That which does not kill you, makes you stranger: *E. coli* populations selected for 560 generations in randomly fluctuating stressful environments could become superior invaders

A thesis submitted towards the partial fulfilment of
BS-MS dual degree programme

By

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20091054

Under the guidance of
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Dedicated to the point of singularity of whose I am one of the inevitable ramifications.

Certificate

This is to certify that this dissertation entitled **"That which does not kill you, makes you stranger: *E. coli* populations selected for 560 generations in randomly fluctuating stressful environments could become superior invaders"** towards the partial fulfilment of the BS–MS degree at the Indian Institute of Science Education and Research, Pune represents original research carried out by Mr. Somendra Singh Kharola at IISER, Pune under the supervision of Dr. Sutirth Dey, Associate Professor, Biology Department during the academic year 2013-2014.

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Declaration

I hereby declare that the matter embodied in the report titled **"That which does not kill you, makes you stranger: *E. coli* populations selected for 560 generations in randomly fluctuating stressful environments could become superior invaders**" are the results of the studies 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.

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Abstract. —Populations with a history of living in fluctuating environments are predicted to be successful invaders. Enhanced invasive abilities can stem from capacity to inhabit novel environments. A recent study has shown that bacterial populations selected under fluctuating temperatures perform better when confronted with novel environments. But experimental evidence for evolution of better competitors/invaders, however, is still sparse. Moreover, the question as to how the nature of selection regime, i.e. complexity and predictability, affects the potential to respond to novel environments is largely unexplored. Here I compare the invasive ability of replicate *E. coli* populations – selected in a randomly fluctuating complex environment – with control populations that have not experienced such environmental fluctuations. For this purpose, relative fitness was assessed in three different biotic scenarios: (a) separate competition with two different bacteria, *Serratia*, and *Staphylococcus*; (b) the ability to infect *Drosophila melanogaster* (fruit fly). Results show that selected populations are better than the controls when competed against *Serratia*, without any disadvantage in any of the other novel biotic challenges. In the second part of the study, I checked whether the observed advantage in the competition with *Serratia* exhibits a correlation with an improvement in fitness in any of the component environments, i.e. environmental variables which were part of the selection. To answer this, I compared the fitness of control and selected populations over the duration of selection in all the three component environments. The results of these fitness assays in the component environments reveal absence of adaptation over time for selected populations. Therefore, my results suggest that complex randomly fluctuating environments can select for populations which can potentially be better invaders/competitors in some novel environments, and evolution of this ability need not be correlated with the adaptation to the component environments.

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I would like to thank my mentors Dr. Sutirth Dey and Shraddha Karve for their guidance and support; they have fathered me through all these years.

Introduction

The natural environment of any organism is a mixture of different environmental conditions, each with varying effects on the potential fitness of the population (reviewed in Kassen 2002). The environments that influence the population growth potential can either be constant or fluctuating. Several studies have shown that fluctuating environments can affect an organism's fitness, selecting for generalists with broad niches (see review Kassen 2002; Hughes et al 2007; Ketola 2013). On the other hand, those environments which remain constant, both spatially and temporally, select for specialists with narrow niches (Elena and Lenski 2003;Lenski et al 1993; Bell et al 1997; Leroi et al 1994). Thus, specialists are expected to exhibit a substantial variance in fitness across different habitats, while generalists should show reduced variance in the same (Kassen 2002).

Being a specialist (Alto et al 2010) or a generalist (Ketola et al 2013), however, entails a cost. The specialist populations, evolved to thrive in environmental constancy, are vulnerable to the fixing up of neutral mutations that could prove to be deleterious in other habitats (Futuyama et al 1988; Alto 2010). Studies have also shown that prolonged exposure of microorganisms to a constant environment entails a cost in the form of antagonistic pleiotropy (reviewed in Kassen 2002). In generalists, christened the proverbial "jack-of-all-trades-master-of-none", however, a fluctuating environment dampens the rate at which favourable traits could otherwise fix in a constant environment (Kassen 2002). Thus, the generalists are unable to maximize fitness in one

particular niche (see Ketola et al 2013; Levins 1968). Further, in some cases even neutral mutations, which otherwise do not negatively affect the fitness of the generalist in one particular environment, could prove to be deleterious in other environments (Kawecki 1994).

In this context, a question of particular interest is how the performances of these generalist and specialist bacteria are affected when exposed to novel environments? As far as we know, only one study has dealt with this particular question (Ketola et al 2013). This study shows that generalists boast of higher fitness levels as compared to specialists even in a novel environmental stress. On the other hand, however, there have been studies (Alto et al 2010; Alto et al 2013) where the generalists had lower fitness than the specialists in a novel stress. These contradictory predictions are further complicated by the fact that the experimental environments of these studies involve fluctuations of a single environmental variable – for example pH (Hughes et al 2007) or temperature (Ketola et al 2013). This limits the possible extrapolation of the results, since fluctuations in natural habitats are less likely to be of such singular nature.

To assess the invasive potential after the history of random, complex fluctuations, we subjected selected and control *E. coli* populations to three different biotic stresses. Two of these novel biotic environments were in the form of two different bacterial strains, namely *Serratia* and *Staphylococcus*. The third novel biotic environment was provided in the form of host fruit fly (*Drosophila melanogaster)*. We assessed the relative fitness of selected and control populations for the two biotic competition assays, while the time

taken to kill the host fly was taken as a proxy of fitness for the third. It was observed that the selected populations fared better than the control in the competition assay against *Serratia.* Thus, this observed advantage in one of the novel environments prompted the third part of this study. In which we assessed the extent of adaptation of selected populations to the component environments.

Generalists are known to broaden their tolerance curve in fluctuating environments by actually improving their fitness in one of the components of the fluctuating environments (Leroi et al 1994). Thus, we estimated the fitness of control and selected populations from the growth trajectory in extreme values of each of the three component environments. Contrary to previous selection studies, however, we find no evidence of the selected populations of bacteria broadening their tolerance curve and thus performing better than their ancestor, the control, in the component environments.

Materials and Methods

Competition assay:

The number of bacterial cell divisions has long been used as a measure of bacterial fitness (Lenski et al 1991). Therefore, in order to understand the interaction between our control and 100 days selected *E.coli* populations when confronted with competitors (*Staphylococcus* and *Serratia*), the measure of relative fitness, i.e. the ratio of the total number of cell divisions of each of the competitors after the completion of the competition assay, was used.

In other words, relative fitness was computed using the formula(Lenski et al 1991):

Relative fitness = $\frac{D(E.coli)}{D(competitor)}$

where D is the total number of cell divisions -- the doublings -- by either of the competitors and is given by the formula,

$$D = \frac{\ln\left(\frac{N(t)}{N(o)}\right)}{\ln 2}$$

N(t) is the number of bacterial colonies after the completion of the assay and N(o) is the number of bacteria at the initiation of the assay.

Thus, the relative fitness of the competitors was ascertained by competing two competitors – one of them being either the selected or control populations, while the other being either *Staphylococcus* or *Serratia.* Both these competing populations were first revived in nutrient broth. Then, these competitors were mixed at a particular volumetric ratio, thus initiating the competition assay. At the same time point of initiating the competition, the bacteria were plated on NA to determine the number of bacteria, N(o), just before the initiation of the competition. Then, after allowing the bacteria to compete against one another for a particular time period, the nutrient broth holding the competitors was plated on NA once again so as to ascertain the number of competitors after competition N(t).

The following sections delve deeper into the finer details of the competition assay.

Bacterial strains

This assay comprised the six populations of *E. coli*, which included the three control populations (denoted henceforth as 'S') that were grown for 100 days in NB, and the other three populations grown in fluctuating environments *(*henceforth denoted as 'F') that were selected via serial transfer for 100 days. The fluctuating environments during this 100 day selection period comprised randomly picked sub-lethal concentrations/values of hydrogen peroxide, pH and salt. The details of the maintenance regime of these populations can be found elsewhere (Karve et al 2014).

Other than these *E. coli* strains, this assay also included bacterial competitors. Since both, these competitors and the strains of *E. coli*, would be plated together from the same suspension, it was critical, that the competitors be chosen such that they exhibited a characteristic colony colour distinct from *E. coli.* Thus, *Serratia* (red) and *Staphylococcus* (yellow) were chosen. All these bacterial strains were stored as glycerol stocks at -80ºC.

Further, since this assay involved extensive plating, as a part of the (I) competition assay, a novel nutrient agar plating technique was developed to save on time and labour. The procedure for this novel plating technique is outlined at the end of the section.

Culture conditions

Nutrient broth was used as the culture medium in all the competition assays. The competition assays were carried out in 2ml wells of 24 welled Corning plates. During the course of competition, these plates were placed in an incubator maintained at 37ºC at 150rpm.

Competition (vs *Serratia*)

We followed the growth trajectory of one of the control populations and *Serratia* to fix the suitable duration of competition. Under the experimental conditions, *Serratia* reached saturation faster than *E. coli* (~10 hours), which was treated as endpoint of the competition. Moreover, since it was observed that *Serratia* was a far more aggressively growing bacteria as compared to one of the S populations, it was decided that during the setting up of the competition assay, a 4:1 concentration skew in the favour of F/S

populations would be followed. In other words, in 2 ml of NB 4µl of F/S population and only 1µl of *Serratia* would be inoculated together. Thus, the following protocol was adopted for the competition assays. 100-days selected F/S *E. coli* and *Serratia*, from -80degrees Celsius, were revived in two different wells of a 24 welled plate. Then, the following day, after 20 hours of growth, the revived *E. coli* and *Serratia* cultures were mixed together in a 2ml well to initiate competition. Before the mixing of the cultures, however, the zero hour bacterial count of both these competitors was determined by plating them on NA. After 10 hours of competition, the competition wells, which contained both *Serratia* and *E. coli* were plated on NA and the number of colonies of each type were counted manually.

Competition (vs *Staphylococcus*)

The procedure followed in the *Staphylococcus* competition assay was similar to the earlier *Serratia* competition assay, barring one difference: the nutrient agar plating was done after 20 hours of competition. Once the numbers of the competitors involved in all the above competition assays were determined, then the relative fitness between the S and F populations was measured by using the same procedure delineated in Lenski et al 1991.

Novel nutrient agar plating technique

A novel methodology of plating bacterial colonies on nutrient agar was developed to allow for a high throughput which is far more efficient (less time consuming, less labour intensive) as compared with traditional nutrient agar plating methods.

The following describes the protocol so followed:

1. As traditional nutrient agar plates are prepared by pouring nutrient agar, 3 ml of nutrient agar is poured into each of the wells of a sterile, six welled tissue culture plate.
2. Then while plating bacterial colonies of certain dilution, one does not use a spreader for plating but instead simply drops 200 micro liter of inoculum on the nutrient agar in a well, swirls the plate for a few seconds in one's hands (inside the microbial hood) and then places it in the incubator (37 degrees Celsius, 150 RPM). The 150 RPM allows for an even spread of the inoculum volume over the surface of the nutrient agar.
3. After 15minutes of shaking in the incubator, the plates are opened in a sterile hood and left to dry for another 25 minutes.

   Therefore, by following this method, one has bypassed the tedious spreading by using a spreader, and also can efficiently spread several inoculums without needing to continually sterilize the spreader.

II. Non invasive *Drosophila* infectivity assay:

Bacterial strains and *Drosophila* biotic stress

The materials required for non-invasive *Drosophila* infectivity assay, were as follows: (a) Three replicate F and S populations each and (b) *Canton S Drosophila* flies. Thus, bacterial fitness, in our study, is measured as the time required for the F and S populations to kill the *Drosophila* host, and is an approximate measure of virulence as well (Ketola 2013, Nehme et al 2007).

Bacterial infectivity assay

The procedure to set up this assay is based on the protocol delineated in Bharathi et al 2007. Before initiation of the infectivity assay, 5 to 6 ml of NA were poured into autoclaved glass vials. Twenty hours before setting up the assay, these vials were a smeared with 50 µl of revived SA and FA bacterial suspension. Then, the vials were incubated at 37º Celsius for 24 hours. After 24h, a full grown lawn of bacterial cells was observed. These vials were used as treatment vials. Further, vials with 5 to 6 ml NA, without any bacterial lawn served as control vials. The total number of vials was 32, out of which 16 were inoculated with the S/F bacteria, and the remaining 16 served as control. Eight of the S/F inoculated vials had ten females each, while the other eight had ten males each. After setting up the vials as per above, we checked for dead flies every two hours till all the flies were dead. The checking was done without opening the vial, by visual inspection and tapping. Every 24 hours the live flies were transferred into new vials which contained a 20h old lawn of revived S/F bacterial culture. This demanded the revival of fresh bacterial cultures of corresponding stock (SA and FA) for every day.

III. Fitness in component environments

Growth curves in component environments

The selection stress regime for the F bacteria comprised four different stresses, H_20_2, salt, acidic, and basic environments. The F bacteria were continually exposed to all the four stresses, the levels being randomly fluctuated after every 24 hours. The stocks of these bacteria, after every 24 hours, were flash frozen and stored at -80ºC. Thus, the stocks of the bacteria ranging from 1 day selected to 100 days selected were available

to me. Here, the word ‘component’ refers to one of the four stresses against which the bacteria were selected.

In this experiment, 24-hour growth curves of the three replicates of the S and F bacteria were charted separately in each one of the four stresses that comprised the fluctuating environment of the F bacteria. The level/concentration of each stress was based upon the results of earlier standardization experiments and were as follows: 5g% salt, pH 4.5, pH 10, and 0.5µl H_2O_2. Growth curves were performed in triplicates for each of the F and S populations in each of these environments.

Parameter estimation and statistical design

(a) Competition Assay

As outlined earlier in this section, the relative fitness measurements was computed using the formula:

$$\text{Relative fitness} = \frac{D(E.coli)}{D(competitor)}$$

where D is the total number of cell divisions -- the doublings -- by either of the competitors given by the formula,

$$D = \frac{\ln\left(\frac{N(t)}{N(o)}\right)}{\ln 2}$$

N(t) is the number of bacterial colonies after the completion of the assay and N(o) is the number of bacteria at the initiation of the assay.

These values of relative fitness of all the S and F populations when confronted with the competitors were analyzed using three way mixed model ANOVA where environment (2 levels – *Serratia* and *Staphylococcus*) and selection (2 levels – F and S) were treated as fixed factors crossed with each other. Replication (3 levels) was a random factor nested in selection. Since we were also interested in the performance of S and F populations in the face of individual competitors, we then performed a two way mixed model ANOVA with selection (2 levels – F and S) as a fixed factors and replication (3 levels) as a random factor nested in selection.

(b) Non invasive *Drosophila* infectivity assay

The time to death of *Drosophila* is a proxy for bacterial virulence, and in effect its fitness (Bharathi et al 2007; Ketola et al 2013). Three-way mixed-model analysis of variance (ANOVA) was performed with replicate S and F populations as random factors, and the selection –2 levels, S and F– and sex 2 levels, male and female, as fixed factors.

(c) Fitness in component environments

In each of the four component environments (e.g. salt 5 g%), the growth trajectory of the S and F populations was studied. The 24 hour growth curve trajectories in a component environment were conducted in the same 24 well plate and constituted a trial. Each trial thus had 18 (6 populations X 3 replicates) growth curve measurements. Then, to compare the fitness of F and S in each of the component environments after 100 days, 3-way mixed-model ANOVA was performed with selection (2 levels – S and F) and assay environments (4 levels) as treated as fixed factors crossed with each other.

Replicate (3 levels, nested in selection) was treated as a random factor. For the difference in adaptation over time, we analyzed every assay environment separately. Three way mixed model ANOVA was performed with Selection (two levels F and S) and Time (10 levels, one for every 10^{th} day in selection) as fixed factors and Replication (three levels) as a random factor nested in selection.

All the ANOVAs of the in this study were performed on STATISTICA v5.0 (Statsoft Inc.). Further, independent of this statistical significance testing, we also performed a substantive significance testing in the form of Cohen's d (Sullivan et al 2012) to estimate the effect size of the differences in the relative fitness. Cohen's d was computed as $d_s = (M_1 - M_2)/ s$, where the difference between group means (M) is denoted by M_1-M_2, and s denotes the pooled standard deviation of the two groups. The Cohen's d-statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004). The maximum slope so computed in all the growth curves of the F and S populations in the component environments was considered as an estimate for the max growth rate of the populations.

Results

I. Competition assays:

We found significant effect of competition environment (F = 40.28, p = 0.003) and selection (**Figure 1;** F = 30.05 p = 0.005). F populations performed significantly better with large effect size (d = 0.8). The interaction between environment and selection was marginally insignificant (F = 4.85, p = 0.09).

S and F *E. coli* vs *Serratia*

The F populations had a significantly higher relative fitness (**Figure 2;** F= 18.36005; p = 0.013) with large effect size (d = 3.53) in the competition assays against *Serratia*. Further, there also exists a significant difference in the relative fitness amongst the F and S replicates (F= 9.68679; p = 0.001).

S and F *E. coli* vs *Staphylococcus*

When competed against *Staphylococcus,* F populations had higher relative fitness with medium effect size (d = 0.54) than S but the difference was not statistically significant (**Figure 3;** F= 0.572126; p = 0.491).

II. Non invasive *Drosophila* infectivity assay:

We did not find any significant difference in the virulence of the S and F populations towards the *Drosophila* (**Figure 4;** F = 0.001764; p = 0.970, d = 0.01). Infectivity was significantly different across sexes along with a significant interaction between selection, sex and replicates, (F = 6.476713; p = 0.002).

III. Fitness in component environments after 100 days:

Fitness did not differ significantly across S and F populations when fitness estimates from all the component environments were pooled (**Figure 5;** F = 0.439, p = 0.543, d = 0.19) neither was there any significant interaction between selection and different component environments (F = 0.596, p = 0.629).

IV. Fitness in component environments over the period of selection:

Component environment 5g% salt

Considering selection as the main effect, the fitness of the F populations are significantly higher than the S populations (**Figure 9**; F = 11.7193; p = 0.027) but the effect size was low (d = 0.37). Additionally there was a significant interaction (**Figure 12**;F = 28.27547; p = 9.83E-14) of selection with time but none of the F or S populations show monotonous increase in fitness with time.

Component environment pH 10

Considering selection as the main effect, there is no significant difference between the growth rates of the F bacteria and S bacteria (**Figure 7**; F= 0.005078; p = 0.947; d = 0.01). Further, considering the two way interaction between selection and time period, the max slopes of S and F are not significantly different from one another(**Figure 11**;F = 0.995366; p = 0.461) and neither is there any monotonous increase in fitness with time.

Component environment pH 4.5

Considering selection as the main effect, the fitness of the S bacteria are significantly higher than the F populations although the effect size was small (**Figure 6**; F = 9.183508; p = 0.0388, d = 0.28). Additionally there was a significant interaction of selection with time (**Figure 10**; F = 23.90476; p =1.25E-12) but none of the F or S populations show monotonous increase in fitness with time.

Component environment H_2O_2

Considering selection as the main effect, there is no significant difference between the fitness of the F and S populations (**Figure 8**; F= 4.934362; p = 0.09; d = 0.29). Additionally, there was a significant interaction of selection with time(**Figure 13**; F = 2.261821; p = 0.04) but none of the F or S populations show monotonous increase in fitness with time.

Discussion

I. Improved relative fitness of F populations in the face of *Serratia* but not *Staphylococcus*

F populations display a higher relative fitness in the face of *Serratia* as a competitor. One previous study has investigated the performance of bacterial populations selected in fluctuating environments, in the face of novel biotic stresses (Ketola, 2013). Authors report better performance of the selected populations of *Serretia* in presence of a predator and a virus, but when we computed the effect size for these, it was of small to medium magnitude. Our results with *E. coli* populations underline another possible advantage, that of higher competitive ability, for the populations with the history of fluctuating environments.

There are multiple ways by which a bacterial population can become a superior competitor. These include the modification of their higher maximum growth rate; their ability to reduce the amount of resources required to sustain growth at half maximum growth rate; and their ability to decrease the death rate after the limiting resource is consumed (Vasi et al 1994, Lenski et al 1998). In competition with *Staphylococcus*, though F populations exhibit a higher relative fitness, the difference is not statistically significant (with medium effect size) when compared to relative fitness of S populations. One possible reason for this could be the intensity of the competition. Relative fitness of both S and F populations is high in the competition assay with *Staphylococcus* (**Table 1**) suggesting weak nature of competitor. This can potentially mask the difference. We

can test this hunch in future studies by modifying the odds in the favour of *Staphylococcus*, at the start of the competition assay.

The second way that can confer an advantage to F populations in the face of *Serretia* alone, is the evolution of antagonistic mechanisms which assist in fighting *Serratia* and/or its related species. Such traits include being resistant to different antimicrobial agents secreted by competitors, or the ability to secrete lethal antimicrobial agents effective against certain class of bacteria (Hibbing et al 2010). Earlier investigations on 30 day selected F populations have revealed enhanced energy dependent efflux abilities along with the better performance in the novel abiotic environments (Karve et al 2014). These efflux pumps are known to be involved in the secretion of antibacterial proteins that kill neighbouring competitors, along with the ability to resist multiple drugs by pumping out foreign and toxic material out of the cytoplasm (reviewed in Andersen 2000, 2003 , Hocquet et al 2003, Rosenberg 2000, Kuete et al 2010). If the enhanced efflux ability is found to be retained even after 70 more days of selection, it can be a possible explanation for selective advantage in the face of novel competitors.

It is important to note that though all three F populations show higher relative fitness than any of the replicate S population while competing with *Serratia*, there exists a significant difference in the relative fitness within the replicates of the F populations. Two replicate populations exhibit a significantly higher relative fitness as compared to the third one (data not shown). The reason for such a difference in relative fitness levels amongst the replicates could be that the bacteria belonging to different replicates sit on different fitness peaks of their fitness landscapes (Lenski et al 1991, reviewed in Elena

and Lenski 2003). Such differential positioning can produce difference in fitness when exposed to the novel environments. Therefore, it would be interesting to test whether, F populations, if allowed to grow for several hundred more generations in a fluctuating environment, would maintain, increase, or decrease the difference in relative fitness against *Serratia* (Lenski et al 1991).

II. No difference in the ability to infect *Drosophila*

Drosophila is a model organism that has been widely used to study the virulence of microorganisms and fungi (Apidianakis et al 2009, Fry et al 2004, Lionakis et al 2005, Ketola et al 2013). A previous study reports significant decrease in the survival of fruit flies, when infected with the bacterial populations with the history of fluctuations compared to the populations selected in constant environments (Ketola et al 2013). Contrary to this study, however, we find no significant difference in the virulence between the S and F populations towards the *Drosophila*.

There was a significant difference between the survival times of two sexes, as is well known in the literature (Bharathi et al 207). Additionally we found highly significant difference between the blocks ($F = 136.34$, $p = 4.21E\text{-}27$). This suggests that the fly populations which were used on three different days affected the results significantly. We strictly maintained the rearing protocol for all three blocks, randomly selecting flies from four different bottles (containing 200 to 300 flies). In spite of this, the fly populations seem to have a high contribution in the observed variation. Assaying all the populations together, if made logistically possible, might give us better resolution.

Additionally, different routes of infection can also be tested, since routes of infection can drastically affect the infectivity (Vodovar et al 2005; Lemaitre et al 1997).

III. Fitness in Component Environments:

Better performance in the face of a novel competitor by all the three replicate F populations raises a simple possibility of correlation of this ability with one of the stresses, which were part of the selection. Fitness measurement in the four component environments after 100 days selection does not support this possibility. In fact, control populations performed marginally better in the face of H_2O_2. To ascertain the lack of adaptation by F populations to component stresses, we estimated the fitness for every 10^{th} day during the selection, confirming the lack of adaptation on the part of F populations. Though these results negate the possibility of correlated response in terms of competitive ability, it opens up bunch of new questions in terms of adaptation to component stresses themselves.

Adaptation to single parameter fluctuations are well studied in microbial literature. Several studies have shown that the generalist evolves to match its tolerance curve to the width of the environmental fluctuations (Kassen 2002; Bell et al 1997; Kassen et al 1998; Lenski 1992; Hughes 2007). In some of these studies, (Bell et al 1998; Hughes 2007; Leroi et al 1994) the generalists were found to have a comparable, or even higher fitness as compared to specialists when exposed to a stable environment that was one of the components of the fluctuating environment. The fitness of generalists and specialists in these studies was ascertained by assaying their performances across the range of the same environments that were used during selection. In fact, in one

particular selection study (Leroi et al 1994), it was found that the generalists increase their fitness in the fluctuating environment, by actually improving their fitness in one of the components (stable) of the fluctuating environments. Against this background, it was interesting to trace the adaptation of F populations to the component stresses.

Unlike most other selection studies that used only one fluctuating environment, this study used a total of three environmental variables during the selection. And as the complexity of the selection environment increases, it becomes more difficult to adapt to all the fluctuations, and inevitably results in a cost (Kassen 2002, Dickerson 1955). Such a fitness cost is usually in the form of antagonistic pleiotropy, where genes beneficial in one environmental fluctuation prove to be detrimental to the organism's fitness in another (Cooper et al 2000, Kassen 2002). Moreover, when a genotype is exposed to a fluctuating environment, there might be a strong negative genetic correlation as the number of fluctuating variables increases (Kassen 2002, Dickerson 1955). This reduces the chances of the evolution of an 'ideal' genetic architecture that could perform well across all these environments. In other words, as the number of components of a randomly fluctuating environment increases, an organism must simultaneously adapt to a large number of conditions. This makes it difficult for the organism to increase its fitness w.r.t any one of the environmental conditions(Kassen 2002). Here, the F populations were confronted with multiple stresses during the selection which selected for genes that could have been either antagonistically pleiotropic, and/or strongly negatively correlated. Further, since the fluctuations were random, the selection pressure may simply have been too weak for the fixing of any

beneficial mutations, owing to which the F populations simply did not adapted to any one of the component environments in particular.

Lastly, we observed a pattern of peculiar oscillations in the fitness estimations over time in pH 10 and in pH 4.5. The fact that both S and F populations show such periodicity strongly argues for the presence of setup to setup variation. We need multiple trials to ameliorate this noise(**Figure 10-11**).

Conclusion

Several studies have shown that selection in fluctuating stresses increases the tolerance in component environments (Ketola 2013, Hertz et al 1984, Hoffmann (a) et al 1993, Hoffmann (b) 1993). Our results show that this fact will be largely dependent on the nature of the selection regime and complex environments with stochastic fluctuations might not result in adaptation to the component stresses. But such a selection regime can result in an ability to effectively face novel biotic environments. Further studies can focus on the mechanisms which can lead to such response.

Figures and tables

Data for competition assays pooled for *Serratia* and *Staphylococcus*

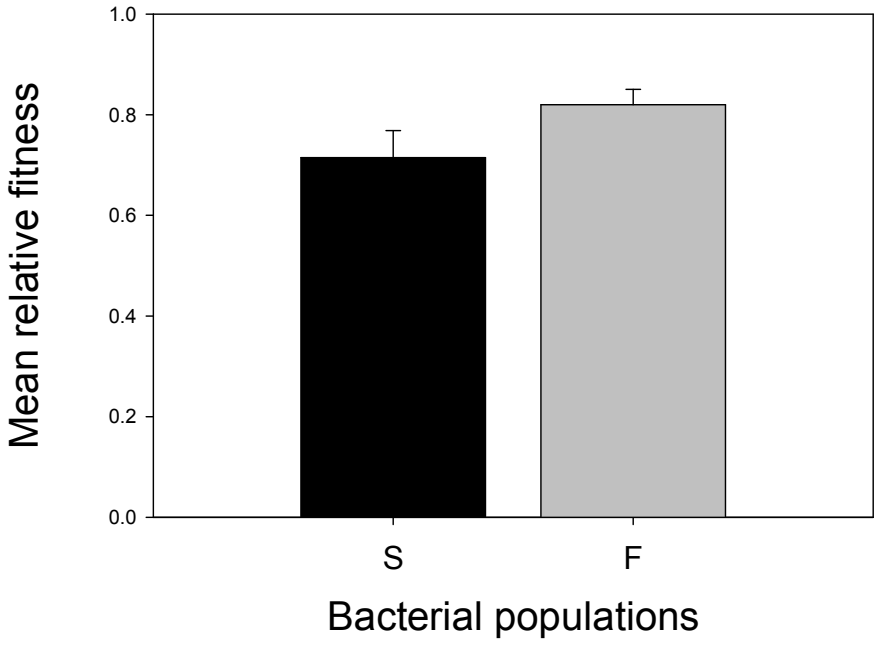

Figure 1. Here the comparison of the mean relative fitness, pooled over both the biotic environments (*Serratia* and *Staphylococcus)*, shows the selected (F) populations show significantly higher relative fitness than the control (S) populations. Error bars denote standard error of the means.

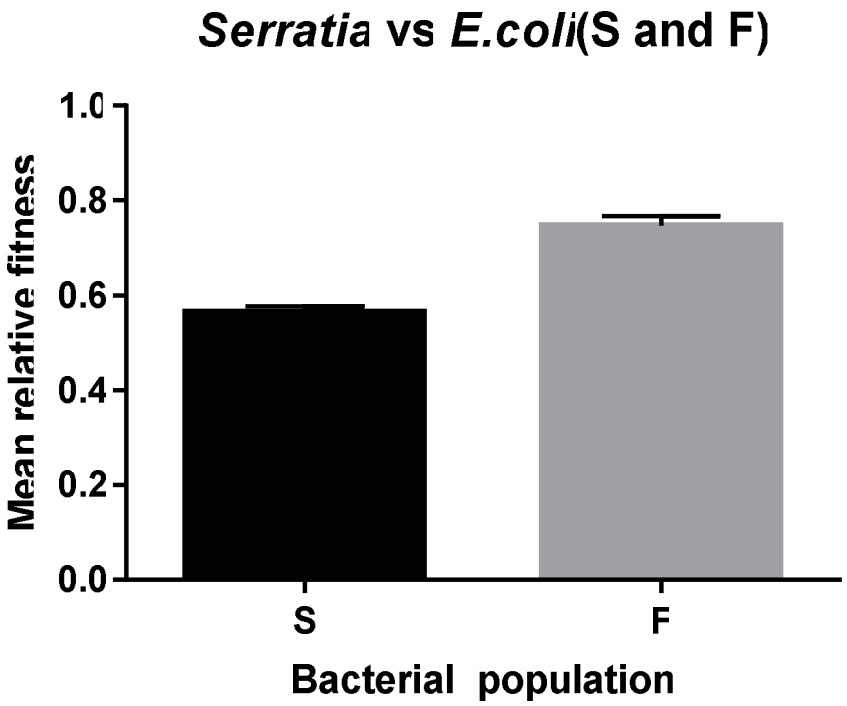

Figure 2. Here the comparison of mean relative fitness of S and F populations when confronted with *Serratia* show the selected (F) populations show significantly higher relative fitness than the control (S) populations. Error bars denote standard error of the means.

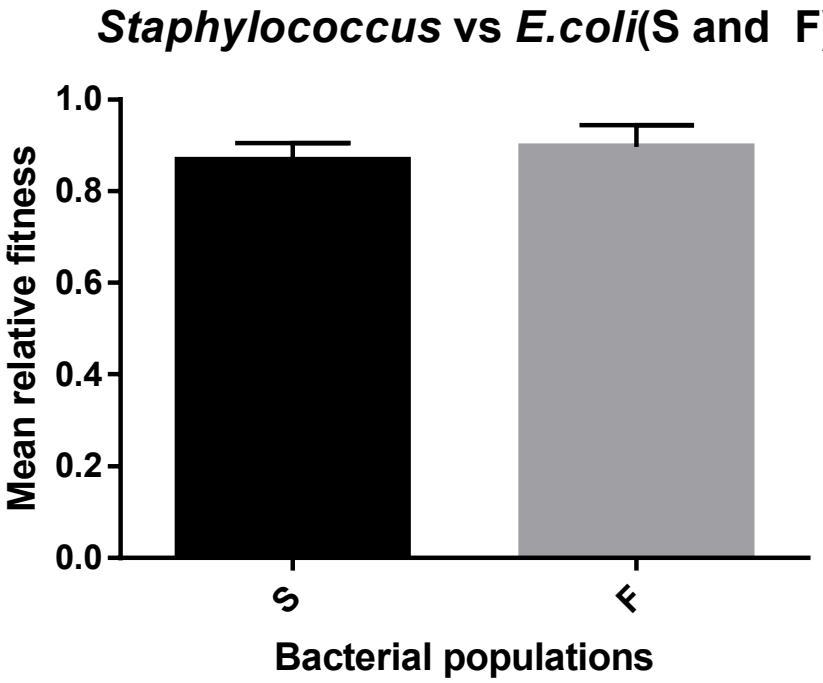

Figure 3. Here the comparison of the mean relative fitness of S and F populations when confronted with *Staphylococcus,* show no significant difference between selected (F) populations and the control (S) populations. Error bars denote standard error of the means

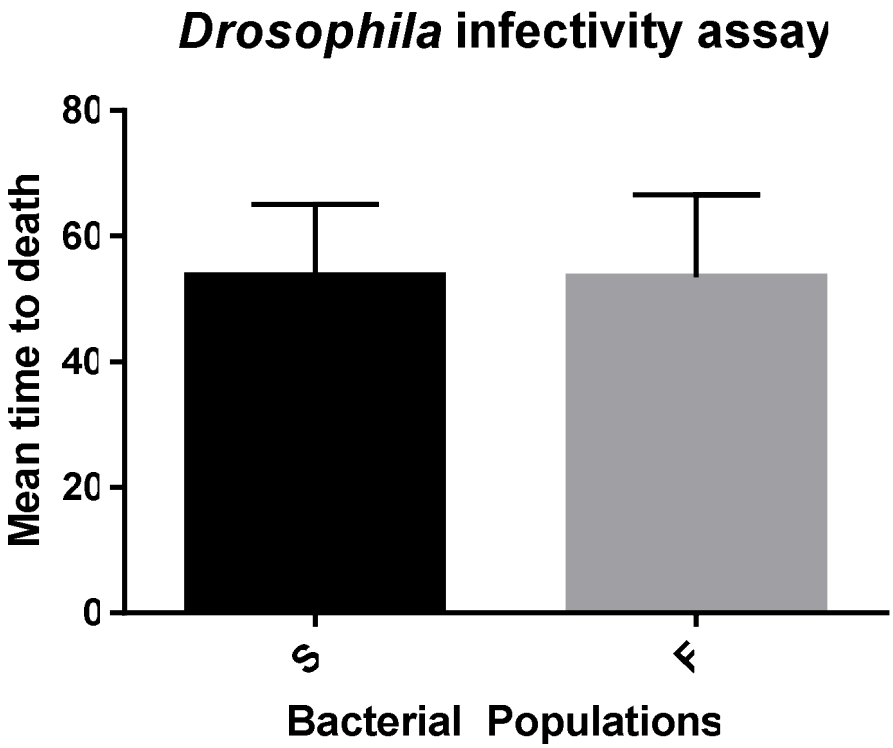

Figure 4. Here the comparison of the mean time to death of *Drosophila* flies when infected with selected (F) and control (S) *E. coli* populations shows no significant difference. Error bars denote standard error of the means.

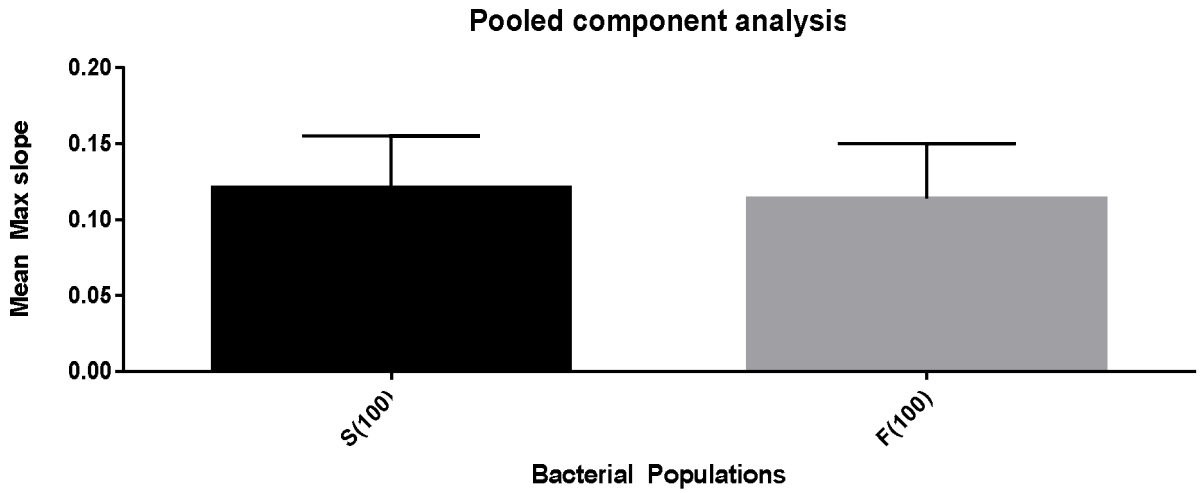

Figure 5. Fitness was measured as maximum slope of the growth trajectory over 24 hours. Here the comparison of the pooled means is over all four component environments, the 100 days selected (F) populations show no significant difference in fitness compared to the control (S) populations. Error bars denote standard error of the means.

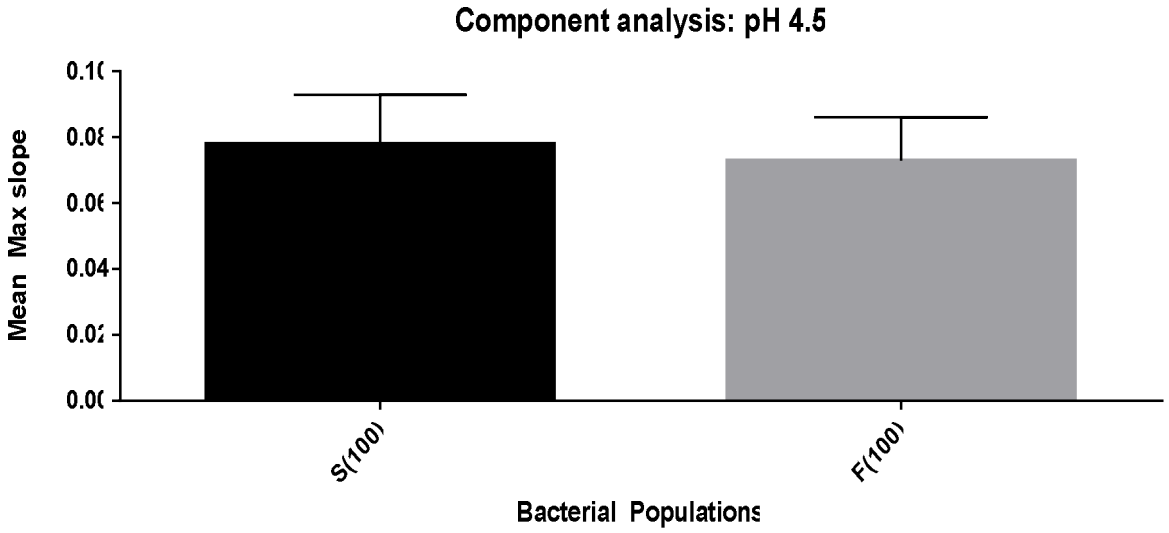

Figure 6. Fitness was measured as maximum slope of the growth trajectory over 24 hours. Here the comparison of the means of growth rates in component environment pH 4.5 shows no significant difference between the 100 days selected (F) populations and the control (S) populations. Error bars denote standard error of the means.

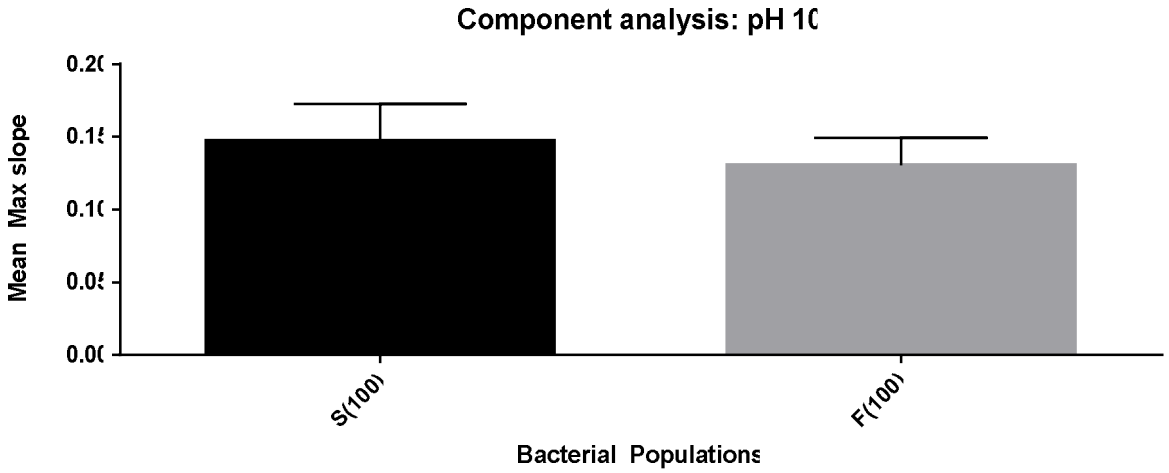

Figure 7. Fitness was measured as maximum slope of the growth trajectory over 24 hours. Here the comparison of the means of max slopes in component environment pH 10 shows no significant difference between the 100 days selected (F) populations and the control (S) populations. Error bars denote standard error of the means.

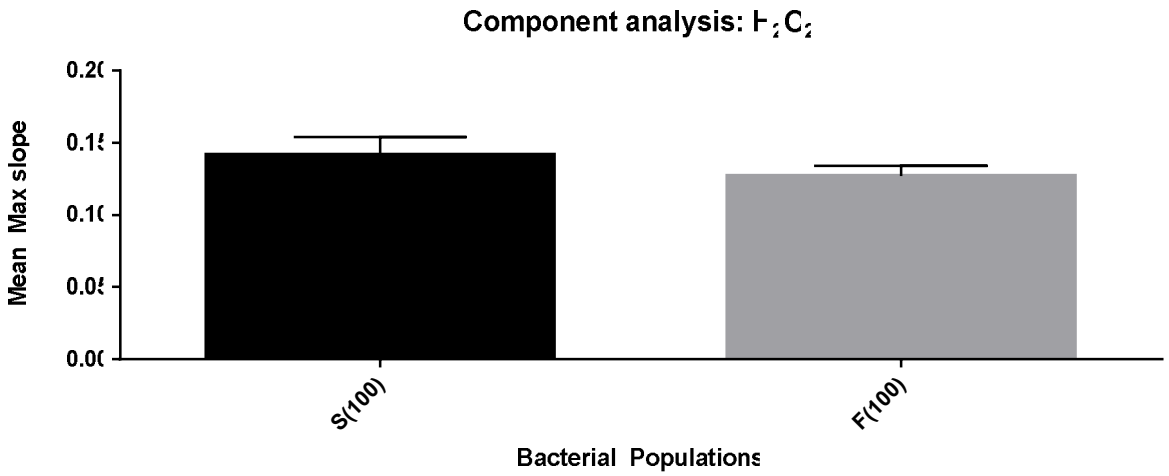

Figure 8. Fitness was measured as maximum slope of the growth trajectory over 24 hours. Here the comparison of the means of max slopes in component environment H_2O_2 shows no significant difference between the 100 days selected (F) populations and the control (S) populations. Error bars denote standard error of the mean.

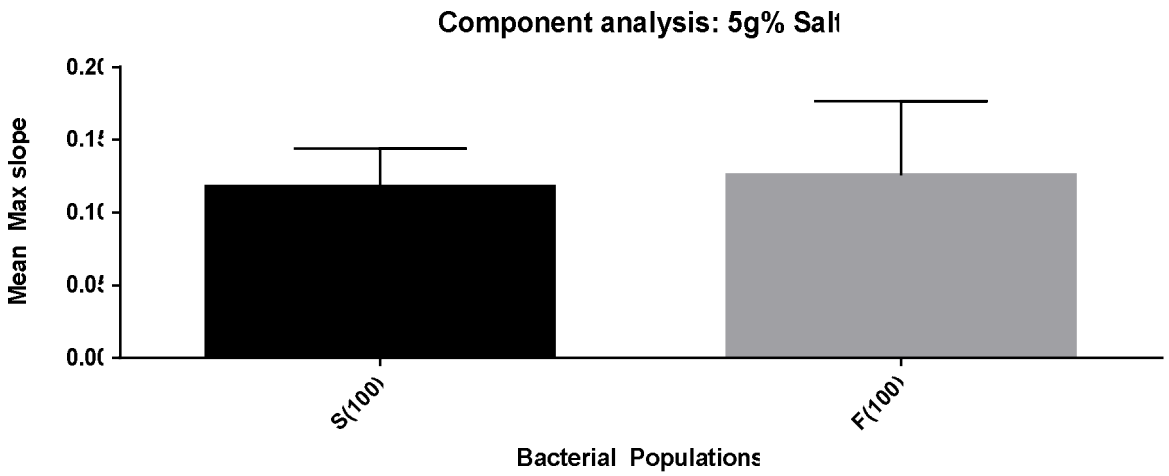

Figure 9. Fitness was measured as maximum slope of the growth trajectory over 24 hours. Here the comparison of the means of max slopes in component environment 5g% salt shows no significant difference between the 100 days selected (F) populations and the control (S) populations. Error bars denote standard error of the means.

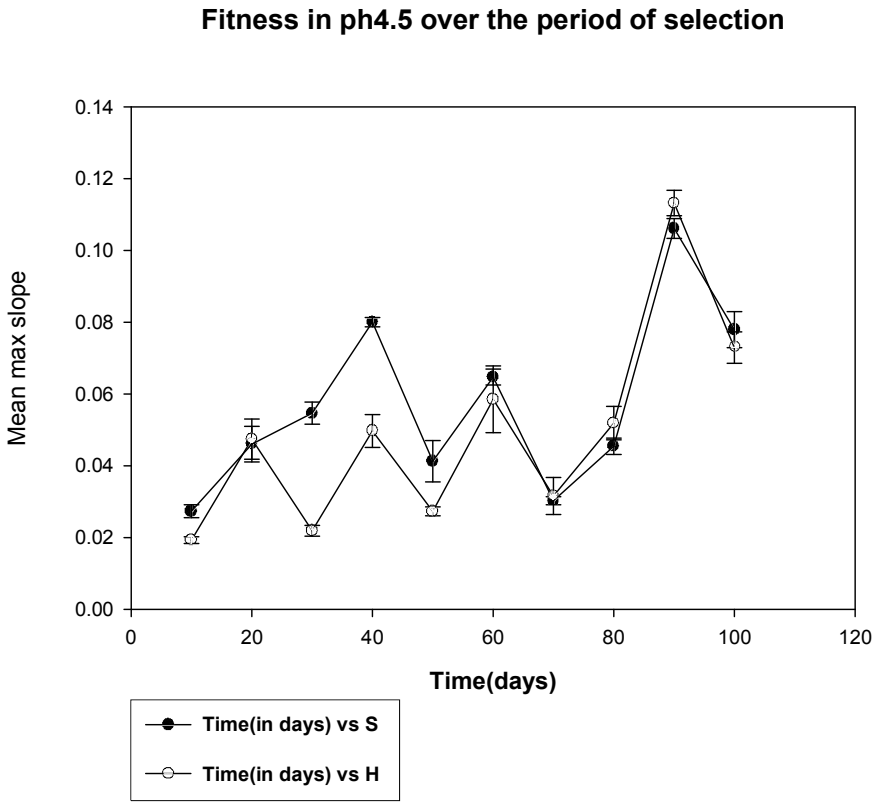

Figure 10. Fitness of control(S) and F(selected) bacteria selected in component environment pH 4.5 over the period of selection(100 days). Fitness was measured as maximum slope of the growth trajectory over 24 hours. Error bars denote standard error of the means.

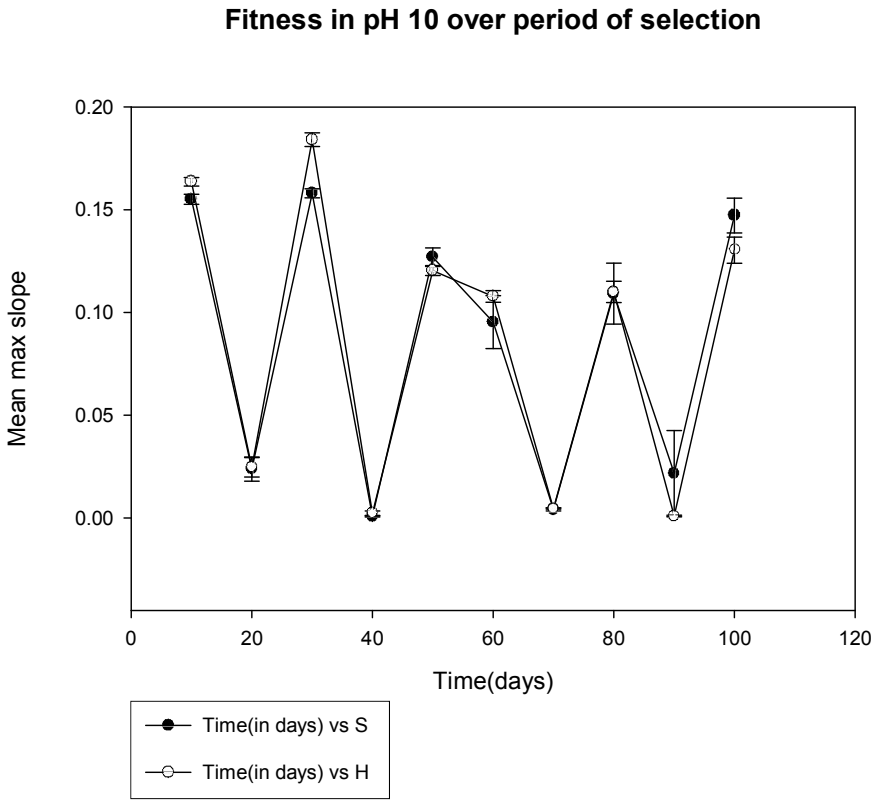

Figure 11. Fitness of control(S) and F(selected) bacteria selected in component environment pH 10 over the period of selection(100 days). Fitness was measured as maximum slope of the growth trajectory over 24 hours. Error bars denote standard error of the means.

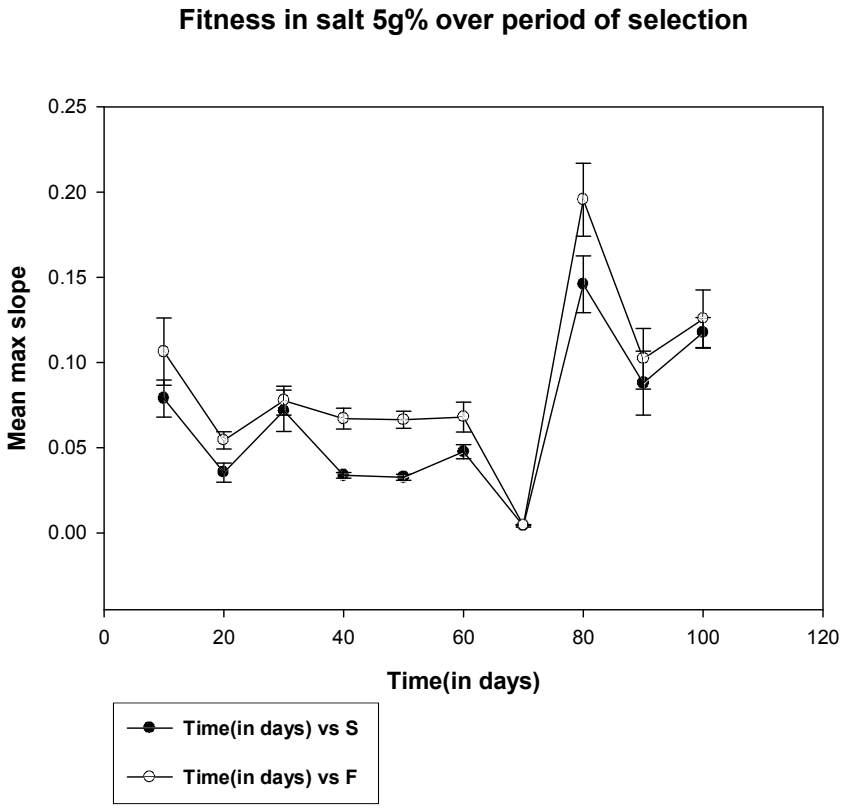

Figure 12. Fitness of control(S) and F(selected) bacteria selected in component environment salt 5g% over the period of selection(100 days). Fitness was measured as maximum slope of the growth trajectory over 24 hours. Error bars denote standard error of the means.

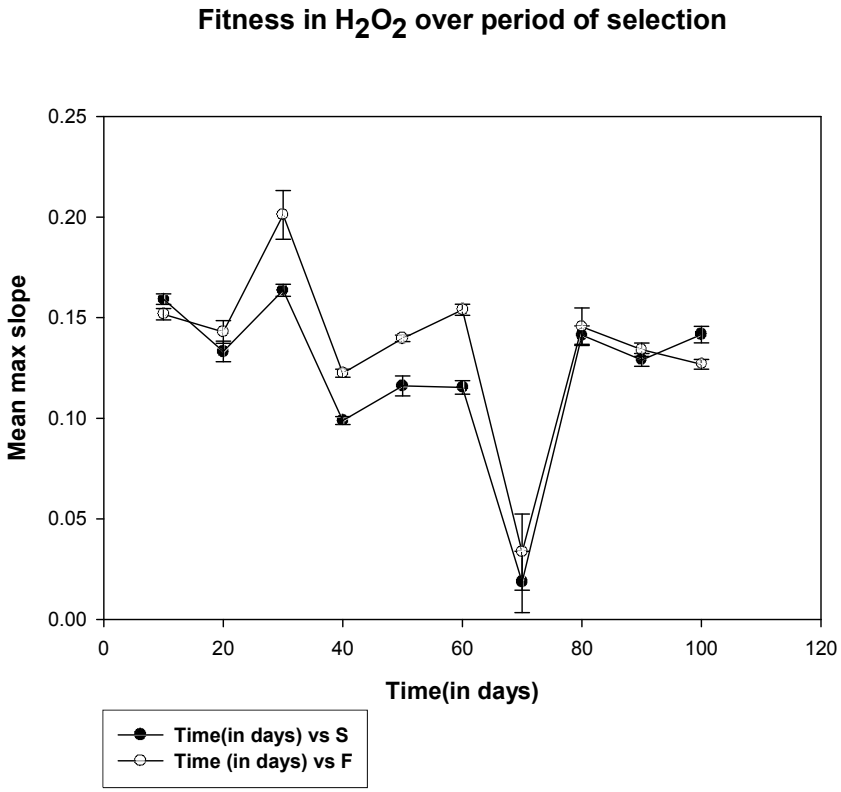

Figure 13. Fitness of control (S) and F(selected) bacteria selected in component environment H_2O_2 over the period of selection (100 days). Fitness was measured as maximum slope of the growth trajectory over 24 hours. Error bars denote standard error of the means.

Assay	Environment	S	F	ANOVA F(1,4)	p values	Effect Size	Inference
Fitness in component environments	pH 10	0.084	0.085	0.005	0.947	0.01	Small
	pH 4.5	0.057	0.049	9.184	0.039	0.28	Small
	Salt	0.066	0.08	11.719	0.027	0.37	Small
	H_2O_2	0.122	0.135	4.913	0.09	0.29	Small
Fitness in novel environments	*Serratia*	0.566	0.747	18.36	0.013	3.53	Large
	Staphylococcus	0.867	0.896	0.572	0.480	0.54	Medium
	Drosophila	53.588	53.448	0.002	0.97	0.01	Small

Table 1. Summary of the main effects of selection in the ANOVAs under individual environments for two sets of fitness measurements, namely, component environments and novel environments. Effect size was measured as Cohen's *d* statistic and interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$ respectively.

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Registration number: 20091054

Title of the project: “That which does not kill you, makes you stranger: *E. coli* populations selected for 560 generations in randomly fluctuating stressful environments could become superior invaders”

Dear Mayurika Ma’am ,

I would like to take advantage of this opportunity and thank you for considering my case, particularly my medical exigency, while allowing me a breather of a few months.

I have incorporated the changes that were highlighted by the TAC reviewer in my thesis. Please note the changes, and I hope you will find these changes satisfactory.

Thanking you

Yours sincerely

Somendra Singh Kharola

Revisions incorporated after thesis review by TAC member

I am grateful to the TAC member for perusing my thesis. All the comments made by the TAC member have been considered, and the changes have been made accordingly.

1. Methods section, especially the competition assay section, has been made more explicit and detailed.
2. The concept of relative fitness has been delineated earlier in the competition assay section, and repeated once again later in the statistical analysis section.
3. Figure 1 has been rechecked, and it has been found that there indeed did exist a mistake on my part, and hence the graphs have been plotted once again. Now, the error bars do not overlap.
4. Figures 10-13: The terminology ‘H’ has been replaced with ‘F’ in order to avoid confusion.