Amp (p = 0.378135) and Nal (p = 0.148717), and for S populations in Amp (p = 0.515282), Kan (p = 0.514384) and Nal (p = 0.075879) (Fig. 3 and 3.7b). Further, a two-way ANOVA to compare between L and S populations showed that the interaction between bottleneck ratio and assay environment was not significant($F_{5,77}$ = 1.833, p = 0.116289), but the main effect of bottleneck ratio was significant($F_{1,77}$ = 6.123, p = 7.9E-05), with S populations having a higher carrying capacity than L populations (Fig. 3.7b).

For Kan-selected populations, L populations showed a decrease in carrying capacity in Chl (p = 0.006775), Nit (p = 7.08E-05), and Nal (p = 0.00573), while showing no change in carrying capacity in Amp (p = 0.663977) and Rif(p = 0.761118). S populations showed no change in carrying capacity in any novel antibiotic (Amp – p = 0.063349, Chl – p = 0.202324, Nit – p = 0.160467, Nal – p = 0.074614, Rif – p = 0.09835) (Fig. 3 and 3.7c). This was also reflected in the two-way ANOVA for comparing L and S populations, where the interaction between bottleneck ratio and assay environment was significant

($F_{5,77}$ = 3.7781, p = 0.004111). In Chl and Nit, S populations had a significantly higher carrying capacity than L populations, while there was no difference in the other novel antibiotics (Holm-Šídák corrected p-values: Chl – p = 0.017444, Nit – p = 0.036081, Amp – p = 0.220418, Nal – p = 0.341138, Rif – p = 0.349996) (Fig. 3.7c).

For Nit-selected populations, both L and S populations showed a significant decrease in carrying capacity in Amp (L – p = 0.000897, S – p = 0.002629). Additionally, L populations showed a decrease in carrying capacity in Nal (p = 0.006787), and S populations showed a significant increase in carrying capacity in Chl (p = 0.031983). There was no change in carrying capacity for L populations in Chl (p = 0.231727), Kan (p = 0.161495) and Rif (p = 0.662791), and S populations in Kan (p = 0.118715), Nal (p = 0.119804) and Rif (p = 0.708496). (Fig. 3 and 3.7d). A two-way ANOVA conducted to further understand the differences between L and S populations showed that the interaction between bottleneck ratio and assay environment was significant ($F_{5,77}$ = 2.5027, p = 0.038321), but none of the comparisons between L and S populations in any novel antibiotics were significant after a Holm-Šídák correction (Holm-Šídák corrected p-values: Amp – p = 0.714793, Chl – p = 0.142826, Kan – p = 0.924272, Nal – p = 0.478674, Rif – p = 0.830432) (Fig. 3.7d).

Taken together, these results show that there were no consistent patterns between resistances in novel environments among populations bottlenecked at large and small bottleneck ratios. Populations selected under exposure to Kan show L populations having lower or equal MICs than the other populations, but do not follow this trend for growth rates and carrying capacities. This lack of a consistent trend is seen for the other antibiotics as well.

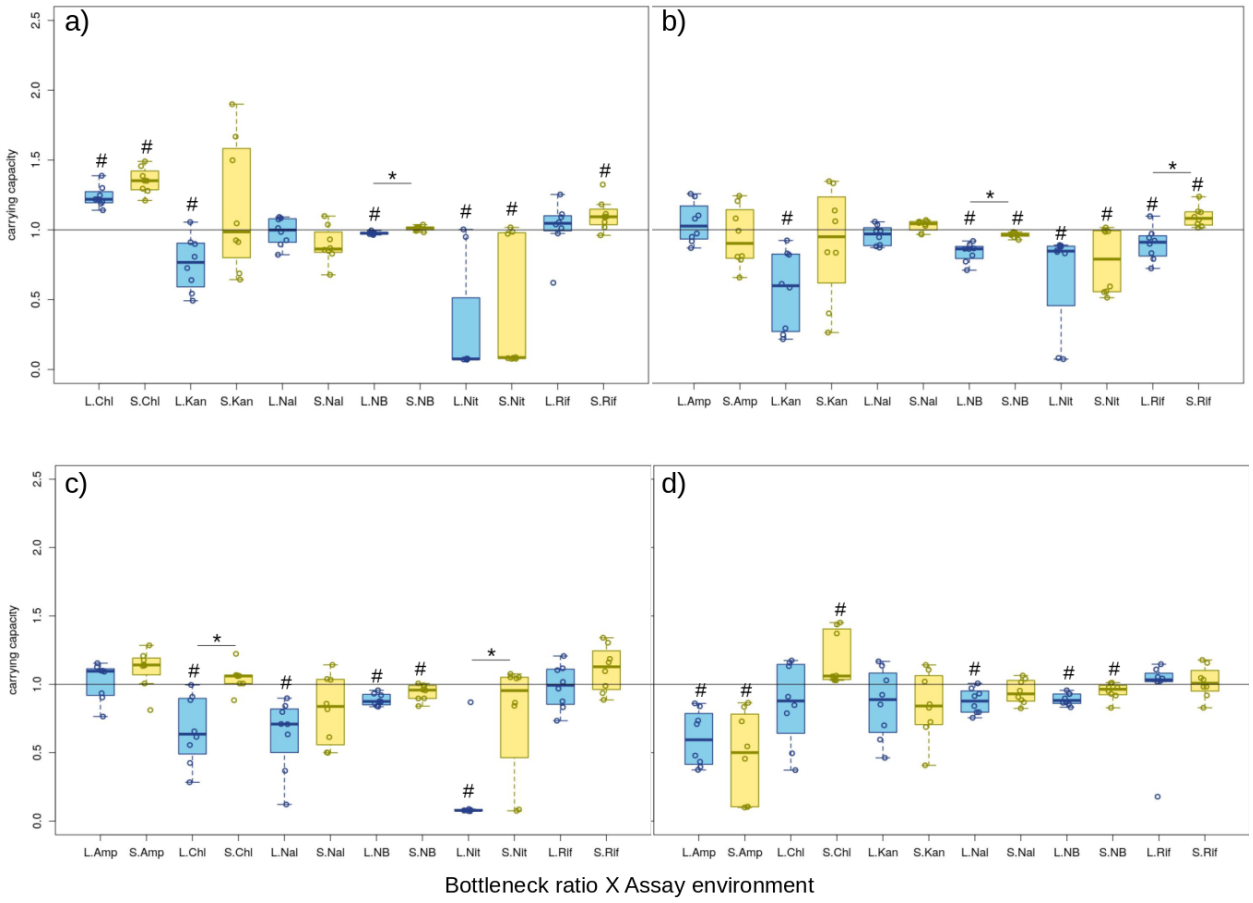


*Figure 3.7. Carrying capacities for a) Ampicillin-selected b) Chloramphenicol-selected c) Kanamycin-selected and d) Nitrofurantoin-selected single antibiotic selected populations in their novel environments. The labels on the x-axis denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes $p < 0.05$ in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant ($p < 0.05$) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

3.3. After being exposed to fluctuations between two antibiotics, small populations are not always able to evolve resistance to the selection environments.

First, we looked at the change in resistance to the antibiotics used for selection for populations selected under exposure to fluctuations between a pair of antibiotics (henceforth referred to as FS populations). This was examined by performing two-tailed t-tests with the ancestor values of MIC. There were six pairs of antibiotics used in the selection, which were – Ampicillin and Chloramphenicol (AC), Ampicillin and Kanamycin (AK), Ampicillin and Nitrofurantoin (NA), Chloramphenicol and Kanamycin (CK), Chloramphenicol and Nitrofurantoin (CN) and Kanamycin and Nitrofurantoin (KN).

Here, all L populations showed a significant increase in MIC to both antibiotics used in the selection compared to the ancestral MIC. However, this was not seen for the corresponding S populations. To further investigate the differences between L and S populations, a two-way ANOVA was conducted to compare the geometric mean MICs in selection environments, which showed a significant interaction between bottleneck ratio and assay environment ($F_{5,70}$ = 11.46, p < 1E-07) (Fig. 1 and 3.8a).

For AC populations, L populations show a significant increase in Amp (p = 3.75E-05) and Chl (p = 1.37E-05) MICs, but S populations only show a significant increase in Chl (p = 1.9E-06) and show no change in Amp (p = 1). This is evidenced by the geometric mean MICs being significantly higher for L populations than S populations (Holm-Šídák corrected p-value = 0.000543) (Fig. 1 and 3.8a). For AK populations, both L and S populations gained a significantly higher resistance to Amp (L – p = 0.000136, S – p = 0.00136) and Kan (L – p = 5.97E-07, S – p = 0.002536). However, the geometric mean MIC for L populations was still significantly greater than that for S populations (Holm-Šídák corrected p-value = 0.00032) (Fig. 1 and 3.8a). For NA populations, both L and S populations gained a significantly higher resistance to Amp (L – p = 0.00122, S – p = 0.000383) and Nit (L – p = 3.41E-07, S – p = 0.000139). In accordance, the

geometric mean MIC for L populations and S populations was not significantly different from each other (Holm-Šídák corrected p-value = 0.141694) (Fig. 1 and 3.8a).
For CK populations, L populations how a significant increase in MIC for Chl (p = 5.97E-07) and Kan (p = 0.000212), but S populations only show a significant increase in Chl (p = 1.14E-05) and not in Kan (p = 0.350617). Similarly, the geometric mean MIC for L populations is significantly higher than that for S populations (Holm-Šídák corrected p-value = 0.000475) (Fig. 1 and 3.8a). For CN populations, both L and S populations show an increase in MIC for Chl (L – p < 1E-07 , S – p = 2.54E-05) and for Nit (L – p = 0.00046 , S – p = 3.75E-06). Surprisingly, the geometric mean MIC for S populations was higher than that for L populations (Holm-Šídák corrected p-value = 0.029252) (Fig. 1 and 3.8a). Lastly, for KN populations, the MICs for L populations in Kan (p = 0.00014) and Nit (p = 0.02113) were both significantly higher than ancestral MICs. However, for S populations, the MIC in Nit was significantly higher than ancestral MIC (p = 0.000455), but not in Kan (p = 0.350617). This difference was reflected in the difference in the geometric mean MIC, where L populations were higher than S populations (Holm-Šídák corrected p-value = 0.027296) (Fig. 1 and 3.8a).

These clear patterns do not emerge when the growth rates and carrying capacities in sublethal concentrations of the selection antibiotic are analyzed. This was examined by performing two-tailed t-tests with the ancestor values of growth rate and K. Also, two-way ANOVAs were carried out to compare the geometric means of growth rates and carrying capacities in selection environments between L and S populations for all FS populations. For geometric mean growth rate, the interaction between bottleneck ratio and carrying capacity was not significant ($F_{5,70}$ = 1.14, p = 0.347386), but the main effect of bottleneck ratio was significant (F1,14 = 11.863, p = 0.00395), with L populations having a higher growth rate than S populations overall. However, for the geometric mean of carrying capacity, the interaction between bottleneck ratio and carrying capacity was significant ($F_{5,70}$ = 4.648, p = 0.001013) (Fig. 2, 3 and 3.8b).

For AC populations, the growth rate in Chl was significantly higher than the ancestral value for both L and S populations (L - p = 7.35E-09, S – p = 5.56E-07). For both L and

S populations, the growth rate in Amp was not different from the ancestral growth rate (L - p = 0.641155, S – p = 0.150488). (Fig. 3.8b) The carrying capacity, however, was higher than the ancestor for both L and S populations, in both the antibiotics used for selection (Amp – L – p = 0.00423, S – p = 0.000916; Chl – L – p = 3.26E-07, S – p = 0.007047). However, the geometric mean carrying capacity was not different between L and S populations (Holm-Šídák corrected p-value = 0.674786) (Fig. 3.8c).

For AK populations, the growth rate in Ampicillin was not different from the ancestor for both L and S populations (L – p = 0.393042, S – p = 0.955382). However, the growth rate in Kan was significantly different from that in the ancestor for L populations, but not for S populations (L – p = 1.85E-05, S – p = 0.602063). (Fig. 3.8b) For carrying capacities, both L and S populations had a higher carrying capacity than ancestor populations in Kan (L – p = 5.17E-08, S – p = 0.019609), but in Ampicillin, only S populations had a higher carrying capacity (L – p = 0.751507, S – p = 0.007658). However, the geometric mean carrying capacity was not different between L and S populations (Holm-Šídák corrected p-value = 0.558705) (Fig. 3.8c).

For NA populations, the growth rate in Amp was not different from the ancestor growth rate for both L and S populations (L – p = 0.363843, S – p = 0.4664). The growth rate in Nit, however, was significantly lesser than the ancestor growth rate for S populations, while for L populations there was again no difference (L – p = 0.206271, S – p = 0.028372). (Fig. 3.8b) Similarly, for carrying capacity in Amp, L populations showed no change, but S populations showed an increase in carrying capacity (L – p = 0.439782, S – p = 0.026502). In Nit, L populations showed no change in growth rate, but S populations showed a significant decrease from the ancestor carrying capacity (L – p = 0.205719, S – p = 0.046129). Because of these different trends, the geometric mean carrying capacity between L and S populations was not different (Holm-Šídák corrected p-value = 0.925839) (Fig. 3.8c).

For CK populations, the growth rate was significantly greater than the ancestor population for both L and S populations in Chl (L – p = 5.95E-08, S – p = 2.36E-07).

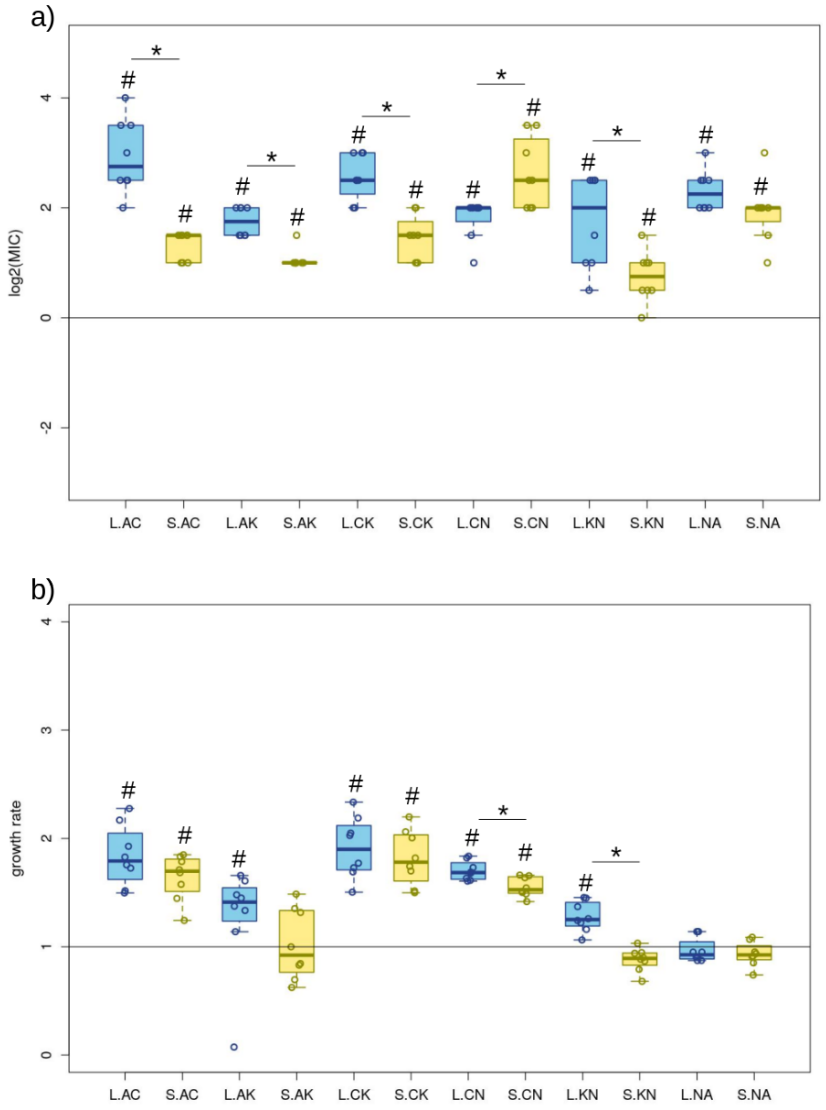
However, for Kan, only L populations had a higher growth rate than the ancestral value (L – p = 0.010969, S – p = 0.079849). (Fig. 3.8b) Both L and S populations had a higher carrying capacity than the ancestor population in Chl (L – p = 3.42E-07, S – p = 2.16E-08) as well as in Kan (L – p = 9.65E-07, S – p = 0.016016). Thus, the geometric mean carrying capacity between L and S populations was not different (Holm-Šídák corrected p-value = 0.677586) (Fig. 3.8c).

For CN populations, the growth rate was significantly greater than the ancestor population for both L and S populations in Chl (L – p = 4.00E-09, S – p = 8.31E-07). However, for Nit, S populations had a lower growth rate than the ancestral value, while L populations showed no change (L – p = 0.06869, S – p = 0.037911). (Fig. 3.8b) Both L and S populations had a higher carrying capacity in Chl (L – p = 2.69E-07, S – p = 3.17E-08), but in Nit, L populations showed a significant decrease in carrying capacity as compared to the ancestor, but S populations showed no change (L – p = 3E-05, S – p = 0.872632). Because of this, S populations had a higher geometric mean carrying capacity than L populations (Holm-Šídák corrected p-value = 0024747) (Fig. 3.8c).

For KN populations, there was no change as compared to the ancestral growth rate in Nit for L populations, while S populations showed a decrease in growth rate (L – p = 0.064222, S – p = 0.695483). For Kan, L populations had a higher growth rate than the ancestral value, while S populations had a lower growth rate (L – p = 0.00048, S – p = 0.018691). (Fig. 3.8b) The carrying capacity in N was not different from the ancestral value for both L and S populations in Nit (L – p = 0.229907, S – p = 0.103756), but in Kan, only L populations had a higher carrying capacity (L – p = 2.58E-07, S – p = 0.387665). Because of these differences, L populations had a higher geometric mean carrying capacity than S populations (Holm-Šídák corrected p-value = 0.000111) (Fig. 3.8c).

Taken together, these results show that populations selected under a large bottleneck ratio showed an increase in resistance to both the antibiotics used in their selection (measured as MIC). However, for three out of the six fluctuating environments, the

populations selected under a small bottleneck ratio did not show an increase in resistance to one of the selection antibiotics. This clear pattern did not show up when fitness (growth rate and carrying capacity) was measured at sublethal concentrations of selection antibiotics, where in a few cases L populations showed no change or even a decrease in fitness as compared to the ancestral values. Conversely, S populations sometimes showed an increase in growth rate and carrying capacity after selection, and in the case of populations selected under exposure to Chl and Nit, even showed a higher geometric mean carrying capacity than L populations.



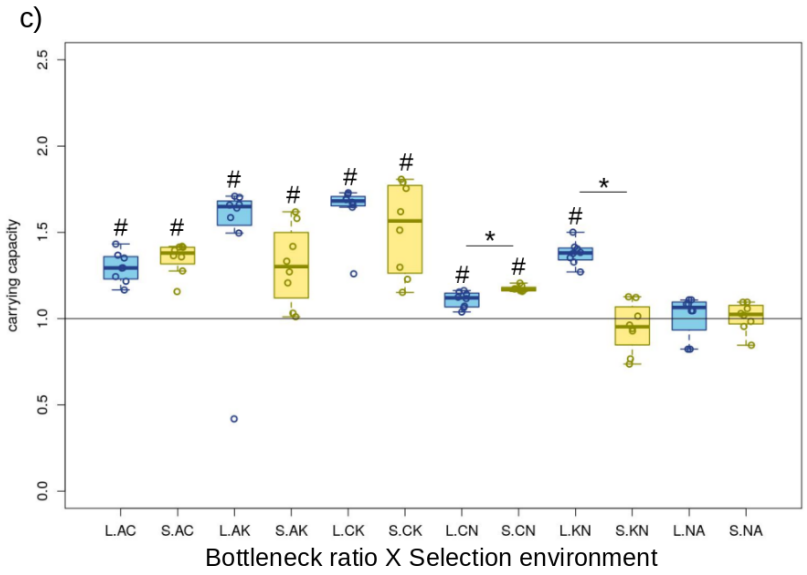


*Figure 3.8. a) log_2(MIC)s b) growth rates c) carrying capacities for populations selected under fluctuations of antibiotics in their selection environment. The geometric means of the values in both selection environments are represented in this graph. The labels on the x-axis denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes p < 0.05 in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant (p < 0.05) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

3.4. Despite being able to increase resistance to both selection antibiotics, large populations do not face higher costs when exposed to novel antibiotics.

The final set of experiments were to see the change in resistance to novel antibiotics for populations selected under exposure to fluctuations between a pair of antibiotics (henceforth referred to as FS populations). This was examined by performing two-tailed t-tests with the ancestral values of MIC, growth rate and carrying capacity. There were four antibiotics used in the selection – Ampicillin (Amp), Chloramphenicol (Chl), Kanamycin (Kan), and Nitrofurantoin (Nit). All six possible pairs of these four antibiotics

were used in the selection - Ampicillin and Chloramphenicol (AC), Ampicillin and Kanamycin (AK), Ampicillin and Nitrofurantoin (NA), Chloramphenicol and Kanamycin (CK), Chloramphenicol and Nitrofurantoin (CN) and Kanamycin and Nitrofurantoin (KN). MIC, growth rate and carrying capacity was measured for all four of these antibiotics, with the addition of two more antibiotics – Nalidixic acid (Nal) and Rifampicin (Rif).

For AC populations, a significant increase in MIC was observed in Nal and Rif for both L and S populations (Nal – L – p < 1E-07 , S – p = 1.41E-06 ; Rif – L – p = 0.000212, S – p = 4.27E-05). Similarly, the MIC in Kan was significantly lower than ancestor MIC for both L and S populations (L – p = 0.000136, S – p = 3.26E-05). However, only L populations showed an increase in MIC for Nit, which was not seen in the S populations (L – p = 0.011201, S – p = 0.079602). A two-way ANOVA conducted to further understand the differences between L and S populations showed that the interaction between bottleneck ratio and assay environment was significant ($F_{3,42}$ = 5.8372, p = 0.001991). The comparison between L and S populations was significant only in Nit after a Holm-Šídák correction (Holm-Šídák corrected p-values: Kan – p = 0.066332, Nit – p = 0.006855, Nal – p = 0.350617, Rif – p = 0.326208) (Fig. 3.9a).

For AK populations, there was no change in MIC for Chl, Nal and Rif as compared to the ancestor populations (Chl – L – p = 0.170471, S – p = 0.350617; Nal – L – p = 0.598331, S – p = 0.079602; Rif – L – p = 0.170471, S – p = 0l70471). In Nit, however, L populations had a lower MIC than the ancestral value (p = 0.000212), while S populations showed no change in MIC (p = 0l70471). This was reflected in the two-way ANOVA conducted to further understand the differences between L and S populations. The interaction between bottleneck ratio and assay environment was significant ($F_{3,42}$ = 3.076433, p = 0.037747), and the comparison between L and S populations was significant only in Nit after a Holm-Šídák correction (Holm-Šídák corrected p-values: Chl – p = 0.108134, Nit – p = 0.037491, Nal – p = 0.405761, Rif – p = 0.591126) (Fig. 3.9b).

For NA populations, both L and S populations showed an increase in resistance to Chl and no change in resistance to Rif with respect to the ancestral MIC (Chl – L – p =

0.001216, S – p = 8.39e-05 ; Rif – L – p = 0.073237, S – p = 0.154642). However, L populations showed a decrease in MIC in Kan, which was not seen for S populations (L – p = 0.033146, S – p = 0.350617). Also, the increase in Nal MIC seen in the S populations was not seen for L populations (L – p = 0.110778, S – p = 0.003816). However, in the two-way ANOVA conducted to further understand the differences between L and S populations, the interaction between bottleneck ratio and assay environment was not significant ($F_{3,42}$ = 2.02037, p = 0.125634), and even the main effect of bottleneck ratio was not significant ($F_{1,14}$ = 1.06028, p = 0.320614) (Fig. 3.9c).

For CK populations, both L and S populations showed an increase in Amp and Nal MIC as compared to the ancestral value (Amp – L – p = 6.47E-07, S – p = 1.37E-05; Nal – L – p = 1.9E-06, S – p = 1.37E-05). However, only L populations showed an increase in resistance to Nit and Rif (Nit – L – p = 0.000212, S – p = 1 ; Rif – L – p = 0.000122, S – p = 0.598331). This overall increase in MIC in all novel environments for L populations was reflected in the two-way ANOVA conducted to understand the differences in MIC between L and S populations. The interaction between bottleneck ratio and assay environment was not significant ($F_{3,42}$ = 0.1901, p = 0.902539), but the main effect of bottleneck ratio was significant ($F_{1,14}$ = 30.1659, p = 7.9E-05) (Fig. 3.9d).

For CN populations, both L and S populations showed an increase in resistance to Amp, Nal and Rif (Amp – L – p = 4.27E-05, S – p = 0.000805 ; Nal – L – p = 0.000122, S – p = 0.000383; Rif – L – p = 0.000122, S – p = 0.005266). However, L populations had a significantly lower MIC than ancestor in Kan, while S populations showed no change (L – p = 0.000122, S – p = 0.094976). Despite this, the interaction between bottleneck ratio and assay environment was not significant ($F_{3,42}$ = 0.6329, p = 0.597912), but the main effect of bottleneck ratio was significant ($F_{1,14}$ = 14.9747, p = 0.0017) (Fig. 3.9e).

For KN populations, L populations showed an increase in resistance to Amp and Rif, while S populations showed a significant increase in Chl MIC as compared to the ancestral value (Amp – L – p = 4.27E-05, S – p = 0.103552 ; Chl – L – p = 0.563028, S

– p = 0.002536 ; Rif – L – p = 0.033146, S – p = 0.079602). Neither of the two populations showed an increase in Nal MIC (L – p = 0.350617, S – p = 0.350617). This difference in the resistance increases for L and S populations was reflected in the two-way ANOVA conducted to understand the differences in MIC between L and S populations. The interaction between bottleneck ratio and assay environment was significant ($F_{3,42}$ = 2.96364, p = 0.042854), but after a after a Holm-Šídák correction, there was no significant difference between MICs for L and S populations (Holm-Šídák corrected p-values: Amp – p = 0.220603, Chl – p = 0.638974, Nal – p = 0.663984, Rif – p = 0.641987) (Fig. 3.9f).

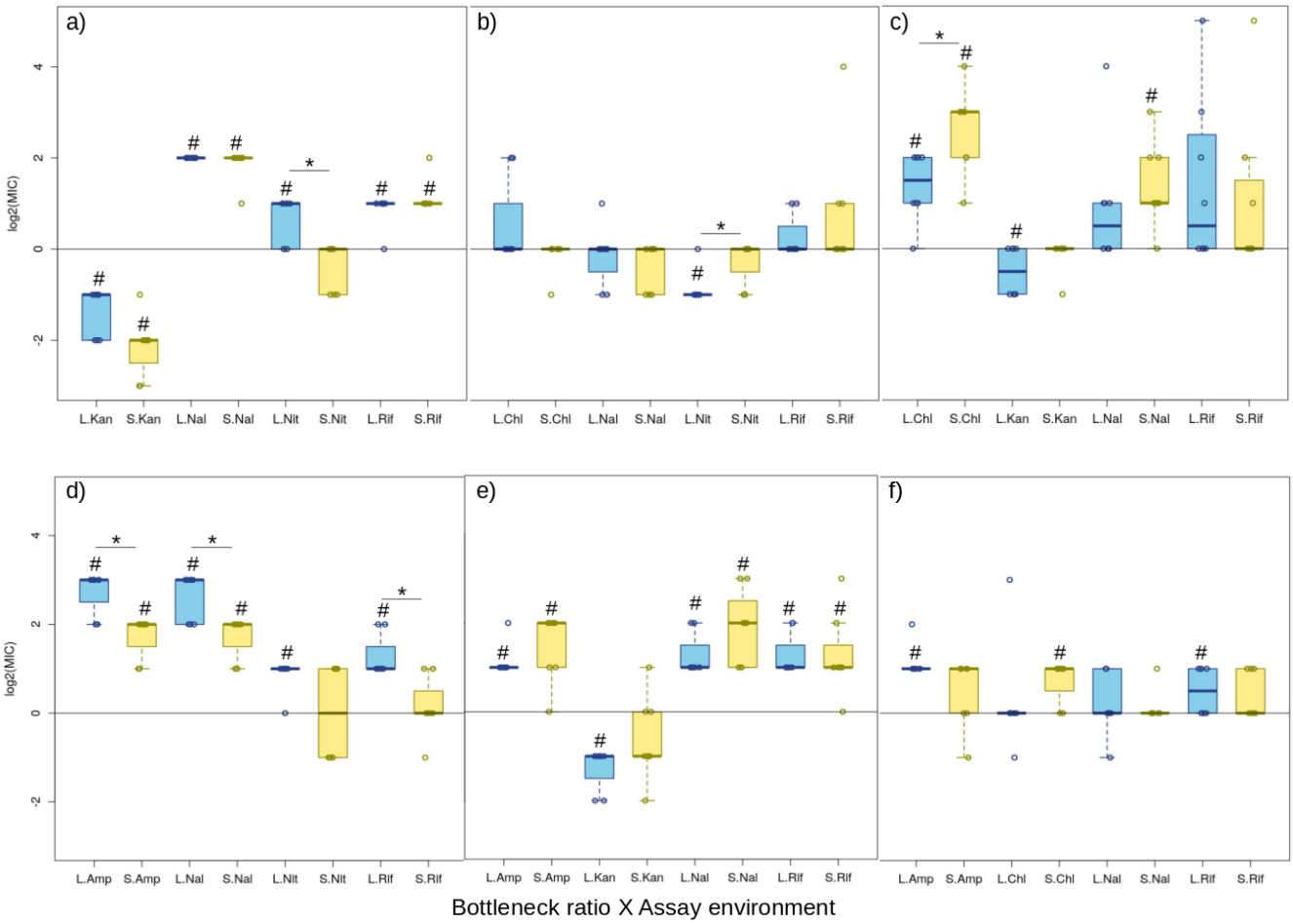


Figure 3.9. log_2(MIC)s for populations selected under exposure to a) Ampicillin and Chloramphenicol, b) Ampicillin and Kanamycin, c) Ampicillin and Nitrofurantoin, d) Chloramphenicol and Kanamycin, e) Chloramphenicol and Nitrofurantoin, and f) Kanamycin and Nitrofurantoin in their novel environments. The labels on the x-axis

*denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes p < 0.05 in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant (p < 0.05) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

When we looked at the fitness in sublethal concentrations of novel environments, we once again saw no consistent patterns emerge. None of the selected populations showed an increase in growth rate and carrying capacity in Nutrient broth (NB), as compared to the ancestral values of growth rate and K. There was no difference between the growth rates as compared to the ancestor value for both L and S populations for AC, NA and KN populations. (AC – L – p = 0.507494, S – p = 0.522413; NA – L – p = 0.48138, S – p = 0.636484 ; KN – L – p = 0.530631, S – p = 0.236167). For CK and CN populations, both L and S populations show a reduction in growth rate (CK - L – p = 1.42E-05, S – p = 0.000742; CN – L – p = 0.005768, S – p = 0.005896). Only for AK populations, the growth rate in NB was less than that of the ancestor for S populations but not for L populations (L – p = 0.142538, S – p = 0.000707). Most of the populations showed a decrease in carrying capacity in NB – only L populations selected under exposure to Ampicillin and Nitrofurantoin and S populations selected under exposure to Kanamycin and Nitrofurantoin did not show any change in carrying capacity. (AC – L – p = 0.014493, S – p = 0.023679 ; AK- L – p = 0.006016, S – p = 8.67E-05 ; NA – L – p = 0.188082, S – p = 0.00946; CK - L – p = 9.89E-05, S – p = 0.00981 ; CN – L – p = 8.56E-06, S – p = 0.000302 ; KN – L – p = 0.004707, S – p = 0.057129) (Fig. 3.10).

Looking at the growth rates in individual populations, again no consistent patterns are observed. For AC populations, both L and S populations show a reduction in growth rate in Kan, but no change in Nal and Rif (Kan - L – p = 0.003786, S – p = 0.005366 ; Nal - L – p = 0.368316, S – p = 0.572293 ; Rif - L – p = 0.464399, S – p = 0.101377).

Only L populations showed an increase in growth rate in Nit (L – p = 0.014248, S – p = 0.115224). A two-way ANOVA conducted to understand the differences in growth rate between L and S populations showed that the interaction between bottleneck ratio and assay environment was not significant ($F_{4,56}$ = 1.9982, p = 0.107291), and even the main effect of bottleneck ratio was not significant ($F_{1,14}$ = 0.2407, p = 0.631283) (Fig. 3.10a).

For AK populations, the growth rate in Chl and Rif was not different from ancestor for both L and S populations (Chl - L – p = 0.417156, S – p = 0.105804; Rif - L – p = 0.65004, S – p = 0.201623). Also, both L and S populations showed a significantly lower growth rate than ancestor in Nit (L – p = 0.005599, S – p < 1E-07). In Nal, however, L populations showed no change in growth rate, but S populations had a significantly lower growth rate (L – p = 0.143765, S – p = 0.000418). Despite these differences, a two-way ANOVA conducted to understand the differences in growth rate between L and S populations showed that the interaction between bottleneck ratio and assay environment was not significant ($F_{4,56}$ = 0.2688, p = 0.896818), and even the main effect of bottleneck ratio was not significant ($F_{1,14}$ = 1.7441, p = 0.207802) (Fig. 3.10b).

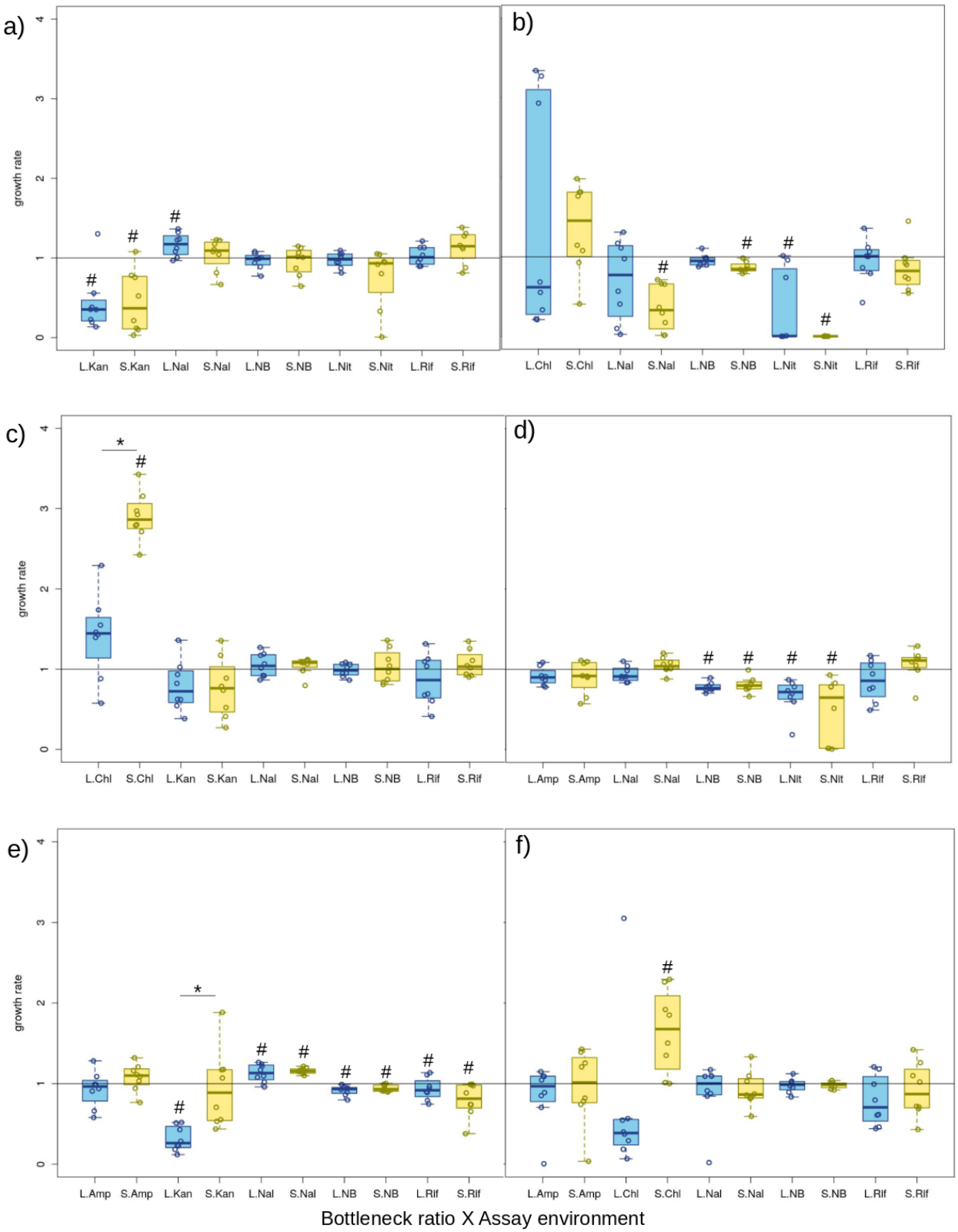
For NA populations, there was no change in the growth rate for any novel antibiotic for L populations. S populations, however, showed a significantly higher growth rate in Chl than the ancestral value (Chl - L – p = 0.057753, S – p = 4.22E-07 ; Kan – L – p = 0.098757, S – p = 0.119501 ; Nal – L – p = 0.529778, S – p = 0.310293 ; Rif - L – p = 0.351628, S – p = 0.278349). This difference was reflected in a two-way ANOVA conducted to understand the differences in MIC between L and S populations. The interaction between bottleneck ratio and assay environment was significant ($F_{4,56}$ = 17.3152, p < 1E-07).The growth rate in Chl, however, was significantly higher for S populations than for L populations after a Holm-Šídák correction. (Holm-Šídák corrected p-values: Chl – p = 0.0001, Kan – p = 0.90037, Nal – p = 0.873096, Rif – p = 0.258512) (Fig. 3.10c).

For CK populations, the growth rate in Amp, Nal and Rif was not different from ancestor for both L and S populations (Amp - L – p = 0.057886, S – p = 0.224013; Nal - L – p =

0.113798, S – p = 0.191867; Rif - L – p = 0.154379, S – p = 0.393609). Additionally, both L and S populations showed a decrease in growth rate in Nit (L – p = 0.003334, S – p = 0.009046). These results were reflected in the results of a two-way ANOVA, where the interaction between bottleneck ratio and assay environment was not significant ($F_{4,56}$ = 2.209, p = 0.079683), and even the main effect of bottleneck ratio was not significant ($F_{1,14}$ = 0.395, p = 0.539912) (Fig. 3.10d).

For CN populations, the growth rate in Amp was not different from ancestor for both L and S populations (L – p = 0.407369, S – p = 0.230563), and both populations showed a significant increase in growth rate in Nal (L – p = 0.012114, S – p = 7.47E-06). L populations showed a significant decrease in growth rate as compared to the ancestral value in Kan, and S populations had a lower growth rate in Rif (Kan - L – p = 4.37E-06, S – p = 0.742684; Rif - L – p = 0.215724, S – p = 0.029511). To further compare the differences in growth rate between L and S populations, a two-way ANOVA was conducted. The interaction between between bottleneck ratio and assay environment was significant ($F_{4,56}$ = 7.876, p = 4.1E-05), but after a Holm-Šídák correction, only L populations had a lower growth rate in Kan as compared to the S populations (Holm-Šídák corrected p-values: Amp – p = 0.404632, Kan – p = 0.037324, Nal – p = 0.549263, Rif – p = 0.145378) (Fig. 3.10e).

For KN populations, L populations did not show any change in growth rate. S populations only showed an increase in growth rate in Chl, and no change in any other novel antibiotic (Amp - L – p = 0.307152, S – p = 0.808437 ; Chl – L – p = ,0.390636 S – p = 0.00899 ; Nal - L – p = 0.4243, S – p = 0.39203 ; Rif - L – p = 0.093551, S – p = 0.515795). In a two-way ANOVA, the interaction between bottleneck ratio and assay environment was significant ($F_{4,56}$ = 4.1125, p = 0.005431). However, after a Holm-Šídák correction, none of the novel antibiotics showed a significant difference between L and S populations (Holm-Šídák corrected p-values: Amp – p = 0.948464, Chl – p = 0.146515, Nal – p = 0.959775, Rif – p = 0.1896484) (Fig. 3.10f).



Bottleneck ratio X Assay environment

*Figure 3.10. Growth rates for populations selected under exposure to a) Ampicillin and Chloramphenicol, b) Ampicillin and Kanamycin, c) Ampicillin and Nitrofurantoin, d) Chloramphenicol and Kanamycin, e) Chloramphenicol and Nitrofurantoin, and f) Kanamycin and Nitrofurantoin in their novel environments. The labels on the x-axis denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes p < 0.05 in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant (p < 0.05) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

Again, for the carrying capacities, we do not see any discernible trends. For AC populations, a significant decrease with respect to the ancestral value was seen in Nal and Rif for L populations, and no change was seen for the other novel antibiotics. S populations showed an increase in carrying capacity in Rif and a decrease in carrying capacity in Kan, while no change was seen for the other novel antibiotics (Kan - L – p = 0.090312, S – p = 0.007047 ; Nit – L – p = 0.130626, S – p = 0.106893 ; Nal - L – p = 3.56E-06, S – p = 0.148374 ; Rif - L – p = 0.000528, S – p = 0.009968). A two-way ANOVA conducted to understand the differences in growth rate between L and S populations showed that the interaction between bottleneck ratio and assay environment was not significant ($F_{4,56}$ = 1.7818, p = 0.145349), and even the main effect of bottleneck ratio was not significant ($F_{1,14}$ = 0.1715 p = 0.685047) (Fig. 3.11a).

For AK populations, the carrying capacity in Chl and Rif was not different from ancestor for both L and S populations (Chl - L – p = 0.627718, S – p = 0.459884 ; Rif - L – p = 0.611931, S – p = 0.649903). Also, both L and S populations showed a significantly lower carrying capacity than ancestor in Nit (L – p = 0.007841, S – p < 1E-07). In Nal, however, L populations showed no change in carrying capacity, but S populations had a significantly lower carrying capacity (L – p = 0.123232, S – p = 0.008194). Despite these differences, a two-way ANOVA conducted to understand the differences in carrying

capacity between L and S populations showed that the interaction between bottleneck ratio and assay environment was not significant ($F_{4,56}$ = 1.837731, p = 0.134421), and even the main effect of bottleneck ratio was not significant ($F_{1,14}$ = 1.268774 p = 0.278935) (Fig. 3.11b).

For NA populations, L populations only showed a decrease in carrying capacity in Nal and no change in other novel antibiotics. S populations showed an increase in carrying capacity in Chl compared to the ancestral value. (Chl - L – p = 0.596488, S – p = 7.51E-06 ; Kan – L – p = 0.794797, S – p = 0.970786 ; Nal – L – p = 0.01898, S – p = 0.8043 ; Rif - L – p = 0.060866, S – p = 0.940196). This difference was reflected in a two-way ANOVA conducted to understand the differences in MIC between L and S populations. The interaction between bottleneck ratio and assay environment was significant ($F_{4,56}$ = 2.6263, p = 0.044428).The carrying capacity in Chl, however, was significantly higher for S populations than for L populations after a Holm-Šídák correction. (Holm-Šídák corrected p-values: Chl – p = 0.004384, Kan – p = 0.99593, Nal – p = 0.238725, Rif – p = 0.923068) (Fig. 3.11c).

For CK populations, both L and S populations showed no change in the carrying capacity in Nit and Rif, and an increase in the carrying capacity in Amp (Amp - L – p = 4.55E-05, S – p = 0.008878 ; Nit - L – p = 0.062158, S – p = 0.071367 ; Rif - L – p = 0.092681, S – p = 0.744784). However, S populations showed an increase in the carrying capacity in Nal, which was not seen for L populations (L – p = 0.214131, S – p = 3.83E-06). These results were not entirely reflected in the results of a two-way ANOVA, where the interaction between bottleneck ratio and assay environment was not significant ($F_{4,56}$ = 1.292, p = 0.284106), and even the main effect of bottleneck ratio was not significant ($F_{1,14}$ = 0.097, p = 0.760465) (Fig. 3.11d).

For CN populations, both L and S populations showed an increase in the carrying capacity in Amp and a decrease in the carrying capacity in Rif (Amp - L – p = 0.00506, S – p = 0.011091 ; Rif - L – p = 0.004956, S – p = 0.021586). However, L populations showed a decrease in carrying capacity in Kan and Nal, while S populations actually

showed an increase in Nal and no difference in Kan (Kan - L – p = 7.25E-06, S – p = 0.914391 ; Nal - L – p = 0.03666, S – p = 0.002278). To further compare the differences in growth rate between L and S populations, a two-way was ANOVA conducted. The interaction between between bottleneck ratio and assay environment was significant ($F_{4,56}$ = 8.127, p = 3.1E-05), but after a Holm-Šídák correction, L populations had a lower growth rate in Kan and Nal as compared to the S populations (Holm-Šídák corrected p-values: Amp – p = 0.933275, Kan – p = 0.025309, Nal – p = 0.00247, Rif – p = 0.755718) (Fig. 3.11e).

For KN populations, a significant increase in the carrying capacity in Chl was seen in S populations, but instead a significant decrease was seen in the L populations. L populations also showed a significantly lower carrying capacity in Rif as compared to the ancestral value (Amp - L – p = 0.429073, S – p = 0.919356 ; Chl – L – p = 0.018713, S – p = 0.013753 ; Nal - L – p = 0.272165, S – p = 0.869088 ; Rif - L – p = 0.012772, S – p = 0.593514). In a two-way ANOVA, the interaction between bottleneck ratio and assay environment was significant ($F_{4,56}$ = 4.9903, p = 0.00164). However, after a Holm-Šídák correction, S populations had a significantly higher carrying capacity in Chl than L populations (Holm-Šídák corrected p-values: Amp – p = 0.646654, Chl – p = 0.017396, Nal – p = 0.537264, Rif – p = 0.223803) (Fig. 3.11f).

Taken together, we see that a higher bottleneck size does not particularly lead to higher costs, as for the populations selected under exposure to a single antibiotic. This lack of a consistent trend is seen for all pairs of antibiotics as well.

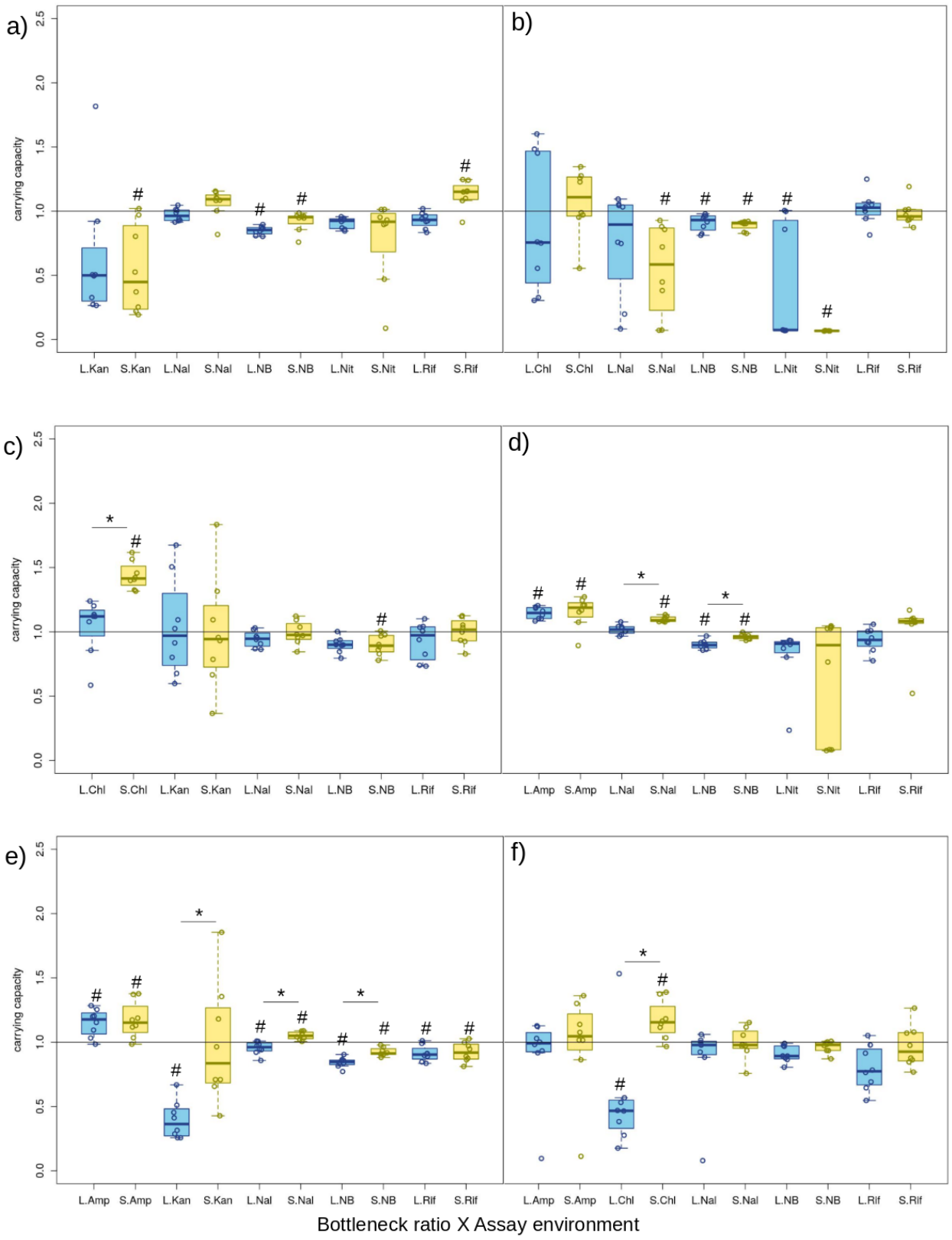

a)
b)
c)
d)
e)
f)
carrying capacity
0.0
0.5
1.0
1.5
2.0
2.5
L.Kan S.Kan L.Nal S.Nal L.NB S.NB L.Nit S.Nit L.Rif S.Rif
L.Chl S.Chl L.Nal S.Nal L.NB S.NB L.Nit S.Nit L.Rif S.Rif
L.Chl S.Chl L.Kan S.Kan L.Nal S.Nal L.NB S.NB L.Rif S.Rif
L.Amp S.Amp L.Nal S.Nal L.NB S.NB L.Nit S.Nit L.Rif S.Rif
L.Amp S.Amp L.Kan S.Kan L.Nal S.Nal L.NB S.NB L.Rif S.Rif
L.Amp S.Amp L.Chl S.Chl L.Nal S.Nal L.NB S.NB L.Rif S.Rif
Bottleneck ratio X Assay environment

*Figure 3.11. Carrying capacity for populations selected under exposure to a) Ampicillin and Chloramphenicol, b) Ampicillin and Kanamycin, c) Ampicillin and Nitrofurantoin, d) Chloramphenicol and Kanamycin, e) Chloramphenicol and Nitrofurantoin, and f) Kanamycin and Nitrofurantoin in their novel environments. The labels on the x-axis denote the assay environment for both the L and S populations separately. L and S denote the populations with a large (1/10) and small (1/10000) bottleneck ratio respectively. Box plots show interquartile range (IQR) ± 1.5*IQR. Open circles show all biological replicates. # denotes $p < 0.05$ in a two-tailed t-test between selected populations and ancestor populations. * denotes a significant ($p < 0.05$) difference between L and S populations in a two-tailed t-test after a Holm-Šídák correction. The black horizontal line shows the ancestral values for all three plots.*

Discussion

The aim of this study was to understand the influence of population size on the evolution of antibiotic resistance and consequently fitness at sublethal concentrations of antibiotics. In addition, we also wanted to understand whether fluctuating environments would influence the evolution of antibiotic resistance. The mechanisms that give rise to antibiotic resistance in bacteria are many, varied and antibiotic-specific (Reygaert, 2018; Windels et. al., 2020). Consequently, the extent to which bacteria develop resistance to antibiotics can be specific to the antibiotic in question. This dependence is also evident from the results of this study.

Population size has been shown to affect a variety of evolutionary phenomena, as it influences the availability of mutations, among other effects (Lachapelle et. al., 2015; Chavhan et. al., 2019a). At every subculture, large populations have access to more mutations, and consequently they have a greater chance to access rare mutations that have a large positive effect (Desai and Fisher, 2007; Chavhan et. al., 2019a). Conversely, these mutations are expected to have higher fitness costs in novel environments, and hence in such a scenario, large populations would show a decrease in fitness as compared to smaller populations (Chavhan et. al., 2020). Based on previous studies (Chavhan et. al., 2020), for populations selected under exposure to a single antibiotic, we expected large populations to perform better than small populations when assayed in the selection environment, and the opposite to be true when assayed in novel environments.

However, our results did not match these expectations. Instead, we observed that large populations do not consistently show a higher MIC than small populations, and do not have a higher growth rate and carrying capacity at sublethal concentrations of antibiotics (Results 3.1). L populations selected under Ampicillin consistently had a lower MIC and a lower growth rate and carrying capacity than S populations. Only in populations selected in Kanamycin did L populations consistently fare better than S

populations. Similarly, when assayed in novel environments, L populations did not consistently have lower MICs, growth rates and carrying capacities than S populations (Results 3.2). In many cases, there were no differences seen between the L and S populations, except in Ampicillin where L populations selected in Ampicillin had a higher MIC than S populations in Nalidixic acid and Rifampicin (Figure 3.5a). This lack of costs in novel environments for L populations is surprising.

These disparate results might be due to different strategies for antibiotic resistance being employed in populations selected under exposure to different antibiotics. Some mechanisms for survival at high concentrations of antibiotics involve a small number of cells that drives the resistance for the entire population (Lee et. al., 2010; Bakkeren et. al., 2020), which have been linked to multi-drug resistance (Levin-Reisman et al., 2017). These small subpopulations are more likely to be propagated at a large bottleneck ratio, and hence might lead to a higher MIC for L populations than S populations in novel environments. However, this does not explain why S populations sometimes show greater resistance to the antibiotics used in the selection.

Additionally, the molecular basis of resistance and dose of antibiotics has been shown to influence the distribution of fitness effects for beneficial mutations (MacLean et. al., 2009; reviewed in MacLean et. al., 2010). Single exposure studies have shown the presence of the inoculum effect, where the effects of the same dose of antibiotics on large and small populations is different (Karslake et. al., 2016). Hence, it is possible that the distribution of fitness effects for large and small populations is different, and L populations do not have access to mutations that confer a higher resistance than S populations.

Fitness costs that are imposed in constant environments might be circumvented in fluctuating environments (Bono et. al., 2017), but again these mutations that have a large, beneficial effect might be available only to large populations. This has been experimentally validated in a previous study with fluctuations between different carbon sources (Chavhan et. al., 2021). Hence, we expected large populations to reach a

higher MIC, growth rate and carrying capacity when selected under fluctuating exposure to two antibiotics.

Indeed, under a fluctuating selection, L populations managed to evolve an increase in MIC for both selection environments, while not every S population managed to do so (Results 3.3). L populations also mostly had a higher MIC than S populations, except for the populations selected under fluctuating exposure to Chloramphenicol and Nitrofurantoin (CN) (Figure 3.7a), where S populations evolved a higher MIC than L populations. Additionally, not all L populations managed to show an increase in growth rate and carrying capacity in the selection environments.

The observation that L populations are better able to evolve resistance under fluctuating exposure to antibiotics might indicate that they are better able to navigate the higher constraints placed by the fluctuating environments. Indeed, multiple studies have shown that bacterial populations evolve antibiotic resistance slower when selected under a sequential antibiotic exposure (Kim et. al., 2014; Evgrafov et. al., 2015; Jiao et. al., 2016). This, taken together with the previous results, might suggest that fluctuating exposures to antibiotics slow down antibiotic resistance evolution for small populations, but not for large populations.

Interestingly, an increase in MIC under both selection environments did not lead to a decrease in MIC in novel environments for L populations (Results 3.4). For populations selected under exposure to Chloramphenicol and Kanamycin, L populations showed a higher or equal MIC to S populations for every novel antibiotic (Figure 3.9d). For other populations, this trend was exactly reversed. For populations selected under exposure to Ampicillin and Kanamycin, L populations had a lower MIC than S populations in Nitrofurantoin (Figure 3.9b). For populations selected under exposure to Ampicillin and Nitrofurantoin, L populations had a lower MIC than S populations in Chloramphenicol (Figure 3.9c). At sublethal concentrations of antibiotics, however, there was either no difference between L and S populations, or L populations had lower growth rates and carrying capacities (Figure 3.10 and 3.11).

This seeming lack of costs faced by L populations in novel environments might be explained by a few possible mechanisms. The first one is that the distribution of fitness effects for the antibiotics used in the selection might have overlapped with some of the novel environments. In other words, rare mutations that are beneficial in one antibiotic might be similarly beneficial in another, leading to the presence of collateral resistance to other antibiotics (Imamovic and Sommer, 2013). Additionally, theoretical studies have shown that fluctuations between environments that have similar fitness landscapes can help populations to overcome local fitness minima (Maltas et. al., 2021). This might explain the lack of costs faced by L populations in novel environments after selection in a single antibiotic, or in fluctuations between two antibiotics. However, it does not adequately explain why S populations do better than L populations in multiple assays in selection as well as novel environments.

Another explanation might be an increase in efflux activity, which is the active transport of unwanted molecules out of bacterial cells, and is an important component of bacterial metabolism (Sun et. al., 2014). It is one of the mechanisms by which bacteria gain antibiotic resistance, and as it is not specific to one antibiotic, an increase in efflux might lead to decreased costs in a variety of other environments. An increase in efflux activity has been implicated in multidrug resistant bacteria that have caused infectious outbreaks (Kumar and Schweizer, 2005). Furthermore, increased efflux activity has been shown to be behind an increased fitness in novel environments after selection in unpredictably fluctuating environments (Karve et. al., 2015) and regulators of efflux pumps were upregulated in populations subjected to fluctuations between antibiotics (Yen et. al., 2017). Also, an increase in antibiotic resistance due to mutations in efflux pumps or regulators of efflux pumps has been frequently observed (Webber et. al., 2003). Hence, the lack of costs for L populations in novel environments after selection in antibiotics might be due to an increase in efflux activity.

The presence of costs for resistance to antibiotics is exploited clinically as a treatment strategy for pathogenic bacteria. As the battle against multidrug resistant bacteria goes

on, and in the absence of new drugs against these bacteria, the ability of utilising existing drugs in more targeted treatment regimes is crucial. Increasingly, experimental evolution of antibiotic resistance is seen as an important tool to understand the implications of treatment strategies before deploying them for actual use. This study explores one facet of the multiple factors that influence the development of antibiotic resistance, and it is concerning to note the increase in fitness due to the seeming lack of costs in multiple novel environments, even after exposure to a fluctuating antibiotic treatment. An exploration into the mechanisms behind this phenomenon is necessary to understand how to combat the rapid evolution of multidrug resistance.

References

1. Abdi, H. (2010). Holm's sequential Bonferroni procedure. Encyclopedia of research design, 1(8), 1-8.
2. Bakkeren, E., Diard, M., and Hardt, W. D. (2020). Evolutionary causes and consequences of bacterial antibiotic persistence. Nature Reviews Microbiology, 18(9), 479-490.
3. Barbosa, C., Römhild, R., Rosenstiel, P., and Schulenburg, H. (2019). Evolutionary stability of collateral sensitivity to antibiotics in the model pathogen *Pseudomonas aeruginosa*. Elife, 8, e51481.
4. Baym, M., Stone, L. K., and Kishony, R. (2016). Multidrug evolutionary strategies to reverse antibiotic resistance. Science, 351(6268).
5. Bono, L. M., Smith Jr, L. B., Pfennig, D. W., and Burch, C. L. (2017). The emergence of performance trade‐offs during local adaptation: insights from experimental evolution. Molecular ecology, 26(7), 1720-1733.
6. Boyer, S., Hérissant, L., & Sherlock, G. (2021). Adaptation is influenced by the complexity of environmental change during evolution in a dynamic environment. *PLoS Genetics*, *17*(1), e1009314.
7. Brook, I. (1989). Inoculum effect. Reviews of infectious diseases, 11(3), 361-368.
8. Chavhan, Y. D., Ali, S. I., and Dey, S. (2019). Larger numbers can impede adaptation in asexual populations despite entailing greater genetic variation. Evolutionary Biology, 46(1), 1-13.
9. 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(4), 836-846.
10. Chavhan, Y., Malusare, S., and Dey, S. (2020). An interplay of population size and environmental heterogeneity explains why fitness costs are rare. bioRxiv.
11. Chavhan, Y., Malusare, S., and Dey, S. (2020). Larger bacterial populations evolve heavier fitness trade-offs and undergo greater ecological specialization. Heredity, 124(6), 726-736.

12. Dean, Z., Maltas, J., and Wood, K. (2020). Antibiotic interactions shape short-term evolution of resistance in *E. faecalis*. PLoS pathogens, 16(3), e1008278.
13. Desai, M. M., and Fisher, D. S. (2007). Beneficial mutation–selection balance and the effect of linkage on positive selection. Genetics, 176(3), 1759-1798.
14. Garoff, L., Pietsch, F., Huseby, D. L., Lilja, T., Brandis, G., and Hughes, D. (2020). Population bottlenecks strongly influence the evolutionary trajectory to fluoroquinolone resistance in *Escherichia coli*. Molecular biology and evolution, 37(6), 1637-1646
15. Imamovic, L., and Sommer, M. O. (2013). Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. Science translational medicine, 5(204), 204ra132-204ra132.
16. Jiao, Y. J., Baym, M., Veres, A., and Kishony, R. (2016). Population diversity jeopardizes the efficacy of antibiotic cycling. BioRxiv, 082107.
17. Karslake, J., Maltas, J., Brumm, P., and Wood, K. B. (2016). Population density modulates drug inhibition and gives rise to potential bistability of treatment outcomes for bacterial infections. PLoS computational biology, 12(10), e1005098.
18. 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. Journal of evolutionary biology, 28(5), 1131-1143.
19. Kim, S., Lieberman, T. D., and Kishony, R. (2014). Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. Proceedings of the National Academy of Sciences, 111(40), 14494-14499.
20. Kohanski, M. A., Dwyer, D. J., and Collins, J. J. (2010). How antibiotics kill bacteria: from targets to networks. Nature Reviews Microbiology, 8(6), 423-435.
21. Kumar, A., & Schweizer, H. P. (2005). Bacterial resistance to antibiotics: active efflux and reduced uptake. Advanced drug delivery reviews, 57(10), 1486-1513.
22. Lachapelle, J., Reid, J., and Colegrave, N. (2015). Repeatability of adaptation in experimental populations of different sizes. Proceedings of the Royal Society B: Biological Sciences, 282(1805), 20143033.

23. Lee, H. H., Molla, M. N., Cantor, C. R., and Collins, J. J. (2010). Bacterial charity work leads to population-wide resistance. Nature, 467(7311), 82-85.
24. Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. Science, 355(6327), 826-830.
25. MacLean, R. C., and Buckling, A. (2009). The distribution of fitness effects of beneficial mutations in *Pseudomonas aeruginosa*. PLoS Genet, 5(3), e1000406.
26. MacLean, R. C., Hall, A. R., Perron, G. G., and Buckling, A. (2010). The population genetics of antibiotic resistance: integrating molecular mechanisms and treatment contexts. Nature Reviews Genetics, 11(6), 405-414.
27. Mahrt, N. (2020). Periodic bottlenecks in experimental antibiotic resistance evolution of *Pseudomonas aeruginosa* (Doctoral dissertation).
28. Maltas, J., McNally, D. M., and Wood, K. B. (2021). Evolution in alternating environments with tunable interlandscape correlations. Evolution, 75(1), 10-24.
29. Martinez, M. N., Papich, M. G., and Drusano, G. L. (2012). Dosing regimen matters: the importance of early intervention and rapid attainment of the pharmacokinetic/pharmacodynamic target. Antimicrobial agents and chemotherapy, 56(6), 2795-2805.
30. Pál, C., Papp, B., and Lázár, V. (2015). Collateral sensitivity of antibiotic-resistant microbes. Trends in microbiology, 23(7), 401-407.
31. Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. AIMS microbiology, 4(3), 482.
32. Rodriguez de Evgrafov, M., Gumpert, H., Munck, C., Thomsen, T. T., and Sommer, M. O. (2015). Collateral resistance and sensitivity modulate evolution of high-level resistance to drug combination treatment in Staphylococcus
33. Römhild, R., and Schulenburg, H. (2019). Evolutionary ecology meets the antibiotic crisis: Can we control pathogen adaptation through sequential therapy?. Evolution, medicine, and public health, 2019(1), 37-45.
34. Sniegowski, P. D., and Gerrish, P. J. (2010). Beneficial mutations and the dynamics of adaptation in asexual populations. Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1544), 1255-1263.

35. Sun, J., Deng, Z., & Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochemical and biophysical research communications, 453(2), 254-267.
36. Turnidge, J., Kahlmeter, G., and Kronvall, G. (2006). Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clinical Microbiology and Infection, 12(5), 418-425.
37. Udekwu, K. I., Parrish, N., Ankomah, P., Baquero, F., and Levin, B. R. (2009). Functional relationship between bacterial cell density and the efficacy of antibiotics. Journal of antimicrobial chemotherapy, 63(4), 745-757.
38. Webber, M. A., and Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. Journal of antimicrobial chemotherapy, 51(1), 9-11.
39. Windels, E. M., Fox, R., Yerramsetty, K., Krouse, K., Wenseleers, T., Swinnen, J., and Michiels, J. (2021). Population bottlenecks strongly affect the evolutionary dynamics of antibiotic persistence. Molecular Biology and Evolution.
40. Yen, P., and Papin, J. A. (2017). History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. PLoS biology, 15(8), e2001586.