

LETTER

Interplay of population size and environmental fluctuations: A new explanation for fitness cost rarity in asexuals

Yashraj Chavhan  | Sarthak Malusare | Sutirth Dey 

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

Correspondence

Sutirth Dey, Department of Biology, Indian Institute of Science Education and Research Pune, Dr Homi Bhabha Road, Pune, Maharashtra 411 008, India.
Email: s.dey@iiserpune.ac.in

Present address

Sarthak Malusare, Gaia Doctoral School, Institut des sciences de l'évolution (ISEM), 1093-1317 Route de Mende, Montpellier, 34090, France

Funding information

Department of Biotechnology, Ministry of Science and Technology, India, Grant/Award Number: BT/PR22328/BRB/10/1569/2016; Senior Research Fellowship, Council of Scientific and Industrial Research; IISER Pune

Editor: Tim Coulson

Abstract

Theoretical models of ecological specialisation commonly assume that adaptation to one environment leads to fitness reductions (costs) in others. However, experiments often fail to detect such costs. We addressed this conundrum using experimental evolution with *Escherichia coli* in several constant and fluctuating environments at multiple population sizes. We found that in fluctuating environments, smaller populations paid significant costs, but larger ones avoided them altogether. Contrastingly, in constant environments, larger populations paid more costs than the smaller ones. Overall, large population sizes and fluctuating environments led to cost avoidance only when present together. Mutational frequency distributions obtained from whole-genome whole-population sequencing revealed that the primary mechanism of cost avoidance was the enrichment of multiple beneficial mutations within the same lineage. Since the conditions revealed by our study for avoiding costs are widespread, it provides a novel explanation of the conundrum of why the costs expected in theory are rarely detected in experiments.

KEYWORDS

costs of adaptation, ecological specialisation, experimental evolution, fluctuating environments, maladaptation, mutation fixation, mutation supply, whole-genome whole-population sequencing

INTRODUCTION

Costs of adaptation, also known as 'fitness costs' and 'true trade-offs', entail that fitness gains in one environment lead to fitness reductions in another (Bono et al., 2017). Such costs are instrumental in understanding why species tend to favour some particular environments over others (Bono et al., 2017; Fry, 1996). Apart from answering such fundamental questions in evolutionary ecology, understanding fitness costs can also help in combating practical challenges like the rampant spread of antibiotic resistance (Andersson & Hughes, 2010) and forecasting how species would respond to climate change (Wallenstein & Hall, 2012). Although such costs are a key assumption of numerous models of ecological specialisation (Fry, 1996; Futuyama & Moreno, 1988; Levins, 1968), a large number of experimental evolution studies spanning diverse taxa have failed to detect

them (Coustau et al., 2000; Friman & Buckling, 2013; Rausher, 1984; Vasilakis et al., 2009; Vila-Aiub et al., 2009). Consequently, explaining this rarity of detectable fitness costs has been a major challenge over the last few decades (Fry, 1996; Remold, 2012).

Here we investigate the evolutionary emergence and avoidance of fitness costs in asexual microbial populations, which have proven to be convenient model systems for experimental evolution studies over hundreds of generations (Kassen, 2014). Whereas numerous microbial experimental evolution studies have reported the absence of detectable fitness costs altogether, several others have found such costs in some populations but not in others (Table S1).

An important but trivial explanation for the failure to find costs is the absence of any real costs altogether (Coustau et al., 2000). Indeed, some recent investigations have found the pleiotropy of new mutations to be largely

positive and not negative (i.e. costly) (Sane et al., 2018). More importantly, the extant literature offers three distinct explanations as to why fitness costs may exist but remain undetected in empirical studies: First, costs can be detected only under certain environmental conditions which the experimental setup may fail to provide (Coustau et al., 2000; Kassen, 2014). Second, it is a statistically demanding task to detect antagonistic pleiotropy, the very foundation of fitness costs, which entails that a mutation that is beneficial in one environment is deleterious in another. This is because detecting costs requires establishing the statistical significance of both the beneficial and deleterious effects simultaneously, and the experiment may not have enough statistical power to do so (Coustau et al., 2000). Third, the emergence of fitness costs is expected to require a threshold amount of time; such costs may appear only after several thousand generations of microbial evolution (Jasmin & Zeyl, 2013; Satterwhite & Cooper, 2015). However, none of the above explanations sufficiently account for the diversity in cost detection over two decades of experimental evolution studies. Interestingly, a recent meta-analysis of microbial experimental evolution studies provides a new explanation for the emergence of fitness costs based on environmental stability, suggesting that environments imposing a single (constant) selection pressure frequently lead to fitness costs that can be avoided in environments fluctuating across multiple individual selection pressures (Bono et al., 2017). Antagonistic pleiotropy can evolve freely if the environment does not allow the expression of the ensuing fitness costs. Since selection would be blind to antagonistic pleiotropy if the environment does not change, fitness costs are likelier to appear in constant environments than fluctuating ones.

Unfortunately, the above prediction holds only weakly as many microbial experimental evolution studies have failed to find lower costs in fluctuating environments as compared to constant ones (Friman & Buckling, 2013; Jasmin & Kassen, 2007b; Ketola & Saareinen, 2015). This opens the possibility that factors other than environmental stability may be important in shaping fitness costs. One such factor is population size, which has been shown to be important in shaping the correlated changes in populations' fitness in alternative environments (Chavhan et al., 2019a, 2020). For example, a recent study showed that larger populations evolving in a constant environment suffer greater fitness costs in alternative environments (Chavhan et al., 2020). This is because adaptation in large populations is primarily driven by beneficial mutations of large effect sizes (Desai & Fisher, 2007), which are expected to carry heavier disadvantages in alternative environments (Lande, 1983; Orr & Coyne, 1992).

Thus, environmental stability and population size are two important factors that can shape fitness costs. However, the interactive effects of these two factors on costs remain unknown. Interestingly, this interaction can play out in two contrasting ways.

First, if the pleiotropy across environmental states is largely antagonistic, and large benefits in one context entail large costs in another, the multiplicity of selection pressures in a fluctuating environment would prevent the enrichment of costly large effect mutations, even if the latter were accessible to the population. This is akin to Fisher's formulation of micromutationism where adaptation is expected to proceed via mutations of small effects (Fisher, 1930). In this scenario, in fluctuating environments, both large and small populations should pay similar costs.

Second, in a fluctuating environment, evolving in larger numbers can make populations stumble upon a greater number of mutations that are beneficial in one environmental state, but not necessarily in others. The presence of multiple mutations within an individual belonging to a large asexual population can thus offset the costs carried by individual mutations in isolation. In this scenario, adapting in larger numbers in a fluctuating environment would lead to the avoidance of fitness costs. Interestingly, bacterial experimental evolution studies conducted in fluctuating environments agree with this notion: studies on smaller populations tend to detect costs, whereas those using larger populations do not (Figure 1a). Thus, in constant environments, larger populations should pay heavier costs of adaptation (Chavhan et al., 2020). However, in fluctuating environments, larger populations may either pay similar or lower costs as compared to smaller populations, depending upon which one of the above two possibilities prevails. To the best of our knowledge, no studies in the existing literature have tested these contrasting expectations empirically.

Here we use bacterial experimental evolution to address the above questions and find that population size has opposite effects on costs in constant versus fluctuating environments. Interestingly, large population size and fluctuating environments led to cost avoidance only when present together. Mutational frequency distributions obtained by whole-genome whole-population sequencing revealed how environmental fluctuations led to cost avoidance in large populations but not in smaller ones. Finally, we propose a new explanation for the rarity of fitness costs that accounts for several contrasting observations in microbial evolutionary ecology made over the last two decades.

MATERIALS AND METHODS

Experimental evolution

We performed experimental evolution with clonally derived *E. coli* populations in five different nutrient-limited environmental conditions at two different population sizes for ~480 generations (Figure 1b; see Appendix SA1 for ancestral strain details). This resulted in 10 distinct evolutionary regimens (FL, FS, TL, TS, GL, GS, SL, SS,

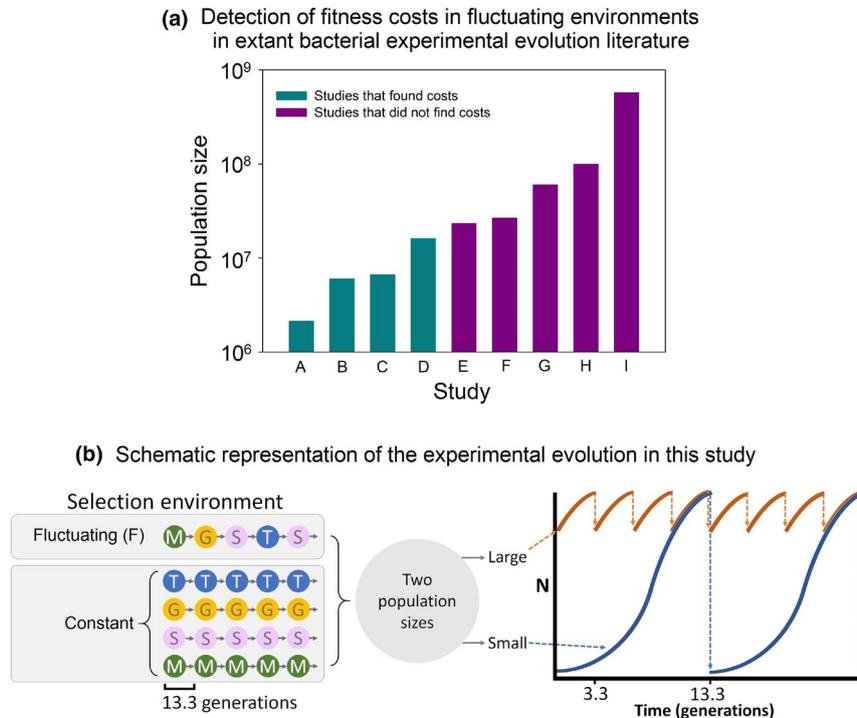


FIGURE 1 (a) The harmonic mean sizes of laboratory populations in existing bacterial experimental evolution studies on fitness costs conducted in fluctuating environments. Reference key: A (Ketola & Saarinen, 2015)*, B (Jasmin & Kassen, 2007a), C (Roemhild et al., 2015), D (Jasmin & Kassen, 2007b), E (Satterwhite & Cooper, 2015), F (Bennett & Lenski, 1999), G (Buckling et al., 2007), H (Buckling et al., 2000), I (Karve et al., 2018)[‡]. *The population size has been calculated indirectly using the stationary phase densities reported for a different species in the selection medium in question and is likely an overestimate; [‡]population size estimate was provided by the authors of Study I. (b) A schematic representation of our evolution experiment. The experimental populations were maintained in five distinct environments at two different population sizes. T, G, S and M refer to thymidine, galactose, sorbitol and maltose respectively. N stands for absolute population size. In the unpredictably fluctuating environment, the identity of the sole carbon source changed every ~13.3 generations. See the text for further details

ML and MS; here the first letter represents the sole carbon source in a regimen's environment (fluctuating (F), thymidine (T), galactose (G), sorbitol (S) and maltose (M); see Appendix SA1), whereas the second letter represents its population size (L (large) and S (small)). Each regimen had six independently evolving replicates. We used the standard batch culture technique to propagate all the 60 populations in 96 well plates (culture volume 300 μ l) shaken at 150 rpm and maintained at 37°C. In the FL and FS regimens, the substrate fluctuated unpredictably across four distinct states (T, G, S and M) approximately every ~13.3 generations (Figure 1b; the substrate was identical across FL and FS at any given time (Table S2)). Our study also involved four distinct constant environmental controls, each with an unchanging sole carbon source corresponding to one of T, G, S or M (Figure 1b). To ensure that the L and S populations spent similar time periods in the stationary phase, we bottlenecked the L populations 1:10 every 12 h (~3.3 generations), and the S populations 1:10⁴ every 48 h (~13.3 generations). Since the culture volume was identical for L and S, this bottlenecking scheme ensured that an S population never had more individuals (and thus access to more variation) than an L population facing the same environment (Figure S1 and Appendix SA2). The harmonic mean population size for our principal treatment (F) was $\sim 1.01 \times 10^8$ for

FL and $\sim 4.04 \times 10^5$ for FS. Moreover, the adaptively relevant population sizes for FL and FS were $\sim 9.13 \times 10^6$ and $\sim 2.28 \times 10^3$ respectively (Chavhan et al., 2019b). The selection protocol pertaining to the T and G populations has been reported in Chavhan et al., (2020).

Fitness quantification and detection of costs

We conducted fitness measurements for all the 60 independently evolving populations in all four carbon sources (T, G, M and S) at the end of the evolution experiment (~480 generations). To this end, we revived the cryo-stocks belonging to each of the 60 experimental populations in a common nutrient-limited environment that was not encountered by any population during the ~480 generations of our experiment (glucose-based M9 minimal medium) and allowed them to grow for 24 h. Using a well-plate reader (Synergy HT, BIOTEK[®] Winooski, VT, USA), we then performed automated growth measurements on each of the 60 revived populations in all four different minimal media, each based on one of T, G, M or S. Ensuring that the physical conditions during the fitness measurements were the same as the culture conditions, we obtained growth readings (optical density (OD) at 600 nm) every 20 min for 24 h.

Since the total number of growth curves was much larger than number of wells in the assay plate, we used a randomised complete block design (RCBD) for growth measurements (Milliken & Johnson, 2009), assaying one replicate of each of the 10 regimens in all four environments on a given day. We used the maximum growth rate (R) as the measure of fitness, computed as the maximum slope of the growth curve over a dynamic window of 10 OD readings (Chavhan et al., 2019a,b; Leiby & Marx, 2014).

We identified the occurrence of significant costs of adaptation in our experimental populations as cases that showed adaptation to one environment and simultaneous maladaptation to another. To this end, we carried out single sample *t*-tests with the ancestral fitness level (scaled to 1) as the reference value. We then corrected for family-wise error rates using the Holm-Šidák procedure (Abdi, 2010). Cases with fitness >1 (corrected $p < 0.05$) were identified as adaptations; analogously, cases with fitness <1 (corrected $p < 0.05$) were identified as maladaptations.

We also investigated the changes in the geometric mean fitness across G, M, S and T for all the 10 evolutionary regimens (see Appendix SA3 for the statistical details).

Whole-genome whole-population sequencing

We performed whole-genome whole-population sequencing with ~100-fold mean coverage on three randomly chosen populations each from FL and FS, and their common ancestor (see Appendix SA4). We subjected the trimmed high-quality sequences to the BRESEQ pipeline (Deatherage & Barrick, 2014) (v0.33.2) to identify mutations enriched during our experiment. We initially compared the ancestral sequence to the reference *E. coli* MG1655 genome to identify differences relative to the latter expected to be found in all the six evolved populations. Next, we adjusted for these differences by using the ancestral sequence as the reference for identifying mutational frequencies in each of the six descendant populations using the ‘polymorphic’ mode in BRESEQ. For our analysis, we considered only mutations that had a frequency ≥10% (Copin et al., 2016; McDonald et al., 2016; Swings et al., 2017). Any mutation rising to frequencies ≥10% within ~480 generations in any of our populations is likely to be beneficial and highly unlikely to be neutral or deleterious (Cooper, 2018).

RESULTS

Large population size and fluctuating environments led to cost avoidance only when present together

We found that 21 out of the 40 possible combinations of regimen and assay environments showed significant fitness changes as compared to the common ancestor

(corrected $p < 0.05$; see Table S3). Using this information, we analysed the effects of population size and environmental stability in shaping fitness costs.

In the fluctuating (F) environment, the large populations (FL) completely avoided costs across all the environmental pairs under consideration (Figure 2; Tables S3 and S4). FL adapted simultaneously to both T and G and did not show a significant change in fitness (vis-à-vis the common ancestor) in S and M (Figure 2). On the other hand, the small populations evolved in the fluctuating environment (FS) adapted only to T, becoming maladapted to (and hence paid costs in) the other three sole carbon sources (G, S and M) (Figure 2; Tables S3 and S4). Taken together, when evolved in the fluctuating (F) environment, the small populations paid greater costs than the large populations, with the latter avoiding all costs altogether.

Interestingly, in the constant (control) environments, the above pattern of costs reversed completely. Here, the large populations paid heavier costs of adaptation than the smaller ones (Figure 3; the fitness changes pertaining to selection in constant T and G environments have been reported previously (Chavhan et al., 2020)). Specifically, when evolved in constant T, both TL and TS paid significant costs. Interestingly, the costs suffered by TL were significantly greater than those suffered by TS, regardless of the environmental pair in question (Figure 3a; Tables S3 and S5). When evolved in constant G, only GL paid costs (GS failed to adapt significantly to its selection environment). None of the populations evolved in constant M and S environments adapted to their respective selection environments, regardless of the population size; hence, there were no costs of adaptation in these regimens (Figure 3; Tables S3 and S5). The lack of adaptation in M and S could be due to the relatively lower scope of adaptation (presumably due to ancestral proximity to fitness optima) in these environments (Couce & Tenaillon, 2015).

Constant T and G environments are known to exhibit reciprocal fitness trade-offs with each other (Chavhan et al., 2020). In other words, adaptation to T is accompanied by maladaptation to G, and vice-versa (Chavhan et al., 2020). Consistent with this notion, we found that when evolved in the fluctuating environment (where the sole carbon source fluctuated unpredictably), the small populations (FS) indeed suffered from the T-G costs. Specifically, FS adapted to T but became significantly maladapted to G (Figure 2). Contrastingly, the large populations evolved in the fluctuating environment (FL) completely bypassed the expected T-G trade-off, adapting simultaneously to both the carbon sources, thereby avoiding costs across this environmental pair (Figure 2).

Taken together, evolution in the 10 regimens of our study reveals that an interplay of environmental stability and population size shaped how fitness costs evolved. We found that population size had opposite effects on costs during evolution in fluctuating versus constant

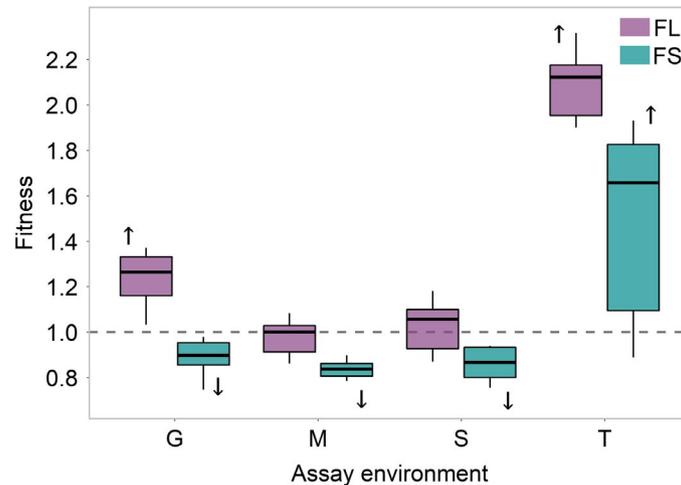


FIGURE 2 Fitness of the large (FL) and small (FS) populations evolved in the fluctuating environment across the four environmental states faced during evolution. G, M, S and T represent galactose, maltose, sorbitol and thymidine respectively. The lower and upper hinges of boxes show the 25% and 75% quantiles respectively. The horizontal line within boxes represents the median. The whiskers denote the two extremes within the data that are less than or equal to 1.5 times the length of the box extending away from the box. The dashed grey line represents the ancestral fitness. ↑ represents significant adaptation, whereas ↓ represents significant maladaptation. FL adapted simultaneously to two environments (G and T) and avoided the costs of adaptation across all the environmental pairs under consideration. Contrastingly, FS adapted to T and paid costs of adaptation in the other three environments (G, M and S). See Tables S3 and S4 for details

environments. In constant environments, larger populations evolved at greater costs. Contrastingly, in fluctuating environments, smaller populations paid greater costs, whereas larger ones avoided them altogether. Importantly, neither environmental fluctuations nor population size could sufficiently explain the emergence (or avoidance) of costs on their own (compare Figures 2 and 3). Finally, costs could be avoided completely only when fluctuating environments and large population sizes were present simultaneously (FL).

Conventional explanations cannot account for cost avoidance in our experiments

Conventional notions about the rarity of detectable fitness costs could not explain our observations. One such explanation is that perhaps the experiment did not provide the relevant conditions for costs to be expressed (Coustau et al., 2000; Kassen, 2014). This was not the case in our experiments as several environmental pairs showed significant costs. Another potential explanation is that the substantial statistical demands of establishing antagonistic pleiotropy were not met (Ågren et al., 2013; Anderson et al., 2013; Bono et al., 2017). However, we were able to statistically detect costs caused by antagonistic pleiotropy in several regimens (Figures 2 and 3). Finally, an often-quoted explanation for the lack of costs is the relatively short duration of experimental evolution studies (Jasmin & Zeyl, 2013; Schick et al., 2015). Contrastingly, our experiment found significant fitness costs across multiple environmental pairs within ~480 generations. Moreover, since such costs could be detected even in small populations (e.g. FS), as discussed

below, the lack of adequate evolutionary change was not sufficient to explain our observations.

Although our temporally fluctuating (F) environment contained only a single carbon source at any given time, the identity of this carbon source fluctuated unpredictably over four states every ~13.3 generations (Table S2). Therefore, the selection was not expected to be blind to the pleiotropic fitness effects of mutations across T, G, M and S. Despite evolving in this fluctuating environment, the FS populations paid significant fitness costs. Thus, Figure 2 shows that contrary to the expectations of the extant literature (Bono et al., 2017), the presence of multiple selection pressures (which prevent 'blindness' to costs) can be insufficient for cost avoidance.

Evolutionary success in fluctuating environments is reflected by the geometric mean fitness (henceforth, GeoMean) across the states about which the environment oscillates (and not necessarily the arithmetic mean fitness) (Orr, 2007). We found that FL had significantly greater GeoMean than both FS and the common ancestor across G, M, S and T (Figure S2a; Table S6). In contrast, the GeoMean of FS was not significantly different from the ancestral value (Figure S2a; Table S6). Furthermore, as expected, evolution in constant environments did not result in increased GeoMean above the ancestral value, regardless of the population size (Figure S2b and Table S6). FL was better prepared to face the fluctuating (F) environment than all the eight constant environmental regimens (Tables S7 and S8). Surprisingly, the preparedness of FS to face the environmental fluctuations across G, M, S and T was similar to most constant environment regimens (Tables S8 and S9). This highlights the key role of population size in shaping fitness across the component states of fluctuating environments.

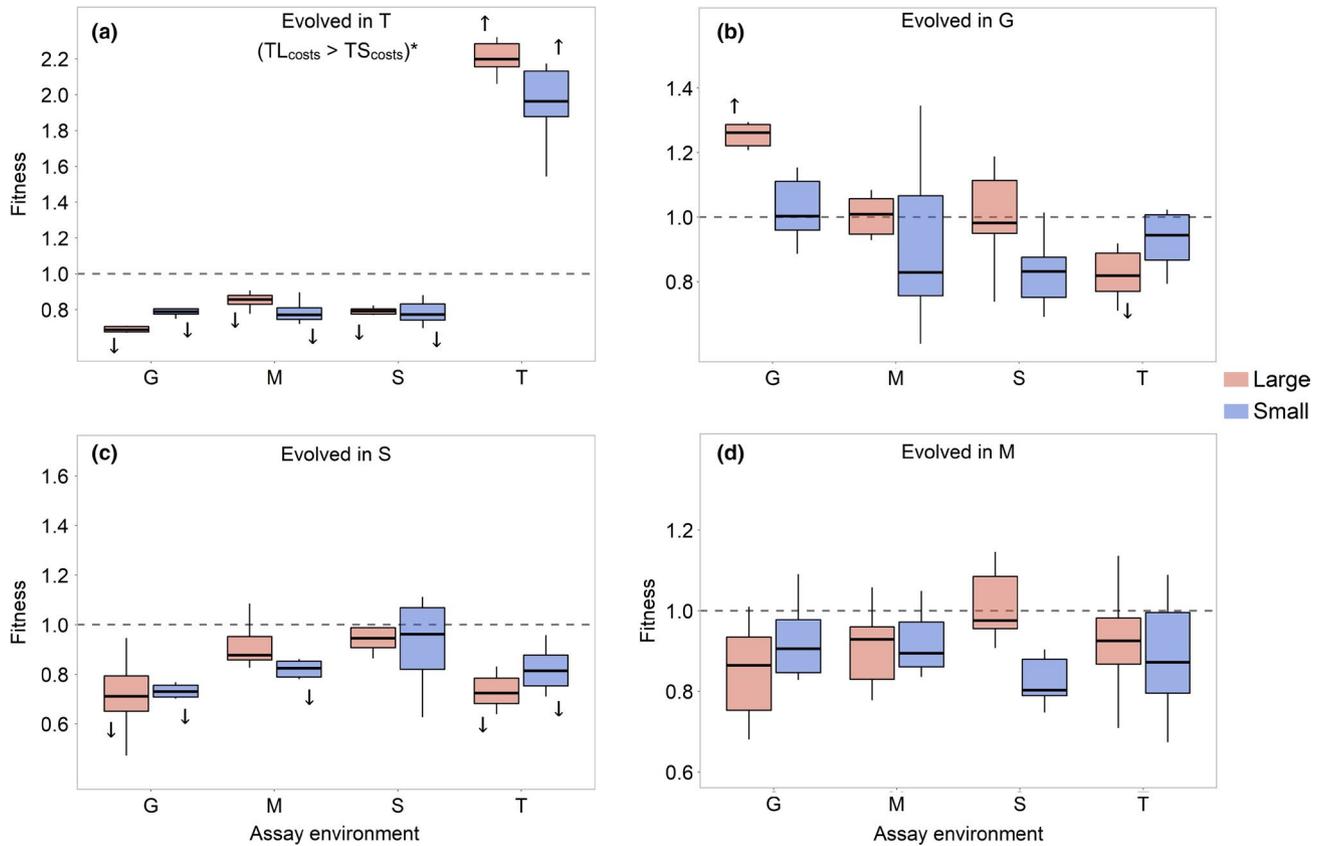


FIGURE 3 Fitness of the large and small populations evolved in constant environments. The lower and upper hinges of boxes show the 25% and 75% quantiles respectively. The horizontal line within boxes represents the median. The whiskers denote the two extremes within the data that are less than or equal to 1.5 times the length of the box extending away from the box. The dashed grey line represents the ancestral fitness. Please note the differences in y-axes across panels. ↑ represents significant adaptation while ↓ represents significant maladaptation. (a) When evolved in T, both the large (TL) and small populations paid costs in the other three environments (G, M and S). The costs paid by TL were significantly greater than those paid by TS (Chavhan et al., 2020). (b) GL paid significant costs in T. GL did not have significantly different fitness relative to the common ancestor in M and S. GS did not adapt significantly to G. Hence there were no costs of adaptation in this case. (c) Both SL and SS failed to show significantly different fitness in S with respect to the common ancestor. Hence there were no costs of adaptation in either SL or SS. (d) Neither ML nor MS had significantly different fitness in M with respect to the common ancestor. Hence there were no costs of adaptation in either ML or MS. See Tables S3 and S5 for details

The lower magnitude of evolutionary change in FS owing to their smaller population size is not sufficient to explain the contrasting fitness patterns of FL and FS. After ~480 generations, FS had significantly different fitness than the ancestor in all four substrates (adaptation to T but maladaptation to G, M and S); contrastingly, FL's fitness was significantly different from the ancestor in only two substrates (adaptation to both G and T (Figure 2)). Thus, FL and FS differed in terms of both the direction and the magnitude of fitness changes. Moreover, hypothetically increasing the magnitude of fitness changes in FS will not make it resemble FL (e.g. FS will have to reverse their direction of fitness change in G to resemble FL (Figure 2)). Importantly, the GeoMean of FL and FS cannot capture such contrasting directions of their fitness changes; FS paid more costs than FL despite having a relatively lower GeoMean. In FS, adaptation to T was offset by maladaptation to G, M and S, keeping their GeoMean at the ancestral level (Figure S2a).

Thus, cost avoidance in fluctuating environments in large populations cannot be trivially explained by their relatively greater magnitude of evolutionary change. Rather, by utilising multiple selection pressures, large population size changed both the magnitudes and the directions of evolutionary trajectories in components of the fluctuating environment, thereby leading to cost avoidance.

The genetic basis of cost avoidance

The observation that FS suffered substantial costs that were completely avoided by FL can be explained by the notion that in the presence of multiple selection pressures, a threshold amount of mutational supply is required to avoid costs. Owing to their relatively larger population size, FL are expected to have much higher mutational supply than FS. We hypothesised that FL enriched more mutations than FS, which made them adapt to multiple

carbon sources, thereby avoiding the costs paid by FS. Indeed, we found that the number of mutations rising to frequencies $\geq 10\%$ was much greater in FL as compared to FS (Figure 4; see Table S10 and Appendix SA5 for detailed descriptions of mutations).

We found that the loci mutated in FL are known to be associated with the uptake/metabolism of either G or T in the extant literature. Contrastingly, the loci mutated in FS had known links to T uptake/metabolism but none with that of G (Figure 4). This agrees with the observation that FL adapted to both T and G, whereas FS adapted to T but not to G.

Since fitness in T has been shown to be negatively correlated with fitness in G (Chavhan et al., 2020), mutations beneficial in T are likely to be deleterious in G, and vice versa. Hence, the presence of several T-associated mutations at high frequencies in FS can explain their maladaptation to G. Moreover, we did not find any known G-associated mutations in FS that could alleviate the putative maladaptive effects of T-associated mutations in G. Had there been no G-associated mutations in FL, the enrichment of

a relatively larger number of T-associated mutations should have led to greater maladaptation of FL in G. However, we found several G-associated mutations at high frequencies in FL that could explain their adaptation to G (and hence their T-G cost avoidance) (Figure 4). We now turn to the potential population genetic drivers of evolution in our study.

Antagonistic pleiotropy can readily explain the positive relationship between population size and fitness costs observed in constant environments (Cohan et al., 1994; Cooper, 2014) (Figure 3). Since these populations faced only one substrate throughout the experiment, their evolution was blind to fitness changes in other substrates. The pleiotropic disadvantages of beneficial mutations are generally expected to be correlated with their direct effects (Lande, 1983; Orr & Coyne, 1992; Otto, 2004). Since larger asexual populations adapt primarily via beneficial mutations with relatively greater direct effect sizes (Desai & Fisher, 2007), adapting to constant environments in larger numbers should lead to heavier costs, as observed in our study (Figure 3) (Chavhan et al., 2020).

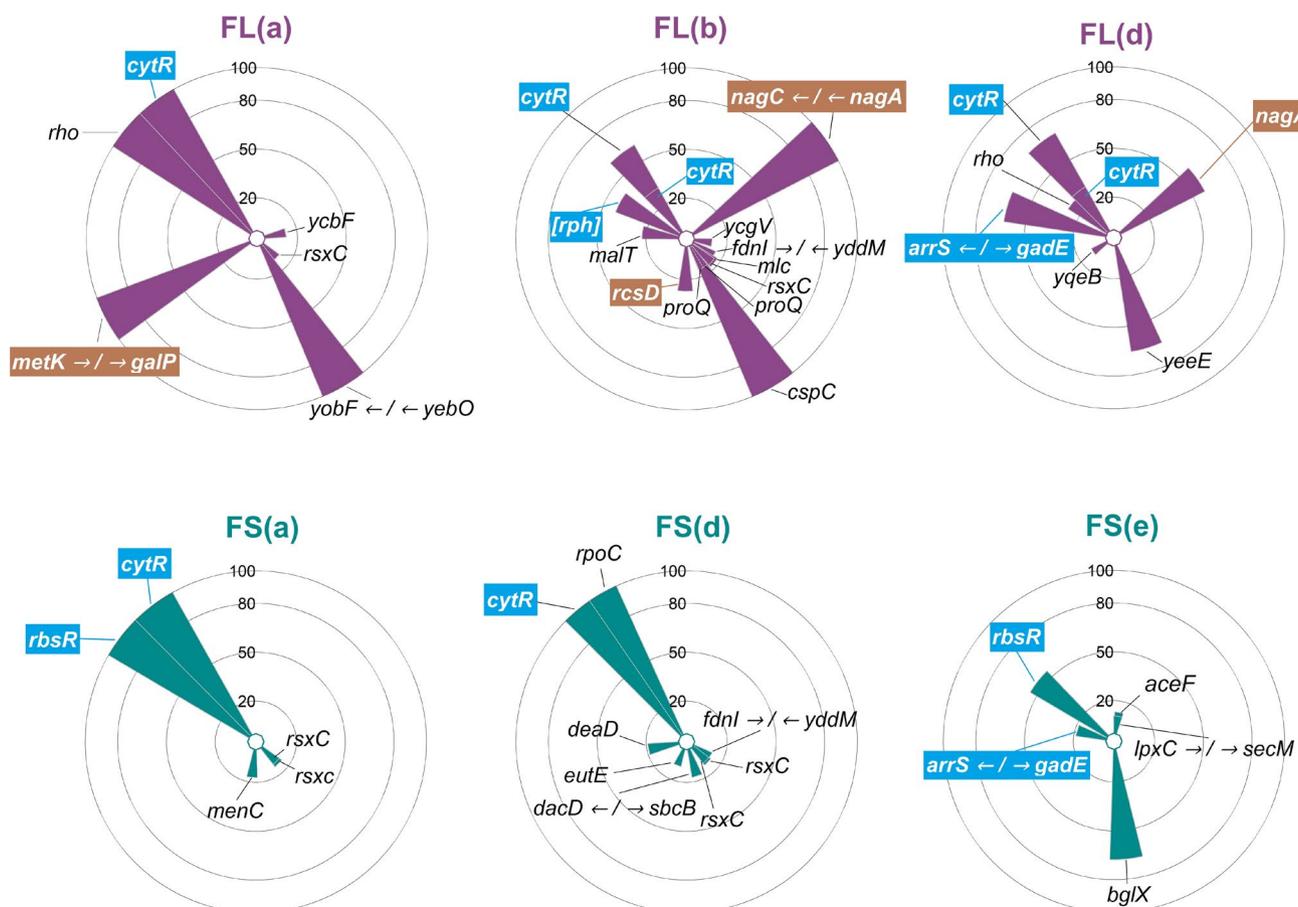


FIGURE 4 The spectrum of mutations in populations of different sizes observed after ~480 generations of evolution in the fluctuating environment. Three randomly chosen replicate populations each of FL (upper row) and FS (lower row) were subjected to whole-genome whole-population sequencing. The radial bars are located at the genomic position of the observed mutations and their heights represent the corresponding mutational frequency. The mutated loci known to be associated with thymidine (T) utilisation are highlighted in blue, whereas those associated with galactose (G) utilisation are highlighted in brown. See Table S10 for details

When evolved in fluctuating environments, the smaller (FS) populations paid significant costs across three distinct environmental pairs under consideration, but the larger (FL) populations avoided costs altogether. As described above, FS suffered significantly from T-G trade-offs, whereas FL bypassed them. Interestingly, despite facing both T and G as the sole sources of carbon for an equal number of generations (~120), the T-G trade-off manifested itself in FS as an adaptation to T and maladaptation to G. The greater scope of adaptation in T as compared to G can explain this asymmetry (Chavhan et al., 2020). Foremost, adaptation to T is expected to have a larger genomic target size than adaptation to G (Sajed et al., 2016). Moreover, despite evolving in constant G for ~480 generations, GS could not adapt significantly to G. Contrastingly, TS increased their fitness in T by >1.5-fold within the same period (Figure 3). Thus, the size of our small-population regimens was sufficient to adapt significantly to T but not to G. Analogous to TS, such adaptation of FS to T also led to significant maladaptation in the other three environments. In contrast to the small populations, the large populations in our study had sufficient supply of mutations to adapt to G within ~480 generations (Figure 3). Curiously, we also found that FL could adapt significantly to G despite encountering this environment intermittently for a total period of ~120 generations (Figure 2). This observation was also supported by the genomics of adaptation in FL, which revealed substantial enrichment of putative G-associated mutations (Figure 4).

An important alternative explanation for cost avoidance in fluctuating environments involves the divergence of the population in question into multiple subpopulations, each one specialised on a different environmental component (Kassen, 2002) (Figure 5a). However, genomic data suggest that this is an unlikely cause of cost avoidance in our study. We used the frequency distributions of observed mutations to calculate the probability that an individual had at least one putative G- and one putative T-associated mutation (see Appendix SA6 for detailed calculations). This probability was 1.00, 0.84 and 0.53 in the three sequenced replicates of FL (the regimen that avoided costs altogether) (Figure 5b). Such high likelihoods suggest that it is unlikely that FL avoided costs by divergent specialisation on individual substrates (T or G) within populations.

DISCUSSION

We offer a novel explanation for an important conundrum in evolutionary ecology, namely the rarity of detectable fitness costs in empirical studies. Specifically, we demonstrate a previously unreported interaction of population size and environmental stability that determines the evolutionary emergence (or avoidance) of costs. These results can explain how evolving populations can

escape costs despite substantial antagonistic pleiotropy across environmental states. We show that the simultaneity of two conditions, namely large population size and environmental fluctuations, can avoid all the costs that potentially evolve when these conditions are not present together.

Theoretical studies expect both severe population bottlenecks and fluctuating environments to impede mutational fixation. To our knowledge, our study presents the first demonstration of rapid fixation of multiple mutations in asexual populations evolving in fluctuating environments in the face of harsh periodic bottlenecks (see Appendix SA7).

The environments of most natural populations of asexual microbes are known to fluctuate temporally (Green & Bohannan, 2006; Muscarella et al., 2019). Moreover, such natural asexual populations also tend to have extremely large sizes (Tenaillon et al., 2010; Torsvik et al., 2002). Our results suggest that if the asexual population under consideration has a history of evolving in fluctuating environments in large numbers, it is expected to have reached its current state after having avoided fitness costs during its evolution. Therefore, if a sample from such a population is now employed to analyse fitness correlations in a single-generation study, such correlations may not be negative, and costs may not be found.

Contrastingly, several laboratory evolution studies using unchanging (constant) environments and large population sizes ($> 10^6$ in terms of the harmonic mean population size) have successfully detected costs (Table S11). This agrees with the interplay of population size and environmental fluctuations revealed here, which predicts such a combination of constant environment and large populations to suffer significant costs.

Thus, apart from explaining why costs may not be detected in single-generation studies with natural isolates, our observations also explain why costs can still be detected if the artificially controlled conditions remain constant over a few hundred generations in an evolution experiment.

Although our study employed nutritional stresses, our explanations are expected to apply to the general notion of fitness costs across environments in asexual populations. Our results offer particularly important implications for understanding the evolution of antibiotic resistance. Mutations that confer resistance to antibiotics have been routinely shown to bear fitness costs in drug-free conditions (Andersson & Hughes, 2010). Interestingly, resistant microbes mostly evolve in an environment that fluctuates unpredictably across antibiotic-laden and antibiotic-free conditions (Baquero et al., 1998). Our results predict that small populations evolving in fluctuating environments will suffer heavy fitness costs, whereas large populations will avoid them (Figure 2). Thus, even if most antibiotic resistance mutations are costly in drug-free conditions, large microbial

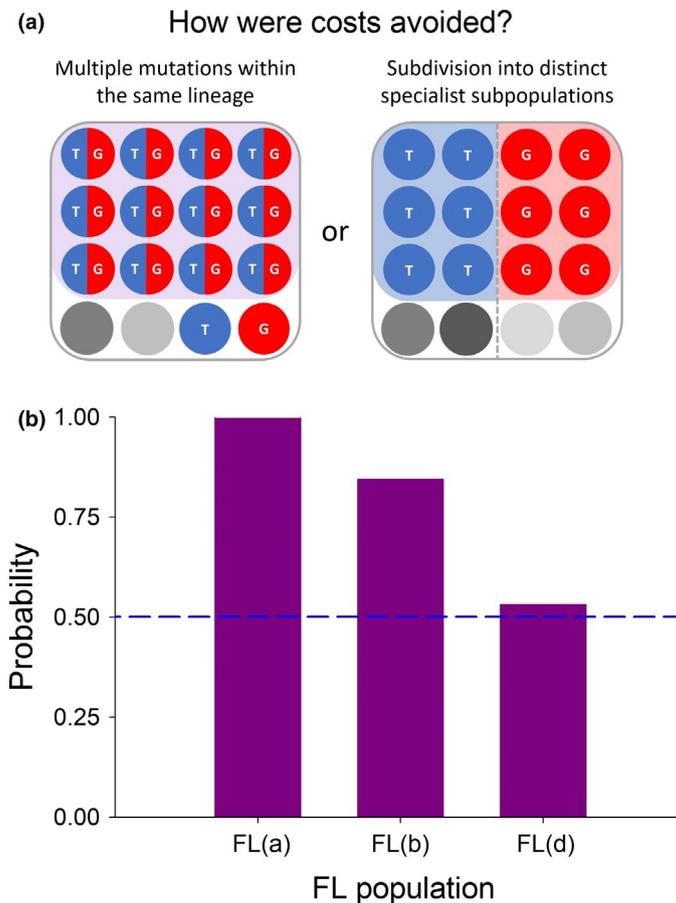


FIGURE 5 The principal mechanism of cost avoidance in our study. (a) There could be two distinct potential mechanisms of cost avoidance in our study: enrichment of multiple beneficial mutations within the same lineage (left) versus subdivision into multiple distinct specialist subpopulations, each specialised on a different environmental state (right). (b) The probability of an FL individual carrying at least one T- and one G-associated mutation, shown for all the three sequenced FL populations. In our experiments, costs were avoided primarily due to the enrichment of multiple mutations within the same lineage under fluctuating selection pressures

population sizes stemming from a lack of sanitary conditions (Cantón et al., 2013) could lead to the vigorous spread of cost-free resistance.

Next, we briefly discuss the extent to which our findings can also apply to sexual macro-organisms in different ecological contexts. Macro-organisms usually have much lower population sizes than micro-organisms. Within short evolutionary timeframes, whereas micro-organisms generally adapt via *de novo* mutations (Arber 2000), macro-organisms tend to do so via the standing genetic variation (Barrett & Schluter, 2008).

In constant environments, both asexual micro-organismal and sexual macro-organismal populations should be oblivious to fitness changes in conditions they do not encounter. Since larger sexual populations generally adapt better (Neher *et al.* 2010), to the extent that greater adaptation to selection conditions is correlated with greater maladaptation in unencountered environments, larger sexual macro-organismal populations will accrue more costs and undergo greater local adaptation (echoing Sanford and Kelly's (2011) prediction for marine invertebrates). Indeed, Jakobsson & Dinnéztz (2005) found greater local adaptation in larger populations of a

perennial plant within constant environments. Similarly, a meta-analysis consisting primarily of studies in constant environments found more local adaptation in larger plant populations (Leimu & Fischer, 2008). This aligns with our observations (Figure 3; Chavhan et al., 2020).

In fluctuating environments, we show that large (but not small) asexual populations avoid fitness costs by enriching multiple beneficial mutations (Figures 4 and 5). Although temporal environmental fluctuations are also expected to reduce fitness trade-offs in sexual macro-organisms (Gilchrist 1995), they are unlikely to do so by rapidly enriching multiple *de novo* mutations. Interestingly, recombination can combine multiple pre-existing inter-locus variants, potentially offsetting the costs they carry in isolation. Such cost alleviation in fluctuating environments via recombination should depend on the availability of standing inter-locus variation. Since sexual diploids can mask recessive alleles and maintain more usable variation than haploids (Crow & Kimura, 1965), cost mitigation in fluctuating environments should be greater in diploids. All else being equal, larger sexual macro-organismal populations would have

greater standing variation, which should make them better at avoiding costs in fluctuating environments. This notion aligns with the insights from several bacterial studies (Figure 1a), including ours (Figure 2).

Notwithstanding the above observations, due caution must be exercised while extrapolating our insights to sexual macro-organisms. For example although not a subject of our study, spatial heterogeneity in the environment can be an important determinant of local adaptation (Blanquart *et al.* 2012), as revealed by a meta-analysis in animals (Hereford, 2009). Interestingly, Leimu and Fischer (2008) did not find any significant effect of either temporal or spatial heterogeneity on local adaptation in plants and speculated that the two factors may cancel each other out. Thus, potentially important confounds like spatial heterogeneity should be explicitly accounted for while studying how population size interacts with environmental fluctuations in natural populations. However, as argued above, despite being driven by fundamentally distinct mechanisms, fitness costs can be shaped by similar interplays of population size and environmental stability in both asexual micro-organisms and sexual macro-organisms. Thus, in principle, our insights can also benefit studies of ecological costs in sexual macro-organisms.

ACKNOWLEDGEMENTS

We thank S. Selveshwari for help with NGS analysis and Milind Watve and M.S. Madhusudhan for their valuable inputs. YDC was supported by a Senior Research Fellowship initially sponsored by IISER Pune and then by the Council of Scientific and Industrial Research (CSIR), Govt. of India. YDC also acknowledges the Department of Biotechnology, Govt. of India for post-doctoral funding for this work. SM was supported by an INSPIRE undergraduate fellowship, sponsored by the Department of Science and Technology (DST), Govt. of India. This project was supported by an external grant (BT/PR22328/BRB/10/1569/2016) from the Department of Biotechnology, Govt. of India, and internal funding from IISER Pune.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

YC and SD designed the study. YC and SM conducted the experiments. YC analysed the data. YC and SD wrote the manuscript with inputs from SM.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/ele.13831>.

DATA AVAILABILITY STATEMENT

The data relevant to this study are publicly available in the Dryad Digital Repository (<https://doi.org/10.5061/dryad.7wm37pvs>).

ORCID

Yashraj Chavhan  <https://orcid.org/0000-0002-6367-821X>

Sutirth Dey  <https://orcid.org/0000-0001-9210-3055>

REFERENCES

- Abdi, H. (2010) Holm's sequential Bonferroni procedure. In: Salkind, N. (Ed.) *Encyclopedia of Research Design*. Thousand Oaks, CA: Sage, pp. 1–8.
- Ågren, J., Oakley, C.G., McKay, J.K., Lovell, J.T. & Schemske, D.W. (2013) Genetic mapping of adaptation reveals fitness tradeoffs in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, 110, 21077–21082.
- Anderson, J.T., Lee, C.-R., Rushworth, C.A., Colautti, R.I. & Mitchell-Olds, T. (2013) Genetic trade-offs and conditional neutrality contribute to local adaptation. *Molecular Ecology*, 22, 699–708.
- Andersson, D.I. & Hughes, D. (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology*, 8, 260–271.
- Arber, W. (2000) Genetic variation: molecular mechanisms and impact on microbial evolution. *FEMS Microbiology Reviews*, 24(1), 1–7. <https://doi.org/10.1111/j.1574-6976.2000.tb00529.x>
- Baquero, F., Negri, M.-C., Morosini, M.-I. & Blázquez, J. (1998) Antibiotic-selective environments. *Clinical Infectious Diseases*, 27, S5–S11.
- Barrett, R.D.H. & Schluter, D. (2008) Adaptation from standing genetic variation. *Trends in Ecology & Evolution*, 23, 38–44.
- Bennett, A.F. & Lenski, R.E. (1999) Experimental evolution and its role in evolutionary physiology. *Integrative and Comparative Biology*, 39, 346–362.
- Blanquart, F., Gandon, S. & Nuismer, S.L. (2012) The effects of migration and drift on local adaptation to a heterogeneous environment. *Journal of Evolutionary Biology*, 25(7), 1351–1363. <https://doi.org/10.1111/j.1420-9101.2012.02524.x>
- Bono, L.M., Smith, L.B., Pfennig, D.W. & Burch, C.L. (2017) The emergence of performance trade-offs during local adaptation: insights from experimental evolution. *Molecular Ecology*, 26, 1720–1733.
- Buckling, A., Brockhurst, M.A., Travisano, M. & Rainey, P.B. (2007) Experimental adaptation to high and low quality environments under different scales of temporal variation. *Journal of Evolutionary Biology*, 20, 296–300.
- Buckling, A., Kassen, R., Bell, G. & Rainey, P.B. (2000) Disturbance and diversity in experimental microcosms. *Nature*, 408, 961.
- Cantón, R., Horcajada, J.P., Oliver, A., Garbajosa, P.R. & Vila, J. (2013) Inappropriate use of antibiotics in hospitals: The complex relationship between antibiotic use and antimicrobial resistance. *Enfermedades Infecciosas y Microbiología Clínica*, 31, 3–11.
- Chavhan, Y.D., Ali, S.I. & Dey, S. (2019b) Larger numbers can impede adaptation in asexual populations despite entailing greater genetic variation. *Evolutionary Biology*, 46, 1–13.
- Chavhan, Y., Karve, S. & Dey, S. (2019a) Adapting in larger numbers can increase the vulnerability of *Escherichia coli* populations to environmental changes. *Evolution*, 73, 836–846.
- Chavhan, Y., Malusare, S. & Dey, S. (2020) Larger bacterial populations evolve heavier fitness trade-offs and undergo greater ecological specialization. *Heredity*, 124(6), 726–736.
- Cohan, F.M., King, E.C. & Zawadzki, P. (1994) Amelioration of the deleterious pleiotropic effects of an adaptive mutation in *Bacillus subtilis*. *Evolution*, 48, 81–95.
- Cooper, V.S. (2014) The origins of specialization: Insights from bacteria held 25 years in captivity. *PLoS Biology*, 12, e1001790.
- Cooper, V.S. (2018) Experimental evolution as a high-throughput screen for genetic adaptations. *mSphere*, 3(3), e00121–e218.
- Copin, R., Wang, X., Louie, E., Escuyer, V., Coscolla, M., Gagneux, S. *et al.* (2016) Within host evolution selects for a dominant

- genotype of mycobacterium tuberculosis while T cells increase pathogen genetic diversity. *PLoS Pathogens*, 12, e1006111.
- Couce, A. & Tenaillon, O.A. (2015) The rule of declining adaptability in microbial evolution experiments. *Frontiers in Genetics*, 6, <https://doi.org/10.3389/fgene.2015.00099>.
- Coustau, C., Chevillon, C. & Ffrench-Constant, R., (2000) Resistance to xenobiotics and parasites: can we count the cost? *Trends in Ecology & Evolution*, 15, 378–383.
- Crow, J.F. & Kimura, M. (1965) Evolution in sexual and asexual populations. *American Naturalist*, 99, 439–450.
- Deatherage, D.E. & Barrick, J.E. (2014) Identification of Mutations in Laboratory-Evolved Microbes from Next-Generation Sequencing Data Using *breseq*. In: Sun, L. & Shou, W. (Eds.) *Engineering and Analyzing Multicellular Systems*. Methods in Molecular Biology (Methods and Protocols), vol. 1151. New York, NY: Humana Press, pp. 165–188.
- Desai, M.M. & Fisher, D.S. (2007) Beneficial mutation–selection balance and the effect of linkage on positive selection. *Genetics*, 176, 1759–1798.
- Fisher, R.A. (1930) *The genetical theory of natural selection*. Oxford, UK: Oxford University Press.
- Friman, V.-P. & Buckling, A. (2013) Effects of predation on real-time host–parasite coevolutionary dynamics. *Ecology Letters*, 16, 39–46.
- Fry, J.D. (1996) The evolution of host specialization: Are trade-offs overrated? *American Naturalist*, 148, S84–S107.
- Futuyma, D.J. & Moreno, G. (1988) The evolution of ecological specialization. *Annual Review of Ecology and Systematics*, 19, 207–233.
- Gilchrist, G.W. (1995) Specialists and generalists in changing environments. I. Fitness landscapes of thermal sensitivity. *The American Naturalist*, 146(2), 252–270.
- Green, J. & Bohannan, B.J.M. (2006) Spatial scaling of microbial biodiversity. *Trends in Ecology & Evolution*, 21, 501–507.
- Hereford, J. (2009) A quantitative survey of local adaptation and fitness trade-offs. *American Naturalist*, 173, 579–588.
- Jakobsson, A. & Dinnétz, P. (2005) Local adaptation and the effects of isolation and population size - the semelparous perennial *Carlina vulgaris* as a study case. *Evolutionary Ecology*, 19(5), 449–466.
- Jasmin, J.-N. & Kassen, R. (2007a) Evolution of a single niche specialist in variable environments. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 274, 2761–2767.
- Jasmin, J.-N. & Kassen, R. (2007b) On the experimental evolution of specialization and diversity in heterogeneous environments. *Ecology Letters*, 10, 272–281.
- Jasmin, J.-N. & Zeyl, C. (2013) Evolution of pleiotropic costs in experimental populations. *Journal of Evolutionary Biology*, 26, 1363–1369.
- Karve, S.M., Bhave, D. & Dey, S. (2018) Extent of adaptation is not limited by unpredictability of the environment in laboratory populations of *Escherichia coli*. *Journal of Evolutionary Biology*, 31, 1420–1426.
- Kassen, R. (2002) The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology*, 15, 173–190.
- Kassen, R. (2014) *Experimental evolution and the nature of biodiversity*. Greenwood Village, CO: Roberts and Company, pp. 65–78.
- Ketola, T. & Saarinén, K. (2015) Experimental evolution in fluctuating environments: tolerance measurements at constant temperatures incorrectly predict the ability to tolerate fluctuating temperatures. *Journal of Evolutionary Biology*, 28, 800–806.
- Lande, R. (1983) The response to selection on major and minor mutations affecting a metrical trait. *Heredity*, 50, 47–65.
- Leiby, N. & Marx, C.J. (2014) Metabolic erosion primarily through mutation accumulation, and not tradeoffs, drives limited evolution of substrate specificity in *Escherichia coli*. *PLoS Biology*, 12, e1001789.
- Leimu, R. & Fischer, M. (2008) A meta-analysis of local adaptation in plants. *PLoS One*, 3, e4010.
- Levins, R. (1968) *Evolution in changing environments: some theoretical explorations*. Princeton, NJ: Princeton University Press.
- McDonald, M.J., Rice, D.P. & Desai, M.M. (2016) Sex speeds adaptation by altering the dynamics of molecular evolution. *Nature*, 531, 233–236.
- Milliken, G.A. & Johnson, D.E. (2009). Analysis of messy data, volume I: Designed Experiments. Chapman and Hall/CRC.
- Muscarella, M.E., Boot, C.M., Broeckling, C.D. & Lennon, J.T. (2019) Resource heterogeneity structures aquatic bacterial communities. *ISME Journal*, 1, 2183–2195.
- Neher, R.A., Shraiman, B.I. & Fisher, D.S. (2010) Rate of adaptation in large sexual populations. *Genetics*, 184(2), 467–481. <https://doi.org/10.1534/genetics.109.109009>
- Orr, H.A. (2007) Absolute fitness, relative fitness, and utility. *Evolution*, 61, 2997–3000.
- Orr, H.A. & Coyne, J.A. (1992) The genetics of adaptation: A reassessment. *American Naturalist*, 140, 725–742.
- Otto, S.P. (2004) Two steps forward, one step back: the pleiotropic effects of favoured alleles. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 271, 705–714.
- Rauscher, M.D. (1984) Tradeoffs in performance on different hosts: Evidence from within- and between-site variation in the beetle *Deloyala guttata*. *Evolution*, 38, 582–595.
- Remold, S. (2012) Understanding specialism when the jack of all trades can be the master of all. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 279(1749), 4861–4869.
- Roemhild, R., Barbosa, C., Beardmore, R.E., Jansen, G. & Schulenburg, H. (2015) Temporal variation in antibiotic environments slows down resistance evolution in pathogenic *Pseudomonas aeruginosa*. *Evolutionary Applications*, 8, 945–955.
- Sajed, T., Marcu, A., Ramirez, M., Pon, A., Guo, A.C., Knox, C. et al. (2016) ECMDDB 2.0: A richer resource for understanding the biochemistry of *E. coli*. *Nucleic Acids Research*, 44, D495–D501.
- Sane, M., Miranda, J.J. & Agashe, D. (2018) Antagonistic pleiotropy for carbon use is rare in new mutations. *Evolution*, 72, 2202–2213.
- Sanford, E. & Kelly, M.W. (2011) Local Adaptation in Marine Invertebrates. *Annual Review of Marine Science*, 3, 509–535.
- Satterwhite, R.S. & Cooper, T.F. (2015) Constraints on adaptation of *Escherichia coli* to mixed-resource environments increase over time. *Evolution*, 69, 2067–2078.
- Schick, A., Bailey, S.F. & Kassen, R. (2015) Evolution of fitness trade-offs in locally adapted populations of *Pseudomonas fluorescens*. *American Naturalist*, 186, S48–S59.
- Swings, T., Van den Bergh, B., Wuyts, S., Oeyen, E., Voordeckers, K. & Verstrepen, K.J. et al. (2017) Adaptive tuning of mutation rates allows fast response to lethal stress in *Escherichia coli*. Adaptive tuning of mutation rates allows fast response to lethal stress in *Escherichia coli*. *ELife*, 6, 6.
- Tenaillon, O., Skurnik, D., Picard, B. & Denamur, E. (2010) The population genetics of commensal *Escherichia coli*. *Nature Reviews Microbiology*, 8, 207–217.
- Torsvik, V., Øvreås, L. & Thingstad, T.F. (2002) Prokaryotic diversity-magnitude, dynamics, and controlling factors. *Science*, 296, 1064–1066.
- Vasilakis, N., Deardorff, E.R., Kenney, J.L., Rossi, S.L., Hanley, K.A. & Weaver, S.C. (2009) Mosquitoes put the brake on arbovirus evolution: Experimental evolution reveals slower mutation accumulation in mosquito than vertebrate cells. *PLoS Pathogens*, 5, e1000467.
- Vila-Aiub, M.M., Neve, P. & Powles, S.B. (2009) Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytologist*, 184, 751–767.
- Wallenstein, M.D. & Hall, E.K. (2012) A trait-based framework for predicting when and where microbial adaptation to climate

change will affect ecosystem functioning. *Biogeochemistry*, 109, 35–47.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Chavhan, Y., Malusare S. & Dey S. (2021) Interplay of population size and environmental fluctuations: A new explanation for fitness cost rarity in asexuals. *Ecology Letters*, 24, 1943–1954. <https://doi.org/10.1111/ele.13831>