Investigating the role of fluctuations and complexity in shaping the evolutionary dynamics of laboratory populations of *E coli*

BS – MS Thesis

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Under the guidance of

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CERTIFICATE

This is to certify that this dissertation entitled “Investigating the role of fluctuations and complexity in shaping the evolutionary dynamics of laboratory populations of *E coli*” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the research carried out by Agrim Saini at IISER Pune under the supervision of Dr. Sutirth Dey, Associate Professor, Biology Department during the academic year 2015 – 2016.

Signature of the Supervisor Date: 28th March, 2016

(Dr Sutirth Dey)

DECLARATION

I hereby declare that the matter embodied in the report entitled “Investigating the role of fluctuations and complexity in shaping the evolutionary dynamics of laboratory populations of *E coli*” are the results of the investigations carried out by me at the Department of Biology, IISER Pune, under the supervision of Dr. Sutirth Dey and the same has not been submitted elsewhere for any other degree.

Signature of the student

Date: 28th March, 2016

(Agrim Saini)

Abstract

In spite of the multitude of studies that deal with the effect of fluctuating environments on bacterial populations, how fluctuations and complexity (multiple stresses silmultaneously) interact with each other is poorly studied. To investigate the interactions of complexity and fluctuations, different combination selection regimes such as Simple Predictable, Simple Unpredictable, Complex Predictable, and Complex Unpredictable were designed and bacterial populations were evolved in these selection regimes for approximately 300 generations. The fitness in terms of growth rate (r) and carrying capacity (K) of these evolved populations were assayed in different novel and component environments. No significant fitness difference was detected between different selection treatments in the novel environments. However, in component environments, Simple Predictable selection treatment showed the highest fitness and Complex Unpredictable had the lowest overall fitness. In general, predictable fluctuations had higher fitness than the unpredictable fluctuations and simple selection treatments (which faced one stress at a time) performed better than the complex selection treatments (which faced two stresses at a time).

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Introduction

In nature, temporally fluctuating stressful environments are faced routinely by different organisms. The environment can either consist of a single stress (simple environment) or multiple stresses (complex environment) changing simultaneously. Moreover, temporal fluctuations can either be predictable or unpredictable. Experimental evolution in laboratory, which uses organisms with shorter generation times, is an excellent way to simulate such temporally fluctuating environments and study their long term evolutionary effects (Kassen 2002). Therefore, there have been many studies that document the evolutionary effects of temporally varying environments on laboratory populations of microorganisms. Studies involving laboratory evolution experiments have demonstrated that predictably fluctuating environments lead to the evolution of generalists (organisms which are adapted to wide range of environments). Constant environments on the other hand, promote the evolution of specialists – organisms with narrower niche width (Ketola et al., 2013; Condon et al., 2013; Kassen 2002). There has also been comparison between the evolutionary outcomes of predictable and stochastic fluctuations suggesting that predictable fluctuations provide higher fitness advantage than the stochastic fluctuations (Alto et al., 2013) and produce superior generalists (Hughes et al., 2007). Additionally, it has been shown that populations evolved in complex environments (containing more than one substitutable resource) were able to adapt to several substrates simultaneously and were neither complete specialists nor complete generalists but “jack of all, master of some” (Barrett et al., 2005). These studies being insightful, fail to describe the complete picture because they deal with only one axis at a time, either fluctuations or complexity. Moreover, most of these studies only deal with fluctuations of a single environment – temperature (Ketola et al., 2013) or pH (Hughes et al., 2007).

In spite of the substantial corpus of studies, how predictability and complexity of environment interact with each other, remains poorly investigated. This is a more

realistic scenario as fluctuations and complexity are intertwined in natural environments. However, a recent study published by Karve et al (2015) have observed that bacterial populations selected in unpredictably fluctuating complex stressful regime composed of pH, salt, and peroxide for 170 generations show fitness advantage in uncorrelated novel environments when compared to the control populations that were selected in nutrient broth. Moreover, the evolved populations did not show any fitness advantage in any of the three selection environments (Karve et al., 2015).

However, it is not clear whether the fitness advantage observed in the novel environments is because of the complexity of the selection regime, unpredictability or a combination of both. Moreover, it is relevant to figure out how these two axes – fluctuations and complexity shape the evolutionary dynamics of bacterial populations and affect their fitness in selection as well as novel environments.

Here, we designed different selection regimes that represent different combinations of fluctuations and complexity along with relevant controls. Replicate bacterial populations were evolved in these selection regimes for approximately 300 generations. The fitness of the evolved populations were assayed in different abiotic novel and selection environments.

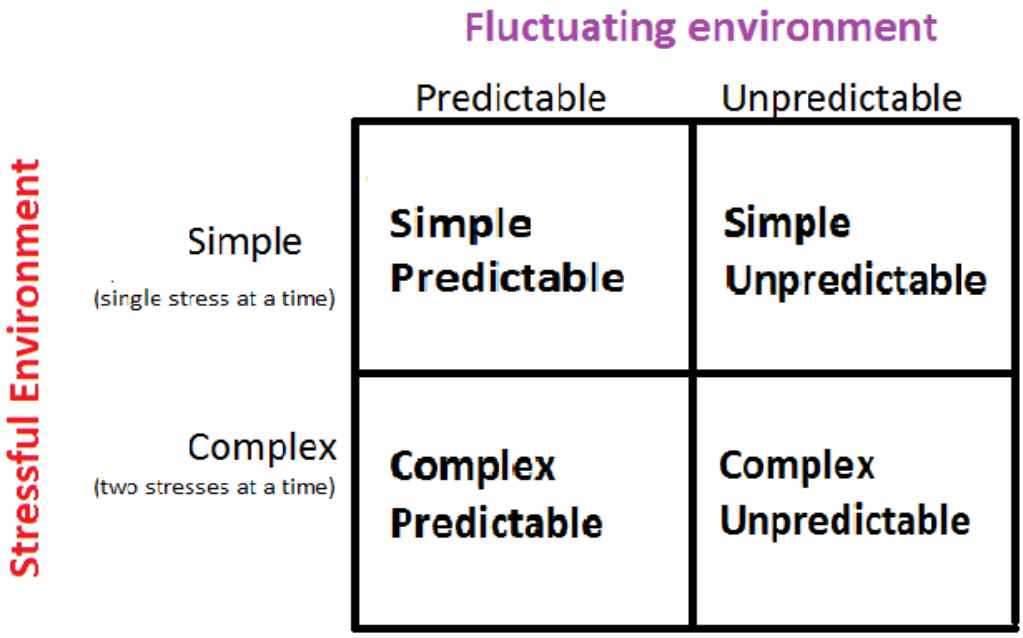

Figure 1: The different selection regimes that were designed.

Materials and Methods

Choice and standardisation of abiotic stresses

Two abiotic stresses were required for the study. Abiotic stresses with broad spectrum of activity but without any antagonistic interactions with each other were considered. By using previous laboratory studies (unpublished data) and relevant literature screening, four abiotic stresses were considered initially: CTAB, (a cationic detergent) SDS, (an anionic detergent) pH, (acidic and basic) and Silver Nitrate. Eventually, CTAB was dropped due to logistic reasons. Pilot studies were done using pH - SDS and Silver Nitrate - SDS as two stressful combinations. Populations of a kanamycin resistant *E coli* MG1655 strain were exposed to a gradient of concentrations of these stresses separately as well as in the combinations mentioned above. 100µL of bacterial volume was inoculated form the glycerol stock in 50mL of NB with relevant stress. The populations were allowed to grow for 24 hour in 100mL conical flasks grown at 37°C and 150rpm. Growth rate (r) (Ketola et al., 2013; Karve et al., 2015) and carrying capacity (K) (highest density reached in 24 hours of growth trajectory) were measured as fitness to estimate the inhibitory action of different concentrations of the stresses. The aim was to determine sub lethal environmental values i.e. values which were stressful but not inhibitory in both simple (one stress) and complex (two stresses) scenarios. Since, the stresses did not have antagonistic effects, a complex combination of two stresses was always more stressful than the subsequent single stresses. Therefore, meeting the above mentioned condition of similar values for the both simple and complex treatments was elusive and could not be achieved in the case of pH. For example, a pH of 9 in conjugation with 0.25% of SDS was lethal/fatal whereas a pH of 9 alone supported the growth comparable to the control. As a result of this, the pH – SDS combination was dropped. Nevertheless, two sub-lethal values each for SDS and Silver Nitrate

were successfully determined that were stressful in both simple and complex scenarios. Therefore, Silver Nitrate (SN) and Sodium Dodecyl Sulphate (SDS) were chosen as stresses for the studies with following sub – lethal concentration values.

SN – 0.08M and 0.112M

SDS – 0.5% and 2% (w/v)

In the pilot studies mentioned above, the individual stress values and their complex combinations (0.08M SN, 0.5% SDS), (0.08M SN, 2% SDS), (0.112M SN, 0.5% SDS), and (0.112M SN, 2% SDS) were all tested for their inhibition and all the environments were sub – lethal, though to the varying degree.

Selection regimes

After determining relevant stressful values, (two for each stress) different selection regimes were designed. The selection experiment was designed for 60 days with sub culturing after every 24 hours.

A sequence of the four stress values (0.08M → 0.112M → 2% → 0.5%) was iterated 15 times to generate the Simple Predictable (SP) selection regime of 60 days in which each stress value appeared for equal number of days i.e. 15 (the arrow represents sub – culturing)

A random sequence of the same four stress values was generated using a random number generator for 60 days such that the values appeared randomly but each value appeared for equal number of times. This selection regime was labelled as Simple Unpredictable (SU).

The four Silver – SDS stressful combinations standardized were arranged in a sequence [(0.112M,0.5%) → (0.08M,0.5%) → (0.08M,2%) → (0.112M,2%)] and this sequence was iterated 15 to generate the Complex Predictable (CP) selection regime. Every combination appeared equal number of times.

A random order of the four Silver – SDS stressful combinations was generated such that each combination appeared for equal number of times but randomly for 60 days. This selection regime was labelled as the Complex Unpredictable (CU) selection regime.

Note: The sequences described in the SP and CP regimes are the ones for which no extinction was observed in the first four days, during which the populations got exposed to every environment sequentially for the first time. In case of any extinction event before the 4^{th} day, I generated a new sequence and restarted the selection. Similarly, in the case of SU and CU, in case of any extinction in the first 5 days, (before the making of the first stocks) it was decided to generate a new random sequence of the environments and restart the selection. Such extinction events happened only once.

Apart from the above mentioned fluctuating selection regimes, three control selection regimes were also designed. Two stressful constant selection regimes were designed, one with a constant environment of 0.112M SN (SIL treatment) and another with a constant environment of 2% SDS (SDS treatment). Finally a selection regime was designed with no stress and thus, 60 days of selection in plain NB. This selection treatment was labelled as NB line.

Selection Protocol

Kanamycin resistant *E coli* strain (MG1655) was used for the study. Thus, kanamycin was always present in the medium to prevent growth of any contaminant. The stored glycerol stock of the strain was inoculated for 18 – 20 hours. This revived culture was used to start the different selection treatments. The same culture was also stored as the ancestral population at -80^0C. Each selection treatment consisted of five biological replicates (populations that faced the same treatment). Thus, a total of 35 populations for seven different selection treatments were evolved. The culture volume was 50mL. Each population was grown in 100mL conical flasks and maintained at 37°C and 150 rpm throughout the selection experiment. Sub culturing was done after every 24 hour. 1mL out of the 50mL grown population was inoculated into a fresh medium with corresponding environmental conditions. Selected populations were stored in 15% glycerol at -80^0C after every 5^{th} day of the selection

for future fitness measurements. 14 randomly chosen populations out of 35 were plated to check for contamination. This was done thrice during the entire duration of the selection. The populations were plated on Nutrient Agar plates with Kanamycin to double check for any contamination. No contamination was observed. In case of any extinction event, which was defined as lack of visible growth in the flask after 24 hours, the selection of the corresponding population was restarted from the previous day's glycerol stock. The glycerol stock was revived in NB for 18 – 20 hours and was subsequently transferred into the corresponding selection environment. Keeping in mind the light sensitive nature of the SN solution, proper care was taken to minimally expose the populations to bright light during the entire duration of the selection. Because of the strong death phase (sharp decrease in optical density) in the growth trajectory of the populations caused by the action of SDS, the measurement of the optical density before every sub – culturing was not done, as it would not have helped in calculating the number of generations. The selection was continued for 55 days i.e. ~300 generations (Bennett and Lenski, 1997).

At the end of the selection, populations were inoculated in plain NB for 18 – 20 hours and used for fitness measurements in different abiotic environments. Moreover, the final selection cultures were also stored in the form of glycerol stocks at -80°C for future fitness measurements.

Fitness measurements of bacterial populations

The selected populations were assayed for their fitness in different abiotic environments. Fitness was measured as the maximum growth rate of the populations (Ketola et al., 2013; Karve et al., 2015) and the carrying capacity of the same. Growth assays were done in different novel (stresses never faced by the populations) and component (stresses faced during the selection) stresses. Selected populations were revived in plain NB for 18 – 20 hours (mentioned in the previous section). These revived cultures were then used to initiate the growth assays. 4µL revived culture was inoculated in 2mL of NB containing the relevant stress. Fitness of each population was assayed twice in every assay environment. Growth assays were done in 24 well plates which were incubated at 37°C and 150 rpm and optical density was measured on a plate reader (Synergy HT BioTek,

Winooski, VT, USA) after every one hour at 600nm. The optical density was measured till a particular population was saturated. Thus, in case of novel treatments and treatments with silver, the OD was measured till the population reached the saturation phase (constant OD for more than 2 hours). In case of SDS treated populations, OD was measured till 4 – 5 hours post death phase. The experiment was terminated at the 36th hour for the populations that did not grow.

a. Fitness measurements in component environments

Fitness measurements were also done in different component environments. These are the environments that were the part of the different selection treatments. Fitness measurements were done in 0.08M Silver, 0.112M Silver, 0.5% SDS, 2% SDS, and NB. These values were used to design the Simple Predictable (SP) and Simple Unpredictable (SU) selection regimes and thus they will be referred as Simple selection environments.

Similarly, fitness measurements were also done in (0.08M, 0.5%), (0.08M, 2%), (0.112M, 0.5%), and (0.112M, 2%). These four combinations were used to design the CP and CU selection regimes and thus will be referred as the complex selection environments.

Therefore, all the evolved populations were assayed for their fitness in the above mentioned nine component environments.

b. Fitness measurements in novel environments

Fitness measurements were done in multiple novel environments. The novel environments chosen for the fitness assays were not encountered by the bacterial populations during the selection. Moreover, these stresses have different mechanisms of action as compared with the component stresses (silver and SDS). Lastly, the multiple novel environments used were uncorrelated to each other as well in terms of their mechanism of actions.

Previous range estimation studies (data nor published) were screened to select the novel stresses. Antibiotics were chosen as the novel stresses. Pilot

studies were performed to standardize a sub-lethal concentration for each novel stress. Ancestral population was used for the standardization.

Finally, Norfloxacin, Rifampicin, and Chloramphenicol were chosen as the novel stresses. Norfloxacin is known to attack DNA Gyrase, an enzyme involved in DNA replication (Drlica and Zhao, 1997). Chloramphenicol inhibits protein synthesis by binding to the 50S ribosomal unit (Wolfe and Hahn, 1965). Rifampicin inhibits transcription by inhibiting RNA polymerase (Calvori et al., 1965).

Fitness estimation

The fitness was estimated using the growth trajectory obtained during the assay in relevant environment. Growth rates (r) and carrying capacities (K) were computed using these growth trajectories. A PYTHON script was used to determine the r and the K of the bacterial populations. The program fits a straight line on overlapping moving windows of four points on the time series data of the optical density values (growth trajectories). The maximum slope obtained through this method was taken as the growth rate of the population. The same script was also used to determine the carrying capacity (K) of the populations. The maximum optical density attained in a growth trajectory was taken as the carrying capacity of the population. Both r and K were used as the measure of fitness for all the populations and in all the tested environments. As mentioned above, each population was measured for the fitness in duplicates. The fitness values of the two measurements were averaged to obtain a single fitness value for each population in each assayed environment. This was done for both r and K measurements. Thus, a total of five fitness values for each r and K represented a selection treatment in an environment.

Statistical Design

a. <u>Component and novel environments</u>

   Pooled data of the fitness (r and K) was analysed using a two way ANOVA. Selection (seven levels: SP, SU, CP, CU, SIL, SDS, and NB) and assay environment were the two fixed factors. Assay environment had nine levels [0.08M SN, 0.112M SN, 0.5% SDS, 2% SDS, (0.08M, 0.5%), (0.08M, 2%),

(0.112M, 0.5%), (0.112M, 2%), and NB] for component environments and three levels (NOR, CHL, and RIF) for novel environments. Separate ANOVA's were done for both r and K.

In case of component environments, fitness of the selection treatments was also analysed separately for simple component environments (0.08M SN, 0.112M SN, 0.5% SDS, and 2% SDS) and complex component environments [(0.08M SN, 0.5% SDS), (0.08M SN, 2% SDS), (0.112M SN, 0.5% SDS), and (0.112M SN, 2% SDS)]. Separate two way ANOVA's were done for both simple and complex component environments.

Similarly, to determine the effects of the fluctuations and complexity on the fitness (r and K), a three way ANOVA was done taking fluctuations (two levels: predictable and unpredictable), complexity (two levels: simple and complex) and assay environment as the fixed factors. As mentioned above, assay environment had nine levels for component environment assays and three levels for novel environment assays.

The effects of fluctuations and complexity were also checked in simple component environments (0.08M silver, 0.112M silver, 0.5% SDS, and 2% SDS) and complex component environments [(0.08M, 0.5%), (0.08M, 2%), (0.112M, 0.5%), (0.112M, 2%)] separately. For this, two separate 3 - way ANOVA's were done, one for the simple component environments and one for the complex component environments. The selection was the common fixed factor in both whereas the assay environment was simple component environments for the first ANOVA whereas it was complex component environment for the other one. Cohen's *d* (Cohen 1988) was also computed as a measure of the effect size to determine the strength of the significance. It was interpreted as small, medium, and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$, and $d > 0.8$, respectively.

Results

Fitness in component environments

Selection affected the fitness (both r and K) significantly in the ANOVA pooled across all the nine component environments ($F_{6,252}$ = 13.294, p < 0.001 for r and $F_{6,252}$ = 26.09, p < 0.001 for K). (Table 1) (Fig 2). Moreover, assay environment i.e. component environments in this case also had a significant effect ($F_{8,252}$ = 9.776, p <0.001 for r and $F_{8,252}$ = 163.67, p < 0.001 for K). This is expected as the component environments had both simple component and complex component environments which are likely to affect the populations in different ways. Similarly, selection × assay environment also had a significant effect ($F_{48,252}$ = 2.909, p < 0.001 for r and $F_{48,252}$ = 4.5, p < 0.001 for K) suggesting that the selection treatments responded differently to different component environments. (Table 1)

The ANOVA pooled across all the simple component environments also showed significant effect of the selection on the fitness ($F_{6,140}$ = 6.033, p < 0.001 for r and $F_{6,140}$ = 15.01, p <0.001 for K). However, the effect of assay environment (simple component environments) was not significant in case of r ($F_{4,140}$ = 2.175, p = 0.0749) but significant for K ($F_{4,140}$ = 163.56, p <0.001). This informs that the five simple component environments were significantly different in the way they affected the carrying capacity and not the growth rate of the bacterial populations. Moreover, Selection × Assay interaction was also significant. ($F_{24,140}$ = 2.878, p < 0.001 for r and $F_{24,140}$ = 4.58, p < 0.001 for K). (Table 3)

The ANOVA pooled across all the complex component environments also showed significant effect of the selection on the fitness ($F_{6,112}$ = 15.491, p < 0.001 for r and $F_{6,112}$ = 22.72, p < 0.001). Similarly, the assay environment (complex component environments) also significantly affected the fitness ($F_{3,112}$ = 14.716, p < 0.001 for r and $F_{3,112}$ = 21.126, p < 0.001 for K). The selection × assay interaction was non – significant in case of r ($F_{18,112}$ = 1.617, p = 0.0675) but significant in case of K ($F_{18,112}$ = 2.004, p = 0.0148). (Table 1)

Since, selection had a significant effect on the overall fitness in all the three 2- way ANOVA's performed, Tukey post hoc test was performed to determine the pair-wise significance of the different selection treatments in all the ANOVA's (Table 2)

ANOVA	**Effect**	**Dependent**	**F - statistic**	**p values**

		Factor		
All component environments	Selection	r	$F_{6,252} = 13.294$	< 0.001
		K	$F_{6,252} = 26.09$	< 0.001
	Assay environment	r	$F_{8,252} = 9.776$	< 0.001
		K	$F_{8,252} = 163.67$	< 0.001
	Selection × Assay	r	$F_{48,252} = 2.909$	< 0.001
		K	$F_{48,252} = 4.5$	< 0.001
Simple component environments	Selection	r	$F_{6,140} = 6.033$	< 0.001
		K	$F_{6,140} = 15.01$	< 0.001
	Assay environment	r	$F_{4,140} = 2.175$	0.0749
		K	$F_{4,140} = 163.56$	< 0.001
	Selection × Assay	r	$F_{24,140} = 2.878$	< 0.001
		K	$F_{24,140} = 4.58$	< 0.001
Complex component environments	Selection	r	$F_{6,112} = 15.491$	< 0.001
		K	$F_{6,112} = 22.72$	< 0.001
	Assay environment	r	$F_{3,112} = 14.716$	< 0.001
		K	$F_{3,112} = 21.126$	< 0.001
	Selection × Assay	r	$F_{18,112} = 1.617$	0.0675
		K	$F_{18,112} = 2.004$	0.0148

Table 1: Summary of the three 2-way ANOVA's performed for the component environments.

Note: Separate ANOVA's were done for r and K. Similarly, a main ANOVA was done pooled over all the component environments followed by two other ANOVA's in simple component and complex component separately (all on the same data set, only the levels of environment variable changed). Since, r and K were not compared in the study and the results of different ANOVA's were interpreted separately, there was no need of controlling for family wise error and hence no correction were performed.

ANOVA	Approximate probabilities of the post hoc test (both r and K)							
All component environments	**SP (r, K)**	**SP**	**SU**	**CP**	**CU**	**SIL**	**SDS**	**NB**
	SU	ns 0.003						
	CP	ns, ns	ns, ns					
	CU	<0.001 <0.001	0.0192 0.0217	0.0162 <0.001				
	SIL	<0.001 <0.001	<0.001 <0.001	<0.001 <0.001	ns, 0.0135			
	SDS	ns, ns	ns, 0.049	ns, ns	0.0032 <0.001	<0.001 <0.001		
	NB	0.0092 0.0012	ns, ns	ns, ns	ns, 0.044	<0.001 <0.001	ns, 0.025	
Simple component environments	**SP (r, K)**	**SP**	**SU**	**CP**	**CU**	**SIL**	**SDS**	**NB**
	SU	ns, ns						
	CP	ns, ns	ns, ns					
	CU	ns, <0.001	0.0074 <0.001	ns, <0.001				
	SIL	<0.001 <0.001	<0.001 <0.001	0.0103 <0.001	ns, ns			
	SDS	ns, ns	ns, ns	ns, ns	ns, <0.001	ns, <0.001		
	NB	ns, ns	ns, ns	ns, ns	ns, <0.001	0.0017 <0.001	ns, ns	
Complex component environments	**SP (r, K)**	**SP**	**SU**	**CP**	**CU**	**SIL**	**SDS**	**NB**
	SU	0.0011 <0.001						
	CP	ns, ns	ns, ns					
	CU	<0.001 <0.001	ns, ns	ns, ns				
	SIL	<0.001 <0.001	ns, 0.0094	<0.001 <0.001	ns, 0.0116			
	SDS	ns, ns	<0.001 <0.001	ns, ns	<0.001 <0.001	<0.001 <0.001		
	NB	<0.001 <0.001	ns, ns	0.0285, ns	ns, ns	ns, ns	<0.001 <0.001	

Table 2: Tukey post hoc pairwise interaction probabilities for both r and K for the different ANOVA's done. "ns" means non-significant probability value.

When pooled across all the component environments, the average fitness (both r and K) of the SP selection treatment was highest among all the selection treatments with significantly higher than CU, SIL, and NB treatment. However, SP had non-significantly higher fitness than SU, CP, and SDS selection treatments (Table 2, Table 3, Figure 2) Surprisingly, SP selection treatment also had significantly higher fitness (both r and K) than SU, CU, SIL, and NB fitness in complex component environments which were not faced by this selection treatment during the selection (Table 2) The mean fitness of all the selection treatments pooled over all the component environments is summarised in Table 3.

	SELECTION	**AVG r**	**SEM**	**AVG K**	**SEM**
All component environments	SP	0.1952	0.006	0.893	0.0339
	SU	0.177	0.009	0.8022	0.04
	CP	0.1775	0.0079	0.836	0.0303
	CU	0.1475	0.0075	0.7248	0.0398
	SIL	0.1262	0.0083	0.644	0.0519
	SDS	0.1816	0.0066	0.8731	0.0339
	NB	0.1637	0.0095	0.7967	0.0507
Simple component environments	SP	0.1972	0.0076	1.0138	0.0444
	SU	0.2052	0.0118	0.9774	0.0423
	CP	0.1874	0.0129	0.9559	0.0368
	CU	0.1588	0.0097	0.84	0.055
	SIL	0.1421	0.0112	0.7968	0.0756
	SDS	0.1717	0.0092	0.9431	0.0531
	NB	0.1936	0.012	1.0307	0.0497
Complex component environments	SP	0.1928	0.0096	0.7421	0.0269
	SU	0.1417	0.0089	0.5832	0.0312
	CP	0.1651	0.0069	0.6861	0.0229
	CU	0.1333	0.0113	0.5808	0.0386
	SIL	0.1064	0.0113	0.4529	0.0394
	SDS	0.1939	0.0087	0.7855	0.0281
	NB	0.1264	0.0127	0.5402	0.0369

Table 3: Mean r and K (±SE) of the selection treatments in component environments. Table 2 can be referred to know the significant pairwise differences.

To determine the effects of fluctuations and complexity on the fitness of the evolved populations, a 3 way ANOVA was done with fluctuations, complexity, and assay environment (all component environments) as the fixed factors.

The growth rate (r) and the carrying capacity (K) of the simple selection treatments (SP and SU) were significantly higher than the complex selection treatments (CP and CU) ($F_{1,144}$ = 13.357, p < 0.001 for r and $F_{1,144}$ = 15.514, p < 0.001 for K) when pooled across all the nine component environments with small effect size. Similarly, the r and K of the predictable selection treatments (SP and CP) were significantly higher than the unpredictable selection treatments (SU and CU) ($F_{1,144}$ = 13.883, p < 0.001 for r and $F_{1,144}$ = 35.004, P < 0.001 for K) when pooled across all the component environments with small effect size. (Table 4 and Figure 3)

This effect was further studied by performing two separate 3 way ANOVA's for simple component environments and complex component environments. Fluctuations and complexity were the two fixed factors that were common for both the ANOVA's whereas the assay environment was simple component environments (five levels) and complex component environments (four levels) for the two ANOVA's respectively.

When pooled across all the simple component environments, the r and K of the Simple selection treatments were significantly higher than the Complex selection treatments ($F_{1,80}$ = 8.22, p = 0.0053 for r and $F_{1,80}$ = 18.126, p < 0.001 for K). In terms of fluctuations, predictably fluctuating selection treatments had higher r and K than the unpredictable fluctuating selection treatments. However, the difference was non – significant in case of r ($F_{1,80}$ = 1.09, p = 0.2996) but significant in case of K ($F_{1,80}$ = 11.013, p = 0.0014). (Table 4)

When pooled across all the complex component environments, Simple selection treatments had higher r and K than the complex selection treatments. However, in this case the difference was significant for r ($F_{1,64}$ = 5.2918, p=0.0247) but non – significant for K ($F_{1,64}$ = 1.305, p = 0.2576). Predictably fluctuating selection treatments had significantly higher r and K than the unpredictably fluctuating selection treatments ($F_{1,64}$ = 27.922, p < 0.001 for r and $F_{1,64}$ = 26.712, p < 0.001 for K). (Table 4)

ANOVA	Effect			Mean	F values	ANOVA p values	Cohen's d	Inference
All component environments	Complexity	Simple (SP & SU)	r	0.1861	$F_{1,144}$ = 13.357	<0.001	0.45	Small
			K	0.8476	$F_{1,144}$ = 15.514	<0.001	0.27	Small
		Complex (CP & CU)	r	0.1625				
			K	0.7804				
	Fluctuations	Predictable (SP & CP)	r	0.1863	$F_{1,144}$ = 13.883	<0.001	0.46	Small
			K	0.8645	$F_{1,144}$ = 35.004	<0.001	0.41	Small
		Unpredictable (SU & CU)	r	0.1622				
			K	0.7635				
Simple component environments	Complexity	Simple (SP & SU)	r	0.2012	$F_{1,80}$ = 8.22	0.0053	0.52	Medium
			K	0.9956	$F_{1,80}$ = 18.126	<0.001	0.43	Small
		Complex (CP & CU)	r	0.1731				
			K	0.898				
	Fluctuations	Predictable (SP & CP)	r	0.1923	$F_{1,80}$ = 1.09	0.2996	0.18	
			K	0.9848	$F_{1,80}$ = 11.013	0.0014	0.33	Small
		Unpredictable (SU & CU)	r	0.182				
			K	0.9087				
Complex component environments	Complexity	Simple (SP & SU)	r	0.1672	$F_{1,64}$ = 5.2918	0.0247	0.39	Small
			K	0.6626	$F_{1,64}$ = 1.305	0.2576	0.19	
		Complex (CP & CU)	r	0.1492				
			K	0.6334				
	Fluctuations	Predictable (SP & CP)	r	0.1789	$F_{1,64}$ = 27.922	<0.001	0.99	Large
			K	0.714	$F_{1,64}$ = 26.712	<0.001	0.98	Large
		Unpredictable (SU & CU)	r	0.1375				
			K	0.582				

Table 4: Summary of the main effects of the three 3 – way ANOVA's done with fluctuations, complexity, and assay environment as the fixed factors.

Note: Separate ANOVA's were done for r and K. Similarly, a main 3 way ANOVA was done pooled over all the component environments followed by two other ANOVA's in simple component and complex component separately (all on the same data set, only the levels of environment variable changed). Since, r and K were not compared in the study and the results of different ANOVA's were interpreted separately, there was no need of family wise error and hence it was not performed

Fitness in the novel environments

The ANOVA pooled across all the novel environments did not show any significant effect of selection on both r and K ($F_{6,84}$ = 1.1715, p = 0.3294 for r and $F_{6,84}$ = 1.913, p = 0.0881 for K) (Fig 4). However, there was a significant effect of the assay environment i.e. novel environments ($F_{2,84}$ = 6.2744, p = 0.0029 for r and $F_{2,84}$ = 3.323, p = 0.0408 for K). This is expected from the fact that the three novel environments are likely to affect the bacterial populations in different ways. There was also a non-significant selection × assay environment interaction ($F_{12,84}$ = 1.4597, p = 0.1561 for r and $F_{12,84}$ = 1.543, p = 0.1251 for K). Table 5 summarises the pooled ANOVA results. Since the pooled ANOVA did not give any significance, ANOVA's were not performed in separate novel environments.

Effect	**Dependent Factor**	**F - statistic**	**p values**
Selection	r	$F_{6,84}$ = 1.1715	0.3294
	K	$F_{6,84}$ = 1.913	0.0881
Assay	r	$F_{2,84}$ = 6.2744	0.0029
	K	$F_{2,84}$ = 3.323	0.0408
Selection × Assay	r	$F_{12,84}$ = 1.4597	0.1561
	K	$F_{12,84}$ = 1.543	0.1251

Table 5: Summary of the ANOVA pooled across all the novel environments

Predictable fluctuations (SP and CP) performed better than unpredictable fluctuations (SU and CU) when pooled across all the novel environments (Fig 5) However, the difference was non-significant in case of r ($F_{1,48}$ = 1.5661, p = 0.2168) but significant for K ($F_{1,48}$ = 5.966, p = 0.0183). The effect of complexity on the fitness was insignificant ($F_{1,48}$ = 1.8978, p = 0.1747 for r and $F_{1,48}$ = 0.392, ns for K). (Table 6 and Fig 5)

All the interaction terms of the ANOVA namely, Complexity × Fluctuations, Complexity × Assay, Fluctuations × Assay, and Fluctuations × Complexity × Assay were insignificant for both r and K.

Effect			Mean	SEM	$F_{1,48}$	ANOVA p values
Complexity	Simple (SP & SU)	r	0.1232	0.0081	1.8978	0.1747
		K	0.9716	0.0337	0.392	ns
	Complex (CP & CU)	r	0.1419	0.0121		
		K	0.9453	0.0314		
Fluctuations	Predictable (SP & CP)	r	0.1411	0.0112	1.5661	0.2168
		K	1.0098	0.0279	5.966	0.0183
	Unpredictable (SU & CU)	r	0.1240	0.0093		
		K	0.9072	0.0343		

Table 6: Main effects of Complexity and Fluctuations on both r and K pooled over all the novel environments.

Discussion

Fitness in the component environments

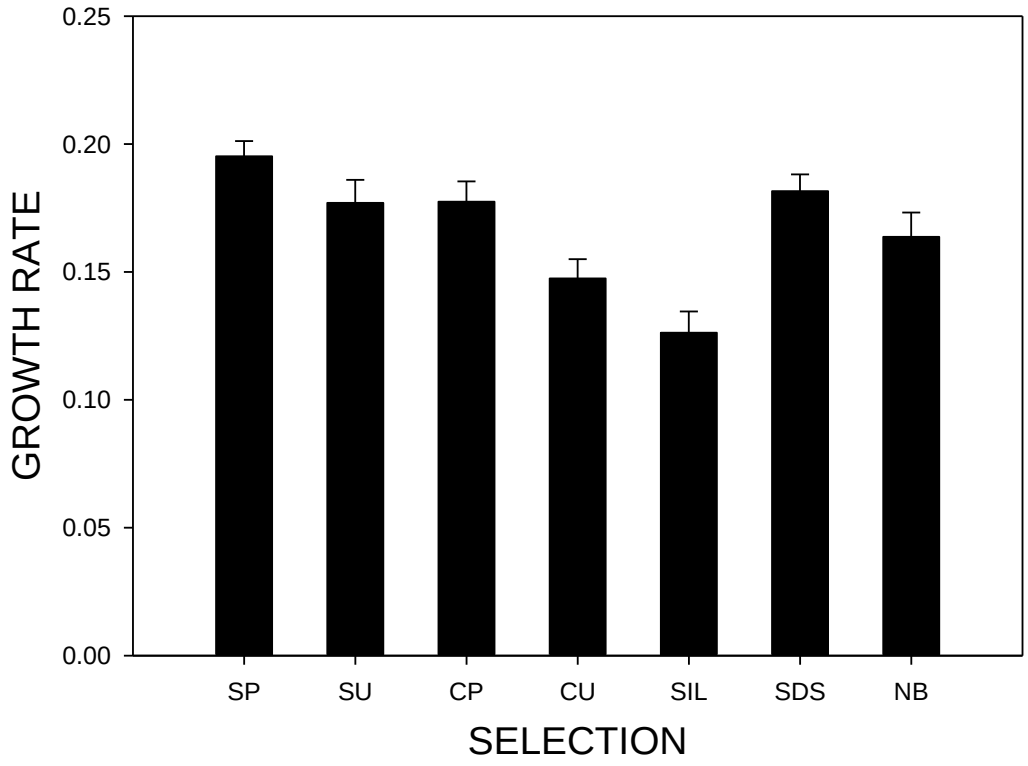

Figure 2 (A): **Mean (±SE) growth rate (*r*) of different selection treatments pooled across all the component environments. (**$p < 0.001$**)** CU is significantly different than the other selection treatments except SIL and NB. SIL is significantly different than the other selection treatments except CU. NB is significantly different than SP. All the other pair-wise interaction are non – significant. (Table 4) SP – Simple Predictable, SU – Simple Unpredictable, CP – Complex Predictable, CU – Complex Unpredictable, SIL – Constant Silver, SDS – Constant SDS, NB – Plain NB.

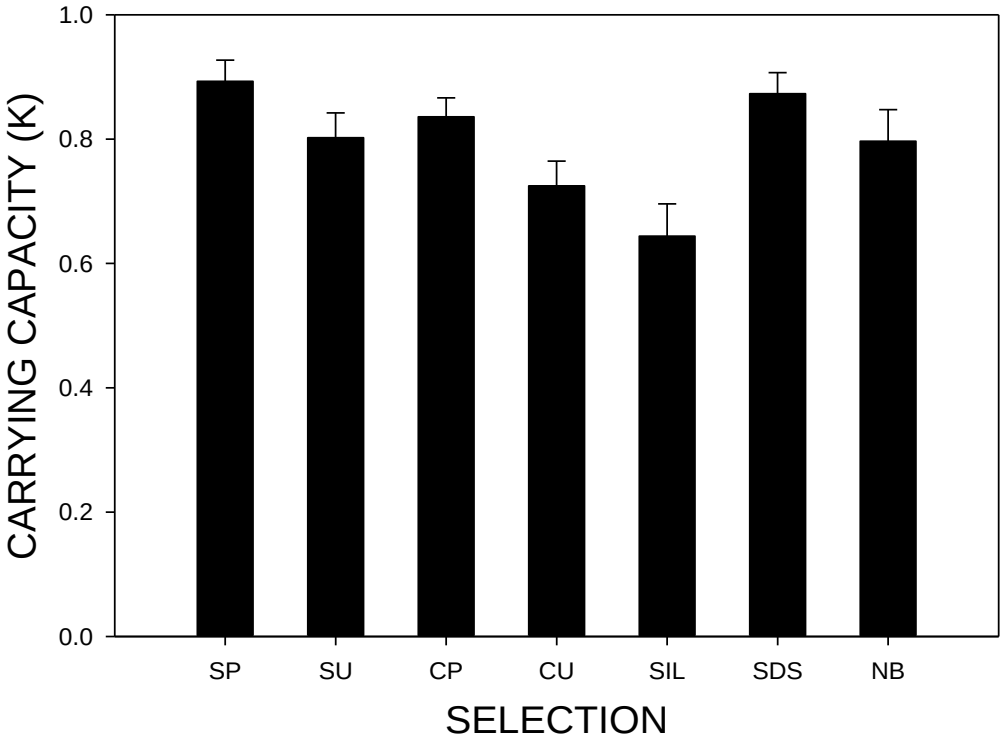

Figure 2 (B): **Mean (±SE) carrying capacity (K) of different selection treatments pooled across all the component environments. (**$p < 0.001$**)** CU is significantly different than all the other selection treatments. Similarly, SIL is also significantly different than all the other selection treatments. SP is significantly different than SU and the NB. SDS is significantly different than SU. NB is also significantly different than SDS. SP – Simple Predictable, SU – Simple Unpredictable, CP – Complex Predictable, CU – Complex Unpredictable, SIL – Constant Silver, SDS – Constant SDS, NB – Plain NB.

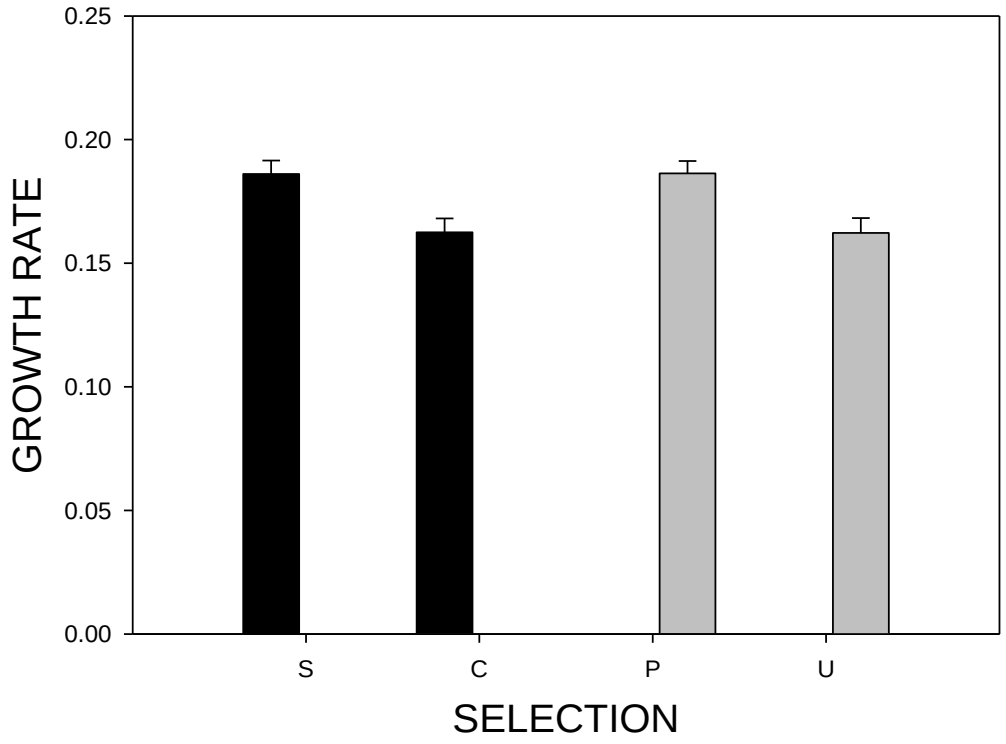

Figure 3 (A): **Mean (±SE) growth rate (r) of Simple (SP and SU), Complex (CP, CU), Predictable (SP and CP), and Unpredictable (SU and CU) selection treatments pooled over all the component environments.** The fluctuating selection treatments have been divided into these groups so as to compare the fitness effects of complexity and fluctuations. These four groups are overlapping in nature and are set on the same axis only for convenience. S must be compared to C and P must be compared to U only. ($p < 0.001$ for S and C and also for P and U)

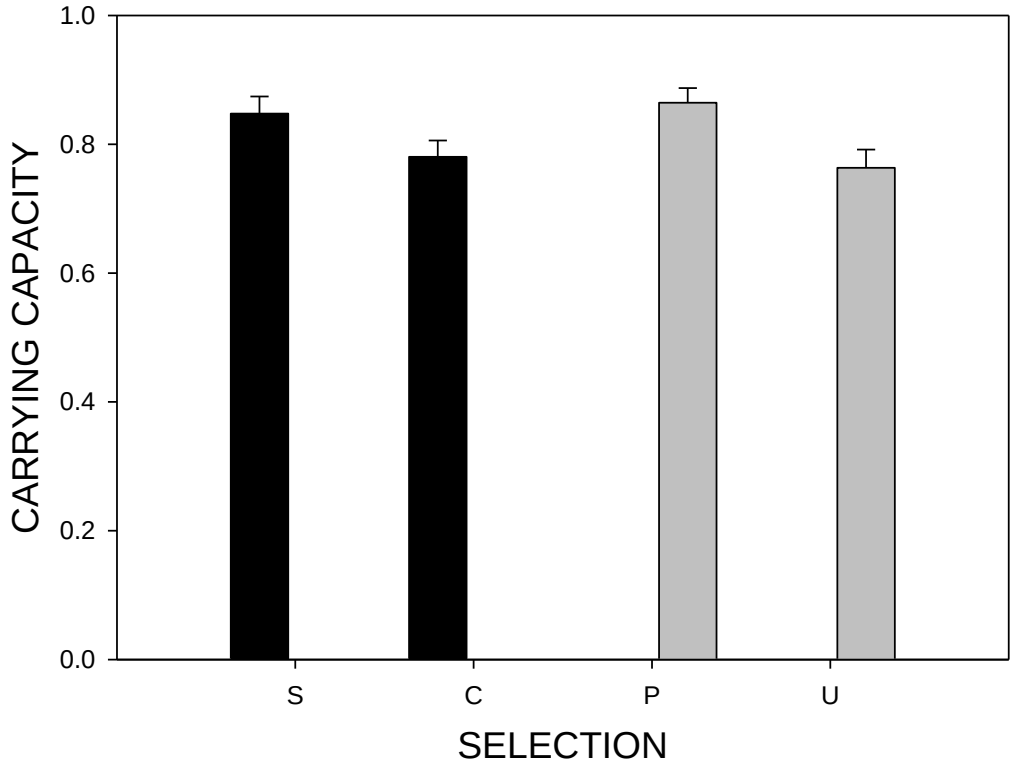

Figure 3 (B): **Mean (±) carrying capacity (K) of Simple (SP and SU), Complex (CP, CU), Predictable (SP and CP), and Unpredictable (SU and CU) selection treatments** ($p < 0.001$ for both S, C comparison and P, U comparison).

In component environments, the different selection treatments were significantly different than each other. When pooled across all the component environments, SP selection treatment had significantly higher fitness (both r and K) than CU and SIL selection treatments (Table 2, Fig 2). Moreover, the fitness of SDS and CP selection treatment is non-significantly different than the SP. Also. CU and SIL have significantly lower fitness than all the other selection treatments. CU and SIL are non – significantly different than each other. Thus, it can be seen that SP, CP, and SDS are the selection treatments with maximum fitness whereas CU and SIL have the least fitness when pooled over all the component environments. (Table 2, Fig 2) The other selection treatments lie in between these two groups in terms of their fitness.

However, during the selection, SP and SU only faced the simple component environments whereas CP and CU only faced the complex component environments. Therefore, it was expected that simple treatments (SP and SU) will

perform better in simple component environments and complex treatments (CP and CU) will perform better in complex component environments. To investigate this, the fitness of the selection treatments was analysed in simple component and complex component environments separately.

Contrary to the expectations, the pattern of fitness and the pairwise interaction between different selection treatments remained fairly constant when analysed in simple component and complex component environments separately (Table 2). SP, SDS, and CP again performed better than CU and D1 in both simple and complex component environments (Table 3). It was surprising to see that CU performed poorly as compare to the SP in complex component environments given that CU was selected for 300 generations in these environments. This indicates that SP, SDS, and CP actually improved their fitness in all the component environments.

The lowest fitness of SIL in all the component environments can be understood from the fact that it was only selected in 0.112M and assayed/analysed in all the component environments.

It is interesting to note that SDS being a constant selection regime which faced only 2% of SDS throughout the selection has the highest fitness comparable to that of SP (Table 2 and 3) . This hints towards the fact that SDS was a stronger component in all the selection regimes than the Silver Nitrate and had a much higher effect than the Silver Nitrate. This is possible as Silver Nitrate, the source of Silver ions in this study is known to release all the silver ions at once (Fox and Modak, 1974). Thus, it might act for shorter durations only. On the other hand, SDS dissolves the plasma membrane and tends to stay for longer duration of time. Therefore, it can play a major role in shaping the evolutionary dynamics of the bacterial populations. This can explain why SDS treatment has higher overall fitness over all the component environments.

The effect of fluctuations and complexity on fitness was further investigated taking only the fluctuating selection treatments into account (SP, SU, CP, and CU). In case of fluctuations, predictable fluctuations (SP and CP) had significantly higher r and K than the unpredictable fluctuations (SU and CU) over all the selection environments. (Figure 3). However, the effect size was small for this significant difference. (Table 4)

However, when analysed over all the complex component environments, predictable fluctuations still had higher r and K than the unpredictable selection treatments. However, the effect size in this case was large (Table 4). This means that most of the effect seen in the pooled analysis is coming from the complex component environments. It also informs that CP performed much better than CU in complex component environments (Table 4) and thus it highlights that predictable fluctuations are better than unpredictable fluctuations in improving fitness of the selected populations in the component environments. Previous studies have shown that populations selected in deterministically fluctuating environments evolve to perform better in their respective selection environments compared to the populations selected in stochastic environments (Alto 2013). Similarly, populations selected in predictable fluctuations are superior generalist than those selected in randomly fluctuating environments (Hughes 2007). However, these studies involves fluctuations of only one environment. In the present case, predictable fluctuations were more complicated and realistic than the ones described in the previous studies as it consisted of both Simple fluctuations (one stress at a time) and complex fluctuations (simultaneous fluctuations of both the stresses).

Moreover, the present study also demonstrates that simple selection treatments have significantly higher r and K than compared to the complex selection treatments across all the component environments. (Figure 3) However, the effect size is small (Table 4). This is in contrast to a previous study that has been published on complex environments which states that populations evolved in complex environments (more than one substitutable resources) were able to adapt to several substrates simultaneously (Barrett et al., 2005). Since the fluctuations were rapid and changed after every 6 generations, it is possible that the complex populations were not able to adapt to two stresses simultaneously. The unpredictable nature of the fluctuations in the case of CU further aggravated the adaptation. This is supported by the fact that CP also had high fitness comparable with that of SP.

Finally, combining the two facts of predictable being better than the unpredictable and simple being better than the complex supported by the fact that CP, SP had the highest fitness and CU had the lowest. Thus, from the present investigation, it seems that fluctuations, especially the predictable ones are far more important in shaping

the evolutionary dynamics of bacterial populations than complexity in component environments.

Further experiments can be done to compare the fitness of these different selected populations with the common ancestor.

Fitness in the novel environments

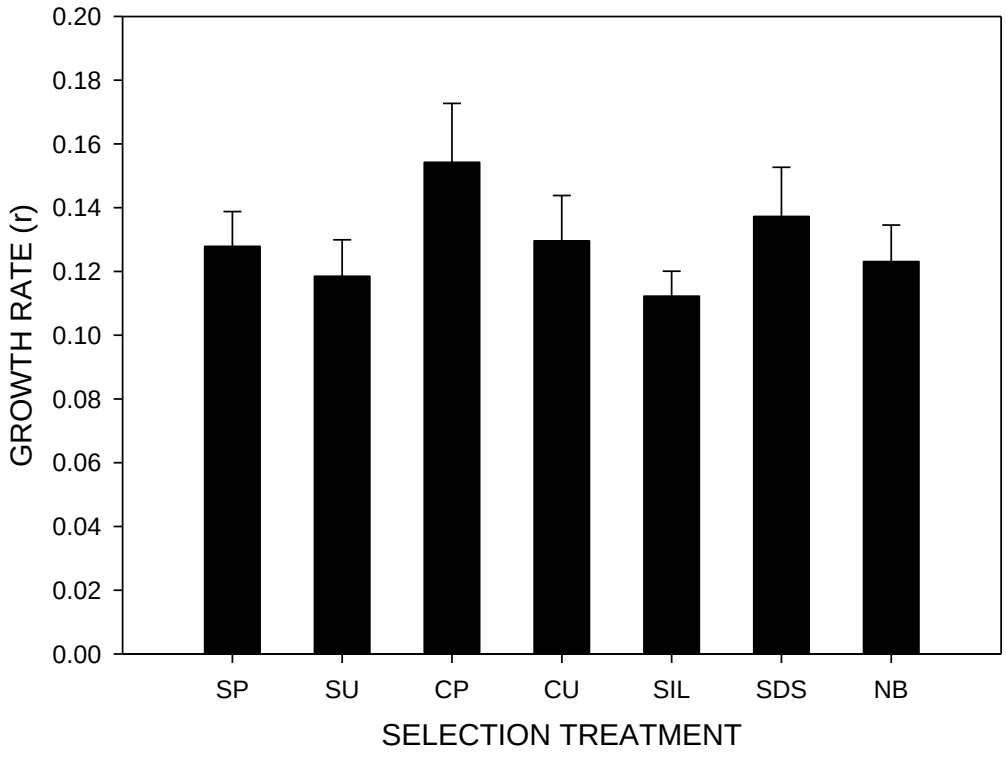

Figure 4 (A): **Mean (±SE) growth rate (r) of different selection treatments pooled across all the novel environments. (**p = 0.1715**)** No significant difference between any treatment pair (p = 0.3294). SP – Simple Predictable, SU – Simple Unpredictable, CP – Complex Predictable, CU – Complex Unpredictable, SIL – Constant Silver, SDS – Constant SDS, NB – Plain NB.

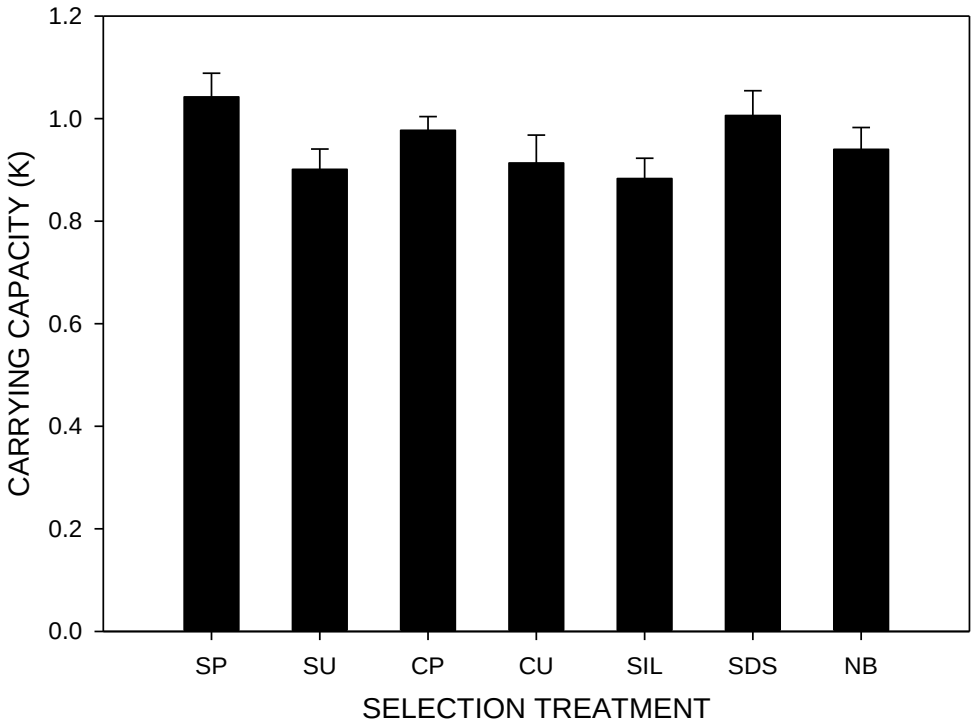

Figure 4 (B): **Mean (±SE) carrying capacity (K) of different selection treatments pooled across all the novel environments.** (p = 1.913) No significant difference between any treatment pair (p = 0.0881). SP – Simple Predictable, SU – Simple Unpredictable, CP – Complex Predictable, CU – Complex Unpredictable, SIL – Constant Silver, SDS – Constant SDS, NB – Plain NB.

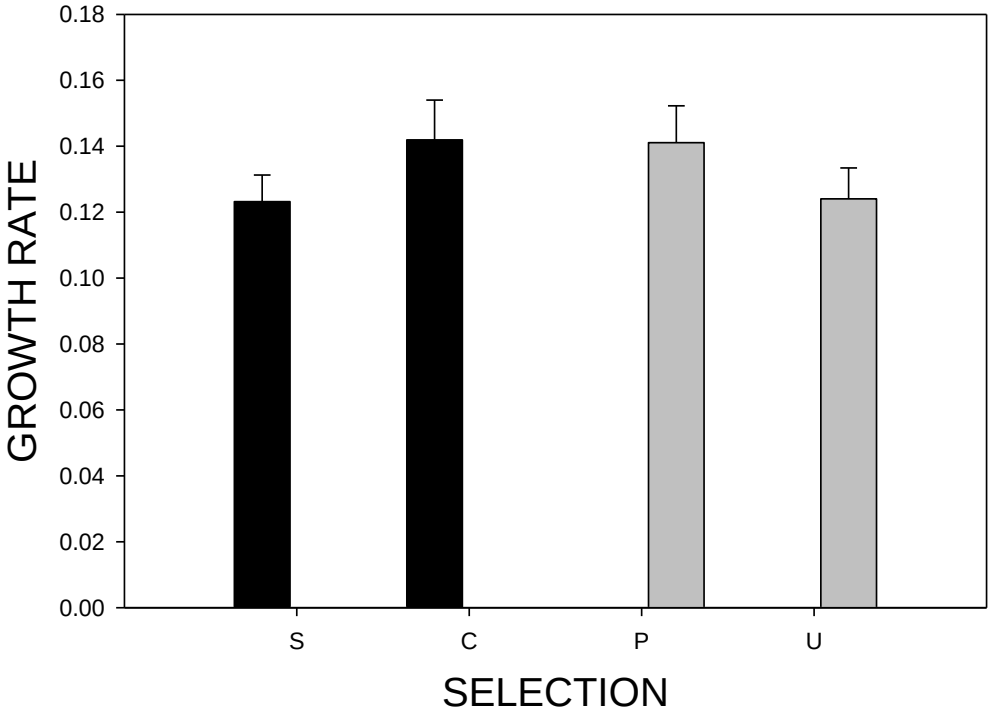

Figure 5 (A): **Mean (±) growth rate (r) of Simple (SP and SU), Complex (CP, CU), Predictable (SP and CP), and Unpredictable (SU and CU) selection treatments pooled across the novel environments**. The fluctuating selection treatments have been divided into these groups so as to compare the fitness effects of complexity and fluctuations. These four groups are overlapping in nature and are set on the same axis only for convenience. However, S must be compared to C and P must be compared to U only ($p = 0.1747$ for S and C comparison and $p = 0.2168$ for P and U comparison)

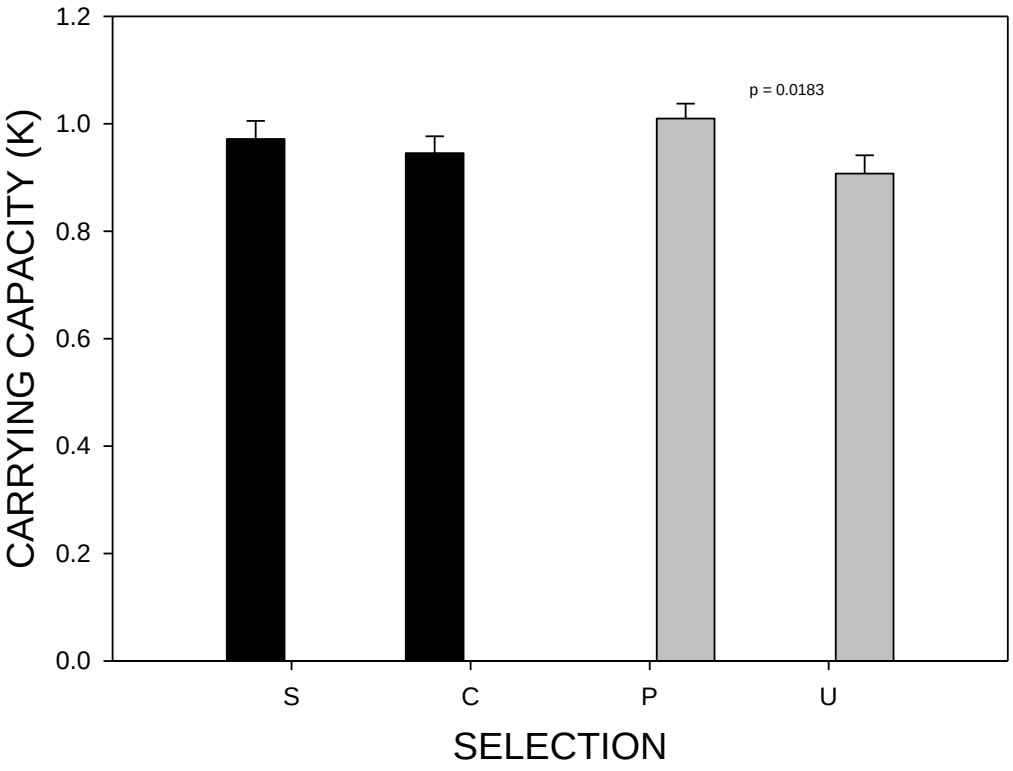

Figure 5 (B): **Mean (±) carrying capacity (K) of Simple (SP and SU), Complex (CP, CU), Predictable (SP and CP), and Unpredictable (SU and CU) selection treatments** (p = ns for S and C comparison and p = 0.0183 for P and U comparison).

In novel environments, there was no significant difference in terms of both r and K between any of the selection treatments (Table 5, Figure 4). This is in contrast to the results published in Karve et al (2015) where bacterial populations evolved in complex unpredictable selection regime had a significant growth rate advantage in novel environments when compared to the controls (selected in NB). However, in this study, the control (NB) also performs equally well compared to the fluctuating selection regimes and no significant difference is seen between the NB line and any other fluctuating selection treatment. However, it should be noted that in Karve et al, the type of unpredictability was different from the one studied here. In Karve et al (2015), there were three abiotic stresses with multiple values and the bacterial populations were randomly exposed to any two stress every day. So, there was an unpredictability arising from the nature of the stress that was used on a given day

and also from the value of the chosen stress (as every stress had multiple values). In the present case, there were only two stresses involved which were faced by the bacterial populations every day. The unpredictability of the selection regime came from the concentrations of the two stresses. However, every stress only had two sub – lethal values. Therefore, the unpredictability in this case was much less than the one used in Karve et al. This could possibly explain the lack of significant fitness different between CU selection treatment and the NB treatment at least.

The lack of significant fitness difference can also be explained from the nature of the abiotic stresses that were used to generate the selection treatments. Silver Nitrate causes severe morphological aberrations such as detachment of cytoplasm from the cell wall which damages the cell wall (Feng et al., 2000). Similarly SDS, being an anionic detergent, dissolves the plasma membrane causing leakage of several proteins, DNA, and RNA out of the bacterial cell (Woldringh et al., 1972) Since, these two abiotic stress severely affect cell membrane/cell wall, it is entirely possible that the different selection treatments evolved robust cell membrane/cell wall which prevents the entry of the novel stresses also. Thus, all the selection treatments perform equally well as all of them were exposed to these stresses during the selection procedure. However, it should be noted that this argument does not explains the similar fitness of the NB line.

Similarly, there is no significant difference between simple (SP and SU) and complex selection treatments (CP and CU). In terms of fluctuations, there is no significant difference between predictable (SP and CP) and unpredictable (SU and CU) in terms of r (Figure 5, Table 6). However, predicable fluctuations had higher fitness than the unpredictable fluctuations in terms of K (Table 6). It has been shown previously that populations evolved in predictably fluctuating environment (of a single environment) have a growth advantage in biotic and abiotic novel environments when compared to the populations evolved in constant environment (Ketola 2013). However, this study deals with the binary fluctuations of a single stress. In the present case, there were two stresses present and it also involved simultaneous fluctuations of two stresses.

However, in the present case, all the fluctuating selection treatments were comprised of two environments and CP and CU selection treatments involved simultaneous fluctuations. This is a novel result which can be investigated further.

Conclusions

The chief result of the study is that populations selected in Simple predictable selection regime have the highest fitness over all the component environments. This selection treatment also performs better than the other selection treatments in complex component environments. On the other hand, the study concludes that the Complex Unpredictable selection treatment has the lowest fitness over all the component environments. In general, the selection treatments that fluctuated predictably perform significantly better than the unpredictable selection treatments. Moreover, contrary to a previous study (Barrett et al., 2005) the study demonstrates that simple selection treatments had higher fitness than the complex selection treatments over all the component environments. In this study, no significant difference in fitness was found between different selection treatments in the novel environments. This is contrary to the results published in Karve et al., 2015.

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