Biophysical constraints determine the selection of phenotypic fluctuations during directed evolution

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Abstract. Phenotypes of individuals in a population of organisms are not fixed. Phenotypic fluctuations, which describe temporal variation of the phenotype of an individual or individual-to-individual variation across a population, are present in populations from microbes to higher animals. Phenotypic fluctuations can provide a basis for adaptation and be the target of selection. Here we present a theoretical and experimental investigation of the fate of phenotypic fluctuations in directed evolution experiments where phenotypes are subject to constraints. We show that selecting bacterial populations for fast migration through a porous environment drives a reduction in cell-to-cell variation across the population. Using sequencing and genetic engineering we reveal the genetic basis for this reduction in phenotypic fluctuations. We offer one interpretation for this reduction by developing a simple, abstracted, simulation model of the evolution of phenotypic fluctuations subject to constraints. We find that directed evolution applied to constrained phenotypes results in a decrease (increase) in phenotypic fluctuations when selection is weak (strong) without explicitly specifying a mechanistic basis for phenotypic fluctuations. This result is a generic property of selection acting on phenotypic fluctuations near a bound on the selected phenotype. We explore the mechanism of the observed reduction of phenotypic fluctuations in our experimental system, discuss the relevance of our abstract model to the experiment and explore its broader implications for evolution.

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1. Introduction

Natural selection acts at the level of the phenotype. Unlike genomes, phenotypes can be highly variable over the lifetime of a single organism or heterogeneous across a genetically identical population. Given the central role of the phenotype in selection, phenotypic fluctuations are believed to play an important role in evolution.

Therefore, understanding the evolutionary origins and impacts of phenotypic fluctuations will be central to any quantitative theory of evolution. Phenotypic fluctuations can arise by stochastic variation in gene expression [1], which can be associated with physiological responses to environmental variation (plasticity)[2]. In bacteria, non-genetic phenotypic variability in a population is critical for survival in the presence of antibiotics[3]. Further, non-genetic variation is present in bacterial swimming behavior[4] and is thought to be adaptive[5].

The role of phenotypic fluctuations in evolution, and how genetic variation alters phenotypic fluctuations, has been the subject of theoretical and experimental investigations since Baldwin [6]. Waddington presented compelling arguments for the role of phenotypic plasticity in facilitating evolution through genetic assimilation [7], and conceptual models of this effect abound [8]. Notably, Kaneko formulated a phenomenological model based on the fluctuation-dissipation theorem, which postulates that phenotypes exhibiting larger fluctuations should evolve more rapidly under selection [9]. The theory was tested in a directed evolution experiment by constructing a diverse population of green fluorescent protein (GFP) expressing Escherichia coli mutants synthetically and then selecting for higher levels of GFP fluorescence. The study showed that directed selection for increasing mean fluorescence resulted in reduced cell-to-cell variability in fluorescence intensity [9]. Conversely, a subsequent series of experimental studies showed that strong selection on the phenotype led to an increase in phenotypic fluctuations [10]. The interpretation of this experiment is complicated, however, because there were only a few clones in the system, and the population seemed to split into two types of mutant distinguished by the width of their phenotype fluctuations [10]. Similarly, in directed evolution experiments of cell size in E. coli a decrease of cell-to-cell variation in size was reported for weak selection whereas little change in cell size fluctuation was observed under strong selection [11].

Phenotypes arise from genotypes through the processes of transcription and translation. Therefore, any generic features of the evolution of phenotypic fluctuations might be illuminated by considering universal aspects of gene expression. Protein copy number distributions have been measured in a variety of microbial species, for example in cultured populations of bacteria [12, 13, 14] and yeast [13, 14] and in single-cells [15, 16, 17]. These studies show that the probability density of protein copy number across a population is consistently non-Gaussian and highly skewed, and reportedly well fit by gamma [15], extreme value (Fisher-Tippett-Gumbel [14] or Frechet [13]) or log-normal [12, 17] distributions, all of which are similar in shape. Regardless of the precise form of the distribution reported, one trend is clear: the standard deviation
σ is a monotonically increasing function of the mean, and the distributions can be collapsed onto a single universal curve using reduced coordinates \((n - \langle n \rangle) / \sigma\) \[^{13, 17}\]. If a phenotype can be associated with a particular dominant protein, then as the phenotype and hence the protein copy number is increased during a directed evolution experiment, one might naively expect the phenotypic standard deviation to increase as well, a result that is not generically found to be true. In reality, the relationship between protein copy number and phenotype is more complex, reflecting regulation, inhibition, and feedback. Therefore, the precise relationship between protein copy number and phenotype remains unclear, with little likelihood of a universal connection, even if the global statistics exhibit universal functional forms.

Direct empirical evidence for the relationship between phenotypic fluctuations and long-term evolution remains limited. Notable exceptions include retrospective studies of hemoglobin binding affinity across mammals\[^{18}\], but even this study does not make direct measurements of phenotypic fluctuations in time or across individuals. While experimental evolution has revealed striking examples of phenotypic evolution\[^{19, 20, 21, 22}\], quantitative measurements of phenotypic fluctuations in many of these experiments have not been made. As a result, conceptual or quantitative models of the evolution of phenotypic fluctuations remain untested.

Here we present a joint theoretical and experimental investigation of how phenotypic fluctuations evolve under selection. We use high-throughput phenotyping to show that the phenotypic variation in the population declines when bacteria are selected for faster migration through a porous environment\[^{22}\]. We then present a simple model of directed evolution which allows us to interrogate how selection strength and mutations result in the evolution of phenotypic fluctuations. We show that, depending on the strength of selection, phenotypic fluctuations can decline when phenotypes are subjected to constraints even when there is no mechanistic link between the mean trait value and phenotypic fluctuations. Finally, we discuss the possible biological mechanisms underlying the experimentally observed reduction in phenotypic fluctuations in the context of our model.

2. Evolution of faster migration in E. coli

Growing populations of motile, chemotactic bacteria migrate outward when inoculated into a low viscosity agar plate containing growth medium and a chemoattractant\[^{23, 24}\]. As cells swim and divide in this porous environment, local depletion of nutrients establishes a spatial nutrient gradient which drives chemotaxis through the three-dimensional agar matrix and subsequent nutrient consumption. Microscopically, cells move through the porous environment by executing runs, at a speed \(|v_r| \sim 20 \mu m s^{-1}\) for a run duration \(\tau_r \sim 1 s\), and tumbles which rapidly reorient the cell in \(\tau_t \sim 0.1 s\). Tumbles are essential for avoiding obstacles in order to successfully navigate the low viscosity agar\[^{23}\]. The result is a macroscopic colony that expands radially through the bulk of the plate at a constant speed after an initial growth phase. We selected populations of
E. coli (MG1655-motile, Coli Genetic Stock Center, Yale University #6300) for faster migration through soft agar by repeatedly allowing a colony to expand for a fixed interval, sampling a small population of cells from its outer edge and using a portion of this sample to inoculate a new plate while preserving the remainder cryogenically (Fig. 1). In rich medium conditions (LB, 0.3% w/v agar, 30°C), we sampled after 12 hours of expansion for a total of 15 rounds of selection. By performing time-lapse imaging on the expanding colonies, we found that the migration rate approximately doubled over the first five rounds of selection and continued to increase marginally in subsequent rounds. We found that this increase was reproducible across replicate experiments.

To understand the mechanism by which faster migration evolved, we performed single cell tracking on hundreds of individuals from the ancestral strain as well as from strains isolated after 5, 10 and 15 rounds of selection. Individual cells were trapped in a circular microfluidic chamber and recorded while swimming in liquid media for 5 minutes per cell[22]. This measurement permitted us to capture the swimming behavior of hundreds of single bacterial cells in the absence of chemical gradients. We found that the average run speed increased approximately by 50% during selection, while the duration of running events declined. The maximum growth rate, which was measured in a separate experiment by monitoring the optical density of a well-stirred liquid culture declined over the course of selection Fig. 2(D). The trade-off between swimming speed and growth rate observed in Fig. 2 is the subject of a separate study[22] and similar trade-offs have been observed elsewhere[25].

3. Phenotypic fluctuations decline with selection

We quantified phenotypic fluctuations in the ancestral population as well as populations isolated during the selection process shown in Fig. 1. Phenotypic fluctuations have previously been characterized in several ways. In some cases, fluctuations refer to the time-dependence of a specific phenotypic parameter during the lifetime of an individual[26]. In other studies, fluctuations refer to cell-to-cell variation in time-averaged phenotypic measurements over a population[27, 28, 4, 29]. Here we use the latter approach, which we define explicitly below.

A single E. coli cell exhibits a series of runs and tumbles. Each run event is described by a run duration (τ_r) and a run speed (|v_r|) and each tumble by a tumble duration (τ_t) and an angular velocity (ω_t). Even in an unstimulated environment where no gradients are present, τ_r will vary between run events, and the distribution exhibited by individual i is given by P(τ_r). Each run event for this individual has a duration drawn from this distribution. Similar distributions exist for |v_r|, τ_t, and ω_t, but ω_t is difficult to measure accurately for single cells, and we omit this parameter from consideration. We consider the phenotype of a single cell to be the mean of these distributions. Thus a complete description of unstimulated swimming behavior of a single cell is captured by the set of phenotypes χ(i) ∈ {⟨τ_r⟩(i), ⟨|v_r|⟩(i), ⟨τ_t⟩(i)}, where ⟨·⟩(i) denotes an average over all
Selection of chemotaxis phenotypes during directed evolution

Events exhibited by individual $i$. In a population, phenotypic traits can be described by a distribution $P(\chi)$ that governs the probability that an individual has a specific value for each trait $\chi$. We quantify phenotypic fluctuations, or cell-to-cell variation, by the standard deviation across the population in each trait, for $N$ cells this is computed as: $\sigma_\chi = \sqrt{\frac{1}{N} \sum_i (\chi^{(i)} - \langle \chi^{(i)} \rangle)^2}$. We note that $\sigma_\chi$ describes phenotypic variation driven by both genetic and non-genetic variation in the population except in cases of clonal populations, where $\sigma_\chi$ is due to non-genetic effects alone.

To experimentally quantify phenotypic fluctuations, we computed average run durations, tumble durations and run speeds on a per cell basis. Explicitly, if cell $i$ executes $M$ runs during the 5 minutes of tracking we compute $\langle \tau_r \rangle^{(i)} = \frac{1}{M} \sum_{j=1}^{M} \tau_{r,j}$. To quantify the cell-to-cell variation we then compute the standard deviation across individuals $\sigma_{\langle \tau_r \rangle}$. We compute identical statistics for the tumble duration $\tau_t$ and the run speed $|v_r|$ for founding populations and populations isolated after 5, 10 and 15 rounds of selection. Fig. 3 shows the standard deviations across the population ($\sigma_\chi$) for $\chi \in \{ \langle \tau_t \rangle, \langle \tau_r \rangle, |v_r| \}$, indicating a significant decline in the cell-to-cell variation during selection. In particular, we observe a significant decline between founding population and rounds 10 and 15 for all phenotypic parameters. We conclude that selection for faster migration results in reduced phenotypic fluctuations in the population.

The common interpretation for the utility of phenotypic variation is that it may increase survival probability under environmental changes by providing variation with every generation as opposed to genetic mutations which occurs less frequently\cite{5, 3}. Whether populations are shaped more by phenotypic variation or genotypic variation depends on the degree of phenotypic variation and on the strength and types of environmental selection. Was this reduction a special feature of the experiment, or could it be understood from general principles? To address this, we describe below an abstract computational model which is independent of the mechanistic details of our particular experiment. We asked how the process of iterated selection, whereby cells from the tail of a phenotypic distribution are propagated to the next round, alters cell-to-cell variation. Our goal with the simulation was to predict how the evolution of cell-to-cell variation depends on the strength of selection.

4. Abstract model of directed evolution of phenotypic fluctuations

The genotype-phenotype map determines the phenotype of an organism with a given genotype. How phenotypic selection is coupled to genetic variation is an important question whose answer illuminates fundamental questions such as the evolutionary rate and the evolvability of organisms. In general, this mapping is a multi-dimensional function that is governed by complex biological features such as gene regulatory and metabolic networks. As such, in laboratory-based directed evolution experiments the evolutionary dynamics of a specific phenotype are difficult to understand in terms of genetic variation alone. Therefore, we seek a framework that does not rely on an explicitly modeled mapping from genotypes to phenotypes. For simplicity, we present
a computational model of adaptation of a single effective phenotype and its associated
genotype, representing a projection of a multi-dimensional phenotype/genotype evolving
under selection. The idea is related to previous population genetics models [30], but
instead of assuming continuous selection due to an assumed fitness landscape, we
specify selection through a population bottleneck that is decoupled from the rate of
growth. We use this model to calculate the evolution of phenotypic variation under
selection. The model is necessarily stochastic in order to capture the dynamics of
fluctuations. We do not specify any explicit mechanism for genotype-phenotype mapping
or how its functional form changes during evolution. Instead, phenotypes are random
numbers generated from a gaussian mapping function whose mean is identified with a
genotype and whose variance reflects phenotypic fluctuations across individuals with
that genotype. The mean and variance change in evolutionary processes such as point
mutations.

Our abstract model captures key features of a fully realistic model built on a
lower-level description such as gene expression. Main experimentally-relevant factors
considered in this abstract model could include strength of bottleneck selection, mother-
daughter correlation and environmental changes. The mother-daughter correlation (or
epigenetic inheritance) describes the degree of gene expression level that is passed
on to descendants and determines how well preserved a phenotype is in subsequent
generations. Environmental changes provide selection stress that affects gene expression
and may induce genetic variations. We focus on the effect of the strength of bottleneck
selection and assume that the mother-daughter correlation does not evolve. Further,
the details of the experiment including the consumption of nutrients and the process of
chemotaxis are not explicitly represented in the model but are captured in the process
of selection.

4.1. Scheme of the abstract model

In our abstract model, each individual $i$ is represented by a random phenotype value
$\chi^{(i)}$ which is determined by the individual’s genotype $g$. $\chi^{(i)}$ is generated from a normal
distribution $P(\chi)$ whose mean is $\mu_{\chi}(g)$ and whose variance is $s_{\chi}^2(g)$ in the absence of
mother-daughter correlations. This abstracted phenotype is intended to represent any
observable phenotypic variable. We assume that the phenotype does not change within
the individual’s lifetime. In our abstract model of directed evolution, the phenotypic
trait $\chi$ is not explicitly stipulated. Instead, our abstract model is intended to explore
the dynamics of phenotypic evolution under generic assumptions about how traits are
passed between generations and respond to mutations.

Individuals reproduce and the offspring acquire mutations with probability $\nu$,
causing the daughter’s genotype $g'$ to be distinct from the mother’s ($g$). Therefore,
the daughter’s phenotype follows another normal distribution with distinct mean $\mu_{\chi}(g')$
and distinct variance $s_{\chi}^2(g')$.

In the absence of mutations (i.e. within a clonal population derived from a single
genotype \( g \), the phenotypes of each new cell are generated based on a bivariate gaussian distribution \( P(\chi^{(i)}, \chi^{(i)'}) \) with mother-daughter correlation coefficient \( \rho \) that captures the fact that daughter cells have phenotypes \( \chi^{(i)'} \) which is correlated with those of their mother \( \chi^{(i)} \) [31]. Phenotypic correlations between generations in clonal populations can arise from protein copy number fluctuations or non-genetic changes in gene expression [32], [33].

For an individual \( i' \), which results from fission of individual \( i \), its phenotype \( \chi^{(i)'} \) follows the conditional distribution of the variable \( \chi^{(i)'} \), given a known value of \( \chi^{(i)} \) [31]:

\[
P(\chi^{(i)'}|\chi^{(i)}) \sim \mathcal{N}(\mu_{\chi}(g) + \rho(\chi^{(i)} - \mu_{\chi}(g)), (1 - \rho^2)s^2_{\chi}(g)),
\]

where \( \mathcal{N}(\mu, \sigma^2) \) is a normal distribution with mean \( \mu \) and variance \( \sigma^2 \).

We calculate these dynamics, along with the procedure for directed evolution through selection, as follows:

(i) \( N_s \) individuals from a single genotype \( g = g_0 \) are generated from \( P(\chi) = \mathcal{N}(\mu_{\chi}(g_0), s^2_{\chi}(g_0)) \), as illustrated in Fig. [x](A). These \( N_s \) clonal individuals are defined as the founder strain, which by construction is a population with a normal distribution of different phenotypes.

(ii) Each individual with phenotype \( \chi^{(i)} \) creates a new individual with phenotype \( \chi^{(i)'} \).

   (a) If it mutates, \( \chi^{(i)'} \) is generated from \( P(\chi) = \mathcal{N}(\mu_{\chi}(g_1), s^2_{\chi}(g_1)) \), where \( \mu_{\chi}(g_1) \) and \( s_{\chi}(g_1) \) are generated from \( \mathcal{N}(\mu_{\chi}(g_0), \eta^2_{\mu_{\chi}}) \) and \( \mathcal{N}(s_{\chi}(g_0), \eta^2_{s_{\chi}}) \) respectively. The variances \( \eta^2_{\mu_{\chi}} \) and \( \eta^2_{s_{\chi}} \) are assumed to be constant for all parent genotypes (\( g_0 \)).

   (b) If the new individual does not mutate, \( \chi^{(i)'} \) updates based on Eq. [1].

An example of the relationship between different phenotypes and the reproduction process is shown in Fig. [x](B-C). During reproduction we neglect the degradation of individuals, and thus the population doubles after one generation. Each individual in the doubled population generates a new individual in the next generation following step (ii)a or (ii)b. We assume that the mother-daughter correlation (\( \rho \)) does not evolve. After \( m \) generations, selection is applied to the whole population with \( N_f = N_s \times 2^m \) individuals.

(iii) To apply selection, \( N_r \) individuals with the largest \( \chi \) values are chosen from the population. The selection fraction \( N_r/N_f \) is defined to be the selection strength. \( N_s \) individuals are further randomly selected from the \( N_r \) individuals to be the seed population for the next round. \( N_r \) is analogous to the outer edge population sampled with a pipette in the experiments, and \( N_s \) represents the individuals that are used to inoculate the new plate. In experiments, \( N_f \sim 10^{10}, N_r \sim 10^8 \) and \( N_s \sim 10^6 \). Thus, in the bacterial chemotaxis experiments \( N_f \gg N_r \gg N_s \).

(iv) In the new round, step (iii) and (iii) are repeated for the \( N_s \) individuals from the previous round.
(v) The phenotypic variance in \( N_s \) individuals at the end of each round is measured by growing a population to \( N_l = N_s \times 2^l \) individuals by repeating step (ii)b without mutations. This mimics the experimental process of single cell tracking in liquid media, where populations are amplified by growth in well-mixed liquid conditions and presumably mutations can be neglected.

The parameters in the simulations are: \( N_s = 20, m = 8, l = 8, \mu_\chi(g_0) = 40, s_\chi(g_0) = 8, \eta_\mu_\chi = 5, \eta_{s_\chi} = 2, \) with \( \nu = 0.2 \). Simulations were run over 40 rounds, and the stochastic values of \( \chi, \mu_\chi \) and \( s_\chi \) are binned with bin sizes equal to 1, 10 and 1 respectively. The parameters were chosen so that the convergence of values of \( \chi \) in \( N_l \) individuals can be reached in the last 10 rounds in order to compare with the evolving trend in the experiments. The selection process is described in Fig. 4[D).

These simulations do not directly stipulate how the phenotypic fluctuations within a given genotype \( s_\chi(g) \) evolve – e.g. these can increase or decrease relative to the parent genotype \( g \). This is intended to avoid any bias on phenotypic fluctuations with respect to the evolving mean trait values. For example, we do not explicitly stipulate that \( s_\chi(g) \) decreases as \( \mu_\chi(g) \) increases. However, a mechanistic link between the mean and variance of a phenotypic trait could occur in more realistic situations where traits are constrained by trade-offs. For example, there is usually a fitness cost for a trait to deviate very far from the mean, especially when the mean trait values are already large or a trait deviates from an optimized value in a given environment.

In addition, traits such as run speed cannot physically evolve to infinitely large values and thus should be bounded by a threshold \( \chi_c \). The threshold on phenotype represents a limitation of the corresponding cellular machinery, and therefore it fluctuates between cells in general. In our simulations we include thresholds on \( \chi^{(i)} \) for each individual and on the mean phenotype \( \mu_\chi \) of each genotype, and the upper thresholds for both are set to be random numbers generated for each individual from \( \mathcal{N}(\mu_\chi_c, \eta_{\chi_c}^2) \). We assumed that the timescale for changes in the threshold \( \mu_\chi_c \) is very long and set this to be a constant in all simulations. Since we perform directed evolution on the largest phenotype values, the lower threshold is insignificant. Therefore the value of the phenotype \( \chi \) for a particular genotype \( g \) is distributed with a truncated normal distribution with an upper bound which is approximately \( \mu_\chi_c \pm \eta_{\chi_c}^2 \). We set \( \mu_\chi_c = 100 \) and \( \eta_{\mu_\chi} = 5 \).

We expect that one of the relevant control parameters is the selection strength \( N_r/N_f \). We note that in the simulation multiple genotypes can coexist in the population at variable frequencies. We denote the distribution of phenotypes for the entire population as \( P_p(\chi) \). Now we present some intuitive arguments that try to predict the behavior of \( P_p(\chi) \) as a function of selection strength. Without any physically-determined threshold on phenotype \( \chi \), individuals who evolve higher \( \mu_\chi \) and larger \( s_\chi \) are expected to preferentially populate the right-most tail of the population trait distribution, and so will have a higher probability of being selected. However, if the distribution is cut off by a threshold \( \chi_c \), and when the phenotype value \( \chi \) approaches the threshold, individuals with larger \( s_\chi \) will no longer provide a higher trait value. Instead these individuals
will have higher weighting at smaller $\chi$ values compared with individuals that have the same $\mu_\chi$ but a smaller $s_\chi$. As a result, once $E[P_p(\chi)] \to \mu_\chi$, individuals with smaller $s_\chi$ will be selected if the selection strength is low (variance in $P_p(\chi)$ will decline). On the other hand, if the selection strength is high, the phenotype value $\chi$ of the selected $N_s$ individuals are confined to be near $\mu_\chi$ no matter what $s_\chi$ values of their genotype are. The $N_l$ individuals generated from these $N_s$ will contain genotypes with a wide range of $s_\chi$, and thus the overall variance in $P_p(\chi)$ will increase. Finally, the mutation rate $\nu$ determines how rapidly the phenotype distribution will reach its threshold. The higher the mutation rate the shorter the time required for the phenotype to reach $\mu_\chi$. However, a high mutation rate will drive phenotype values away from the threshold after it has been reached, which leads to a lower mean $\chi$ and larger variance in phenotypes. A similar conjecture about the way phenotypic variation might evolve as a function of selection strength was made in [34, 35].

Fig. 5(A-B) shows the evolution of the distribution of $\chi$ ($P(\chi)$ of the $N_l$ individuals after different rounds of selection for populations under weak selection (panel A) and strong selection (panel B). The phenotypic fluctuation (or the cell-to-cell variation), $\sigma_\chi$, is defined as the standard deviation of $\chi$ in the $N_l$ individuals (e.g. the standard deviation of $P(\chi)$, the average phenotype $\langle \chi \rangle$ is the expectation value of this distribution). The cell-to-cell variation ($\sigma_\chi$) and $\langle \chi \rangle$ evolve and are shown in Fig. 5(C-D).

The simulation results are consistent with our prediction. Under weak selection, before the distribution tail reaches the threshold $\mu_\chi$, the distribution becomes wider in successive rounds of evolution. Once the distribution of $\chi$ starts to be cut off by $\chi_c$ it becomes more left-skewed and eventually narrower. Fig. 5(C) and (D) compares the effect of low and high selection strength on the average phenotype value over population, $\langle \chi \rangle$ and on the evolution of $\sigma_\chi$. If the selection strength is high, the phenotype distribution evolves faster towards larger $\chi$ values, and thus cell-to-cell variation increases faster as well. Contrary to the case with low selection strength, $\sigma_\chi$ decreases once the distribution tail reaches the threshold but soon increases again. Fig. 5(E) shows that $\sigma_\chi$ at the final round of selection is larger (smaller) than the founder strain if the selection strength is strong (weak). We also observed that if the traits are not bounded by a threshold, i.e. $\mu_\chi \to \infty$, the traits evolve without bound in the simulations, and there is no saturation of trait value after repeated rounds of selection, and there is no decline in the variance in the population.

In conclusion, through the simulations of this abstract model for directed evolution we have shown that an upper bound of phenotype can lead to finite-time saturation of the evolving phenotype, and to the decrease of cell-to-cell variation under temperate selection and with rare mutations. In the case with strong selection and frequent mutations, the decrease of cell-to-cell variation is not a necessary consequence of the directed evolution procedure. Under strong selection, genotypes with large phenotypic variation stand out as long as they are not constrained by the physical thresholds, and the average genotype and phenotype values increase faster (Fig. 5(C)). In this sense,
strong selection can be regarded as increasing the evolvability. In other words, whether phenotypic variation is advantageous or unfavorable depends on the selection strength and constraints on the phenotype.

In general, a reduction in phenotypic fluctuations could be interpreted as stabilizing selection due to canalization [36], but the mechanism in this case is different from ours because there is no explicit threshold present. In the case of canalization specific biological buffering mechanisms such as capacitance [37] are more likely to be at work. Our simulations suggest an alternative mechanism for phenotypic variation, arising as a generic consequence of bounded phenotypic variation under strong or weak selection.

4.2. Comparison between the experiment and the abstract model

The experimental results show that the variance of the run speed decreases with the number of rounds of selection, a result that our model predicts to occur when selection is weak. How can we estimate whether or not our experiment is truly in the weak selection regime? A naive measure of the selection strength is the ratio $N_r/N_f$ which we estimate to be order $10^2$ in the experiment. Does this indicate strong selection then? It is difficult to draw a clear conclusion about this because, in general, selection acts on the phenotype space. The selection strength should be defined including the weighting of phenotype values, and not simply the number fraction that assumes equal weighting of each phenotype. In our experiment selection was applied in real space on agar plates, and thus the real physical phenotype that is being selected is a compound trait of multiple variables. Therefore, the selection fraction in the abstract model might not simply be related to the selection strength in the physical system. Thus, in order to test how the trend of phenotypic variance evolves with selection strength, it would be necessary to perform another set of experiments with different selection strengths, either a smaller selection fraction or selecting at different part of the population profile, to compare with the current experiment result shown in Fig. 5. This is planned for a future publication.

5. Biological mechanisms

Our abstract simulation makes a clear prediction about how phenotypic fluctuations should evolve in the presence of constraints on phenotypes under selection. Fig. 2 shows that over the course of selection the swimming speed of the cell saturates at approximately $28 \mu \text{m s}^{-1}$ and does not change between rounds 10 and 15 of selection. This suggests the possibility that $|v_r|$ is in fact bounded from above in a manner similar to our evolutionary simulations. We note that the precise mechanism of this constraint is not known, but may be hydrodynamic, metabolic or genetic in origin. For example, the swimming speed increases with flagellar bundle rotation rate [38] which depends on the proton motive force and the pH, both of which depend on the metabolic state of the cell. Swimming speed is also under genetic regulation through a braking mechanism.
acting on the flagellar motors \[39\]. These mechanisms likely impart an upper bound on the swimming speed of the cell; indeed such a bound must exist given the finite propulsive force supplied by the flagella. Since we observe a saturation in swimming speed between rounds 10 and 15 of selection (Fig. 2(C)) and a concurrent decline in phenotypic fluctuations for $|v_r|$ (Fig. 3) we speculate that this reduction has as its basis a dynamic similar to our abstract model (Fig. 5), whereby the swimming speed is evolving towards an upper bound.

While swimming speed ($|v_r|$) appears to evolve towards an upper bound we observe a decline in run durations during selection as well as a decline in the phenotypic fluctuations in $\tau_r$ and $\tau_t$ (Figs. 2 and 3). It’s less clear that explicit bounds apply to run and tumble durations. Indeed, mutants which exhibit very long or very short run durations have been isolated. Moreover, phenotypic fluctuations in the temporal statistics of runs and tumbles have been studied in \textit{E. coli} for decades, and the molecular origins of these fluctuations are well understood. Since the seminal work of Koshland and Spudich \[4\], we now know that copy number fluctuations of the enzyme \textit{cheR} and \textit{cheB} drive large fluctuations in the run-tumble statistics at the single motor and single cell level\[26, 5, 40\]. Dufour \textit{et al.} \[40\] measured both gene expression and run-tumble statistics in single-cells to show a reduction in phenotypic fluctuations with increasing $[\text{CheR}]$ and $[\text{CheB}]$ concentrations \textit{in vivo}. Phenotypic fluctuations declined when concentrations of both proteins increased while the ratio $[\text{CheR}] /[\text{CheB}]$ remained constant \[40\]. Furthermore, increasing expression of both genes resulted in an increase in tumble frequency precisely as we observe in our selection experiment \[40\]. In a separate study, Vladimirov \textit{et al.} \[41\] show that the expression levels of both \textit{CheR} and \textit{CheB} are higher at the periphery of a colony expanding through 0.27\% agar than at the center. Taken together these studies suggest that increasing \textit{CheR} and \textit{CheB} expression should reduce phenotypic fluctuations in $\tau_r$ and $\tau_t$ and that this reduction is correlated with distance from the center of the colony.

In light of this understanding we examined the mutations present in strains after selection. We performed whole genome sequencing on the ancestral strain as well as populations isolated after 5, 10 and 15 rounds of selection for four replicate selection experiments\[22\]. In every replicate we observe an identical mutation at $>70\%$ abundance by round 5 and fixed by round 10: a single nucleotide polymorphism which inserted a stop codon at position 185 in the 424 residue \textit{ClpX} protein ($\textit{clpX}\text{E185}^*$). \textit{ClpX} is the specificity subunit of the \textit{ClpX-ClpP} serine protease, which degrades many target proteins including \textit{FlhDC}. \textit{flhDC} is the master regulator of a coherent feedforward motif which governs the expression of motility and chemotaxis genes including \textit{cheR} and \textit{cheB} enzymes, which are determinants of phenotypic fluctuations\[42\].

To investigate the role of the mutation we observed in $\textit{clpX}$ in phenotypic fluctuations, we reconstructed the $\textit{clpXE}185^*$ mutation in the ancestral background using scarless recombineering. We confirmed that this mutation alone is sufficient to drive faster migration through increasing run speed and decreasing growth rate \[22\]. Moreover, this mutation alone causes a decrease in the phenotypic fluctuations in run
duration and tumble duration, but not run speed relative to the ancestral population (Fig. 6).

We considered whether the mutation we observe in clpX might logically result in increased levels of cheR and cheB and therefore the reduced phenotypic fluctuations we observe. Previous studies have shown that mutations in ClpX increase levels of FlhDC in the cell [13]. Zhao et al. [44] show that deleting flhDC results in substantial reduction in expression of the downstream cheR/B genes. However, inducing FlhDC expression above wild-type levels appears not to increase expression of downstream genes substantially [44]. Despite this, single cell measurements show a positive correlation between flhC and cheY expression levels [45]. Since cheY is co-transcribed with cheR and cheB we speculate that increases in FlhDC levels in the cell may drive increases in cheR and cheB expression and that could reduce phenotypic fluctuations. To confirm this, further studies are needed to directly measure the meche operon expression levels in the presence and absence of the clpX mutation we observe.

The clpXE185* mutation alone drives an increase in run speed to 24.2 μm s$^{-1}$ from 18.2 μm s$^{-1}$ for the founder whereas the average run speed of the round 15 evolved strain is 28.7 μm s$^{-1}$ [22]. These results suggest that the mutant run speed is, on average, far from the apparent upper bound in swimming speed. As our abstract model would predict for the mutant, we observe no decrease in $\sigma_{\langle |v_r| \rangle}$ in the mutant relative to the founder – potentially because the mutant phenotype is not constrained by an upper bound on run speed.

6. Discussion

In our measurements we report that selection drives reduction in phenotypic fluctuations associated with chemotactic mobility. We also identified the mutations that appear to be implicated in this evolution of phenotype statistical properties. Are the results surprising, or could they have been predicted on general grounds related to the fluctuation-dissipation theorem and other global properties of stochastic gene expression?

Our abstract model suggests that such a reduction may arise from selection in the presence of a constraint on phenotypes. Thus our data suggest the possibility that swimming speed may be constrained in E. coli by biophysical or metabolic means. Further work is needed to determine whether such a constraint exists and precisely how it impacts phenotypic fluctuations in this system.

However, our abstract model of directed evolution applies to a broad range of potential systems and makes a clear prediction as to how the strength of selection influences phenotypic fluctuations. Genetic, biophysical and chemical constraints play an important role in the dynamics of biological systems from higher organisms such as fungi [16] to limits on the speed of protein translation [17] and enzyme specificity [18]. Our study highlights the potentially important role for these constraints in determining the limits of phenotypic fluctuations. Future experimental evolution work could exploit
known phenotypic constraints and directed evolution to directly test the predictions of our model.

At a lower level of biological organization the mechanisms underlying phenotypic fluctuations remain hard to uncover in general due to the complex relationship between gene expression, protein function and cell-level phenotypes. Despite the difficulty of connecting phenotypes to gene expression recent work has shown universal statistical properties in protein copy number distributions, with monotonically increasing scaling of the variance in protein abundances with mean expression levels [15, 13, 49, 14]. These universal properties of protein abundance fluctuations may provide a basis for understanding the evolution of phenotypic fluctuations in situations where the relevant regulatory architecture is known [10, 9]. However, at present, a molecular accounting for the mechanism of the evolution of phenotype fluctuations requires detailed knowledge of the signaling pathways at work. Our hope is that in studying abstract models such as the one presented here, we may uncover a more general understanding of when and why phenotypic fluctuations evolve.

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Figure 1. Selection for faster bacterial migration: (A) Shows images of *E. coli* colonies in low viscosity agar plates after 12 hours of expansion. After 12 hours a sample of the outer band of cells is taken and approximately $10^6$ cells are used to initiate another identical agar plate (second panel). This process is repeated for 15 rounds of selection where a round consists of colony expansion in a single plate. The color bar to the right applies to all panels, with darker gray indicating higher cell density. Scale bar in left panel applies to all panels in (A). (B) The radius of each colony in (A) as a function of time, lighter shades of gray denote later rounds of selection and correspond to labels in (A). Traces are offset vertically for clarity, note scale bar lower left. (C) The rate of the linear portion of the colony expansion as a function of the round of selection for the plates shown in (A-B). (D) The evolutionary process outlined in (A-C) was carried out in five independent experiments. Each line corresponds to an independent selection experiment. Round 8 for replicate 1 is missing due to failure of the imaging device. The data in panels (A-C) are from replicate 5. Errors in rate of expansion are smaller than the size of markers. Data recapitulated from [22]
Figure 2. Dynamics of phenotypic evolution: (A) An example 50 s long swimming trajectory for a single cell trapped in a microfluidic chamber. The boundary of the chamber is shown by the light black circle. Running events are shown in black and tumble events in red. Scale bar is 50 µm. (B) Aggregate complementary cumulative distribution functions of run durations observed from cells isolated prior to selection (founder, black) and after 5 (blue), 10 (orange) and 15 (green) rounds of selection. Strains tracked were isolated from replicate 1 in Fig. 1. Distributions are constructed from all run events that were not interrupted by collisions with the chamber boundary for 140 (founder), 79 (round 5), 97 (round 10) and 96 (round 15) individuals executing a total of 19,597, 12,217, 18,505 and 15,928 run events respectively. The mean and standard deviation of run durations are (mean:sd) 0.66 s:0.78 s, 0.63 s:0.61 s, 0.58 s:0.51 s and 0.64 s:0.57 s respectively. Shaded regions indicate 95% confidence intervals from bootstrapping. (C) Distributions of run speeds (|v_r|) for the four strains shown in (B), colors from (B) apply. Distributions are constructed by computing an average speed for each run event. Means of these distributions are 18.7 µm s⁻¹ (founder), 24.9 µm s⁻¹ (round 5), 27.6 µm s⁻¹ (round 10), and 28.7 µm s⁻¹ (round 15). The increase in |v_r| is statistically significant between each successive population (p < 0.001, rank sum test). (D) shows triplicate growth rate measurements in well-mixed LB as a function of the round of selection. Error bars are standard errors from regression of log(OD) on time. Growth rate declines with selection at a rate of −0.01 h⁻¹ round⁻¹ (95% confidence interval −0.012, −0.008). Data are recapitulated from [22].
Figure 3. Cell-to-cell behavioral variation declines with selection: Individuality for evolved populations (Fig. 2) for (A) $\tau_r$, (B) $\tau_t$ and (C) $|v_r|$. We compute $\langle \tau_r \rangle$, $\langle \tau_t \rangle$ and $\langle |v_r| \rangle$ for each individual tracked and a standard deviation across individuals for each parameter ($\sigma_*$). $\sigma_*$ was computed for 140 cells (founder), 79 cells (round 5), 97 cells (round 10) and 96 cells (round 15). The circles show the sample $\sigma_*$ for each population. 95% confidence intervals from bootstrapping for each population are given by the error bars. Colormap shows the probability distribution of $\sigma_*$ from bootstrapping. Note distinct colorbars for each panel. All populations exhibit a decline in $\sigma_*$ relative to founder that is significant ($p < 0.05$, permutation test) except for $\sigma(\tau_t)$ and $\sigma(|v_r|)$ in round 5.
Figure 4. Scheme of the abstract model. Illustration of selection procedure (see text for definition of notation): (A) Phenotype distributions for two genotypes ($g_0, g_1$). The phenotype of each genotype $g_i$ is described by a normal distribution with mean $\mu_X(g_i)$ and standard deviation $s_X(g_i)$. (B) Initially the founder strain with $N_s$ individuals whose phenotypes $\chi_i$ are drawn from $\mathcal{N}(\mu_X(g_0), s^2_X(g_0))$ is generated. The $N_s$ individuals reproduce new individuals in the first round with a mutation rate $\nu$. For example, for one of the initial $N_s$ individuals with the founder genotype $g_0$ (circle) and a certain phenotype ($\chi^{(1)}$, in blue color) which is determined by mother-daughter correlation based on Eq. 1, its daughter may have the same genotype but different phenotype ($\chi^{(2)}$, in green color) if it does not mutate. If the daughter mutates, the daughter is assigned a new genotype ($\chi^{(3)}$, in red color) from $\mathcal{N}(\mu_X(g_1), s^2_X(g_1))$ and its phenotype $\chi^{(3)}$ is drawn from $\mathcal{N}(\mu_X(g_1), s^2_X(g_1))$. (C) shows a table of phenotypes ($\chi_i$) and their corresponding genotypes and phenotype distributions. Note that individuals with the same genotype stochastically differ in their phenotypes (first row). After $m$ generations of the process shown in (B), the population becomes $N_f = N_s \times 2^m$. (D) The top $N_r$ individuals are selected from $N_f$ individuals, and $N_s$ individuals are randomly sampled from $N_r$ individuals to start the second round. In the next round, $N_s$ individuals repeat reproduction steps in (A) until the population reaches $N_f$ again. At the end of each round, the selected $N_s$ individuals reproduce for $l$ generations without mutations. These $N_l = N_s \times 2^l$ individuals represent the population of each strain grown in liquid media prior to single-cell tracking.
Figure 5. Simulations of the abstract model: (A) The distribution of $\chi$ of $N_I$ individuals at different rounds under weak selection (e.g. $N_r/N_f = 1/2$): when the distribution $P_p(\chi)$ is away from $\mu_{\chi}$, individuals with larger $s_\chi$ are selected, and the overall variance in $\chi$ increases. When the tail of distribution $P_p(\chi)$ starts to reach $\mu_{\chi c}$ after round 5, $P_p(\chi)$ becomes tilted, and the variance in $\chi$ eventually decreases. $\mu_{\chi c}$ and $\eta_{\mu_{\chi}}$ are denoted by the vertical and horizontal red line. (B) $P_p(\chi)$ under strong selection (e.g. $Nr/N_f = 1/2^n$): $P_p(\chi)$ evolves and reaches $\mu_{\chi c}$ faster, and variance in $\chi$ first increases (before round 3) and then decreases (round 10). Since individuals with smaller $s_\chi$ are not particularly selected under strong selection, $P_p(\chi)$ eventually relaxes to a wider shape due to random mutations (round 40). (C)(D) The overall mean and standard deviation of $\chi$ measured from (A) and (B) and are averaged over 20 replicates. (E) The overall variance in $\chi$ as a function of selection strength. The red dashed line represents the standard deviation of $\chi$ for the founder strain. Parameters in the above simulations: $N_s = 20, m = 8, l = 8, \mu_{\chi}(0) = 40, s_{\chi}(0) = 8, \chi_c = 100, \eta_{\mu_{\chi}} = 5, \eta_{s_{\chi}} = 5, \eta_{\chi_c} = 2, \eta_{\chi c} = 5$, and the stochastic values of $\chi, \mu_{\chi}$ and $s_{\chi}$ are binned with bin sizes equal to 1, 10 and 1 respectively. In (A)-(D) the mutation rate $\nu = 0.2$. 
Selection of chemotaxis phenotypes during directed evolution

Figure 6. Cell-to-cell behavioral fluctuations in clpX mutant: Individuality for a mutant with the clpXE185* mutation compared to the founder. Individuality for each population for (A) $\tau_r$ (B) $\tau_t$ and (C) $|v_r|$. We compute $\langle \tau_r \rangle$, $\langle \tau_t \rangle$ and $\langle |v_r| \rangle$ for each individual tracked and a standard deviation across individuals for each parameter ($\sigma*$). Data from 140 founder cells is reproduced from Fig. 3 and compared to 82 clpXE185* cells. Panels are identical to Fig. 3 with circles showing the sample $\sigma_*$ for each population. 95% confidence intervals from bootstrapping for each population are given by the error bars. Colormap shows the probability distribution of $\sigma_*$ from bootstrapping. Note distinct colorbars for each panel. The clpXE185* strain exhibits a statistically significant decline in $\sigma_\langle \tau_r \rangle$ and $\sigma_\langle \tau_t \rangle$ ($p < 0.01$, permutation test), but not $\sigma_\langle |v_r| \rangle$. 
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