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A Physical foundation of OptiEvo theory

In this article, an organism's population consists of N individuals (the value of N can change during evolution). The genome sequence of the i-th individual is described by an R_i-dimensional vector $g_i = \{n_1, \ldots, n_{r_i}, \ldots, n_{R_i}\}$, where R_i is the length of this individual's genome and each n_{r_i} represents the nucleotide at the r_i-th position of the genome. The population $G = (g_1, \ldots, g_N)$ with $G \in F$ is a collection of the individual members' genotypes g_i, where the entire sequence space F is the union of all possible genotypes g_i, which also can contain genotypes outside the particular population G.

In OptiEvo theory, the organism's population G is represented as a classical stochastic system with probability distribution $\rho_G(\omega)$ over the phase space Ω ($\omega \in \Omega$). Here Ω defines all biologically relevant degrees of freedom of the population and each $\omega \in \Omega$ characterizes a certain microscopic state of the population. $\mathcal{P}_\Omega$ is used to describe the set of all possible probability distributions.

With the above definitions, fitness of an organism's population in a constant homogeneous

environment can be specified by a random function $f(\omega)$, whose average value J is labeled by G: $J(\rho_G) = \int_\Omega f(\omega)\rho_G(\omega)d\omega$. Here a constant environment does not refer to a strictly non-varying environment over any time scale. Fluctuations are allowed during the development of a population. It is only required that the environment be constant within a degree of fluctuations such that the fitness values can be validly compared over the evolution time scale. More generally, OptiEvo theory can be applied to an arbitrary concave fitness functional $J(\rho_G)$ of $\rho_G(\omega)$. During evolution, a change of the population $G \to G'$ (via mutations, insertions, deletions, etc.) leads to a transformation of the population state $\rho_G \to \rho_{G'}$ and consequently a change in average fitness value $J(\rho_G) \to J(\rho_{G'})$.

Assuming that any $\rho \in \mathcal{P}_\Omega$ (on some coarse grained scale) can be produced by members of the set of all available genotypes (i.e., for any $\rho \in \mathcal{P}_\Omega$ there exists at least one genotype G such that $\rho \approx \rho_G$) the fitness $J(\rho_G)$ landscape can be considered as a functional $J(\rho_G)$ over the set of all probability distributions $\mathcal{P}_\Omega$. Specifically, since the genome sequence is discrete, this fitness landscape is a coarse grained image of the optimization landscape.

Several topological properties for general optimization landscapes hold for arbitrary completely optimizable classical and quantum open systems. Only the classical view will be presented here based on the analysis of [1]; the quantum picture is treated in [2, 3]. We use the notation ρ for probability distributions of general classical open systems and P for associated probability measures. The case with an organism's population is a particular circumstance which corresponds to $\rho = \rho_G$. Section A.1 below describes the basic properties of classical stochastic systems. Section A.2 proves the convex structure of $\mathcal{P}_\Omega$. Based on the convexity of $\mathcal{P}_\Omega$ and the linear dependence of physical measurables $J(\rho)$ on ρ (or more generally, on the assumed concavity of $J(\rho)$), Section A.3 proves the trap-free topological character of the landscape $J(\rho)$. Section A.4 shows that satisfaction of the sufficient flexibility condition leads to the absence of traps in the genotypic landscape $J(\rho)$.

A.1 Classical stochastic systems

Classical probability space is defined by a triple $(\Omega, \mathcal{F}, P)$, where Ω is a non-empty set (the phase space), $\mathcal{F}$ is a σ-algebra of subsets of Ω (called the event space; a sigma-algebra $\mathcal{F}$ over a set Ω is a nonempty collection of subsets of Ω that is closed under complementation and the countable union of its elements), and $P : \mathcal{F} \to [0, 1]$ is a probability measure [4]. Each classical stochastic system is characterized by some set of elementary events Ω, where any point $\omega \in \Omega$ denotes a specific microscopic state of the system. For example, for a particle in three-dimensional space, the phase space is $\Omega = \mathbb{R}^3 \times \mathbb{R}^3$ and a point in this space is specified by $\omega = (p, q) \in \Omega$, where p and q are the momentum and the position of the particle, respectively. For more complex systems, as for an organism's population, the phase space refers to all relevant system degrees of freedom. In biological systems, such a phase space would be high dimensional and complex. However, what counts here is the existence of the phase space rather than its details. Any point $\omega \in \Omega$ corresponds to a microscopic deterministic state of the system with certain definite values of the system's degrees of freedom. If the system is stochastic, it can be characterized by more general stochastic states. Each stochastic state is characterized by a probability measure $P(E)$, $E \in \mathcal{F}$ which corresponds to some probability distribution $\rho(\omega)$ through the relation $P(E) = \int\limits_E \rho(\omega) d\omega$ for any $E \in \mathcal{F}$. The descriptions in terms of probability measures P and in terms of probability distributions ρ will be used interchangeably below.

Biologically measurable properties for an evolving organism's population are given by average values of associated random functions. If $f(\omega)$ $(\omega \in \Omega)$ is a random function representing the fitness and if the organism's population is in a state with probability distribution $\rho_G(\omega)$, then the fitness value is $J(\rho_G) = \int_\Omega f(\omega)\rho_G(\omega)d\omega$, which will be maximized during the course of evolution. In this context $J(\rho_G)$ defines the fitness landscape over the sequence space F. Although inherently discrete, J can be effectively considered as continuous when the genome is very large. Genetic changes during evolution will produce successive transformations of the genome $G \to G'$ corresponding to a trajectory over the landscape leading to maximization of $J(G)$. The nature of the landscape topology is crucial for understanding

evolutionary pathways.

An arbitrary abstract probability space $(\Omega, \mathcal{F}, P)$ should satisfy the following three axioms [5, 6]:

A_1. $P(E) \geq 0$ for any $E \in \mathcal{F}$ (i.e., probability of any event is a non-negative number).

A_2. $P(\emptyset) = 0$ (here $\emptyset$ denotes the empty set) and $P(\Omega) \equiv \int_{\Omega} \rho(\omega) d\omega = 1$ (the assumption of unit measure states that the probability that some event will occur is 1).

A_3. For any countable sequence of pairwise disjoint events $E_1, E_2, \cdots \in \mathcal{F}$:

$$P(E_1 \cup E_2 \cup \dots) = P(E_1) + P(E_2) + \dots$$

A.2 Convex structure of the set $\mathcal{P}_\Omega$

A subset $X \subset \mathbb{V}$ of a linear space $\mathbb{V}$ is called a convex set if for any $x_0, x_1 \in X$ and any $\lambda \in [0, 1]$ the point $x_\lambda := (1 - \lambda)x_0 + \lambda x_1$ is in X [7]. This definition says that for two arbitrary points x_0 and x_1 in the set X, the straight line segment connecting these points lies entirely in X. According to this definition, the set $\mathcal{P}_\Omega$ is a convex subset in the linear space $\mathcal{P}_\Omega^{\mathrm{R}}$ of all distributions associated to real-valued signed measures over $(\Omega, \mathcal{F})$ (i.e., measures which can take arbitrary, not necessarily positive, real values). Let P_0 and P_1 be any two probability measures over Ω with associated probability distributions $\rho_0(\omega)$ and $\rho_1(\omega)$, and define $P_\lambda = \lambda P_1 + (1 - \lambda)P_0$ for any $0 \leq \lambda \leq 1$. Then,

$$\begin{aligned}
P_\lambda(E) &= \lambda P_1(E) + (1 - \lambda)P_0(E) \geq 0 && (1)\\
P_\lambda(\Omega) &= \lambda P_1(\Omega) + (1 - \lambda)P_0(\Omega) = \lambda + (1 - \lambda) = 1 && (2)\\
P_\lambda(E_1 \cup E_2 \cup \dots) &= \lambda P_1(E_1 \cup E_2 \cup \dots) + (1 - \lambda)P_2(E_1 \cup E_2 \cup \dots)\\
&= \lambda[P_1(E_1) + P_1(E_2) + \dots] + (1 - \lambda)[P_2(E_1) + P_2(E_2) + \dots]\\
&= [\lambda P_1(E_1) + (1 - \lambda)P_2(E_1)] + [\lambda P_1(E_2) + (1 - \lambda)P_2(E_2)] + \dots\\
&= P_\lambda(E_1) + P_\lambda(E_2) + \dots && (3)
\end{aligned}$$

Here $E_1, E_2, \cdots \in \mathcal{F}$ is any countable sequence of pairwise disjoint events. Equations (1)–(3) imply that P_λ satisfies the axioms A_1, A_2, and A_3, implying that P_λ also a probability measure over $(\Omega, \mathcal{F})$. The associated probability distribution $\rho_\lambda(\omega) = (1-\lambda)\rho_0(\omega) + \lambda\rho_1(\omega)$ is also a probability measure, and this proves the convex structure of the set $\mathcal{P}_\Omega$.

A.3 Absence of local traps for the objective function $J(\rho)$

The objective functional $J(\rho) = \int_\Omega f(\omega)\rho(\omega)d\omega$ (a) is a linear functional of ρ and (b) is defined on the convex domain $\mathcal{P}_\Omega$. The goal of this section is to show that the properties (a) and (b) imply (1) the absence of local maxima and minima of the objective functional J and (2) connectivity of the global optimum manifold as proved in [1]. In general, the functional J has a local maximum at ρ_0 if there exists a neighborhood $U(\rho_0) \subset \mathcal{P}_\Omega$ of ρ_0 such that for any $\rho \in U(\rho_0) : J(\rho) \leq J(\rho_0)$ and ρ_0 is not a global maximum provided that there exists $\rho_1 \in \mathcal{P}_\Omega$ such that $J(\rho_1) > J(\rho_0)$. A *local* maximum is distinguished from global maxima of J. A global maximum is defined as a probability distribution $\rho_0 \in \mathcal{P}_\Omega$ such that $J(\rho) \leq J(\rho_0)$ for all $\rho \in \mathcal{P}_\Omega$. A level set $\mathcal{L}_a$ of the fitness function J corresponding to some value $a \in \mathbb{R}$ is defined as the set $\mathcal{L}_a := \{\rho \in \mathcal{P}_\Omega \,|\, J(\rho) = a\} \equiv J^{-1}(a)$ of all probability distributions for which the objective functional J has the value a.

The absence of local maxima immediately follows from the facts that (a) J is a concave functional and (b) a concave functional does not have local maxima over a convex domain [4, 8]. Moreover, a fitness function of the form in Eq.(1) of the main text is linear and hence also convex; therefore it also can not have local minima over a convex domain. Below we give a proof of the absence of local maxima for a linear fitness function by *reduction ad absurdum*. The proof for the absence of local minima is completely equivalent and generalization to arbitrary concave (for the absence of local maxima) or convex (for the absence of local minima) functions is straightforward.

Suppose there exists a probability distribution function $\rho_0 \in \mathcal{P}_\Omega$ which is a local maximum for J. By the definition of a local maximum, this means that (1) there exists a neighborhood

$U(\rho_0) \subset \mathcal{P}_\Omega$ of ρ_0 such that for any $\rho \in U(\rho_0) : J(\rho) \leq J(\rho_0)$ and (2) there exists $\rho_1 \in \mathcal{P}_\Omega$ such that $J(\rho_1) > J(\rho_0)$. Since $\mathcal{P}_\Omega$ is a convex set, for any $\lambda \in [0,1]$ the probability distribution function $\rho_\lambda := (1-\lambda)\rho_0 + \lambda\rho_1$ is in $\mathcal{P}_\Omega$. Consider the restriction $\tilde{J}$ of the functional J to the line passing through the points ρ_0 and ρ_1. The restriction of a linear function to a line defines either a constant or a strictly monotone function. The restriction $\tilde{J}$ is not a constant because $J(\rho_0) \neq J(\rho_1)$. Thus, $\tilde{J}$ is a strictly monotone functional and $\lambda_2 > \lambda_1 \Rightarrow \tilde{J}(\rho_{\lambda_2}) > \tilde{J}(\rho_{\lambda_1})$. Therefore, $J(\rho_{\lambda_2}) > J(\rho_{\lambda_1})$ if $\lambda_2 > \lambda_1$. In particular, for any $\lambda > 0 : J(\rho_\lambda) > J(\rho_0)$. But, if $\lambda > 0$ is small enough, then $\rho_\lambda \in U(\rho_0)$. Therefore, there exists $\rho_\lambda \in U(\rho_0)$ such that $J(\rho_\lambda) > J(\rho_0)$. The contradiction of this conclusion with the condition (1) proves the absence of local maxima for $J(\rho)$.

The connectivity of the global optimum manifold $\mathcal{L}_{\text{top}}$ for the linear objective $J(\rho)$ is proved as follows. Let ρ_0 and ρ_1 be any two probability distributions on $\mathcal{L}_{\text{top}}$, i.e. $J(\rho_0) = J(\rho_1) = \max_\rho J(\rho)$. By the definition of a convex set, for any $\lambda \in [0,1]$ the point $\rho_\lambda = (1-\lambda)\rho_0 + \lambda\rho_1$ is in $\mathcal{P}_\Omega$. The function J is linear and therefore $J(\rho_\lambda) = (1-\lambda)J(\rho_0) + \lambda J(\rho_1) = J(\rho_0)$. Thus, the segment $\{P_\lambda \,|\, \lambda \in [0,1]\}$ belongs to the same level set as ρ_0 and ρ_1 and connects the probability distributions ρ_0 and ρ_1. This proves the connectivity of the global optimum manifold $\mathcal{L}_{\text{top}}$. This property of connectivity of the global optimum manifold holds also for an arbitrary concave function. Moreover, one can similarly prove that, any level set $\mathcal{L}_a$ is connected for a linear objective any level. However, for more complex non-linear objective functions, level sets other than the global optimum can be disconnected.

The convex structure of the domain $\mathcal{P}_\Omega$ is important in the proofs above. A restriction of the domain to a non-convex set can produce local maxima or local minima for the objective functional J and also can lead to the appearance of non-connected level sets. The linearity of J with respect to $\rho(\omega)$ is not essential in the above consideration for proving the absence of traps. In order to prove the absence of local maxima (resp., minima) it is sufficient to assume that $J(\rho)$ is a concave (resp., convex) function [4].

A.4 Absence of local traps in the genotypic landscape

This section will further explain the biological context of the general properties established in Section A.3. The notions of local minima or maxima and the connectivity of level sets are defined in terms of nearest genotypic neighbors. For any population G, the set of all its nearest neighbors $U_G = \{G'\}$ is the set of all populations G' obtained from G by a single evolutionary step, which can be either a single site mutation or simultaneous mutations at several positions, insertions, deletions, etc. The fitness function J over the genotypic variables is said to have a local maximum at G if $J(G') \leq J(G)$ for all $G' \in U_G$. A level set $\mathcal{L}_a$ of the function J is said to be connected if for any two populations G and G' on this level set (i.e., $J(G) = J(G') = a$) there exists a chain $G_1, G_2, \ldots, G_n$ of genotypes, which all belong to the level set $\mathcal{L}_a$ and $G_1 \in U_G, G_2 \in U_{G_1}, \ldots, G_n \in U_{G_{n-1}}, G' \in U_{G_n}$. In this case the trajectory $G \to G_1 \to G_2 \to \cdots \to G_n \to G'$ in the genotypic space entirely lies on the level set $\mathcal{L}_a$ and is realized by single evolutionary steps.

The absence of local maxima for the objective function $J(\rho)$ means that in a neighborhood of any ρ which is not a global maximum there exists a direction in which the objective $J(\rho)$ increases. Consider a genotype G which does not correspond to a global maximum of the objective. Then around ρ_G in the space $\mathcal{P}_\Omega$ exists at least one direction in which $J(\rho)$ increases. The sufficient flexibility condition states that the neighborhood U_G of G covers, on some coarse grained scale, all directions in the space $\mathcal{P}_\Omega$ around ρ_G; moving around G in the genotypic space F correspondingly allows for moving in all directions around ρ_G in the space $\mathcal{P}_\Omega$. In particular, it allows for moving over F in the direction in which $J(\rho)$ increases. Thus, if ρ_G in the probability distribution function space $\mathcal{P}_\Omega$ does not correspond to a maximum of $J(\rho_G)$, then G is not a maximum in the genotypic space F. Since there are no local maxima for $J(\rho)$ in the space $\mathcal{P}_\Omega$, this means that there are no local maxima in the space F. Significant constraints on the available genotypes G, which reduce the domain in the space of probability distribution functions P_Ω to a non-convex set and which do not satisfy the sufficient flexibility condition, may destroy the absence of traps property in the genotypic landscapes.

B Summary of laboratory studies addressing fitness landscapes

B.1 Direct landscape studies

Direct landscape studies usually apply mutations on a small number of focused positions known to be critical to certain phenotypes. The small mutational space allows for a thorough exploration of particular genotype→phenotype relationships, with the caveat that genetic changes outside the focused positions might also have significant effects on the target phenotypes.

Lunzer et al. [9] characterized the fitness landscape of the six critical amino acids controlling coenzyme use of isopropylmalate dehydrogenase (IMDH) in its evolution from the suboptimal cofactor nicotinamide adenine dinucleotide phosphate (NADP) to the normal cofactor nicotinamide adenine dinucleotide (NAD). The genotype→phenotype map was characterized by the kinetic performance of 164 mutant enzymes differing on all six sites, and the performance was shown to be linearly additive with respect to the gene mutations. The genotype→fitness map, however, is highly nonlinear due to strong epistasis, but its landscape still contains only a single peak where exclusive NAD use resides at the global optimum. The authors commented that "with its single peak, the landscape is far less rugged than those envisioned by Wright [10]". This observation agrees with predictions (a) and (c) of OptiEvo theory. In addition, every genotype was shown to be mutationally accessible from less fit genotypes, implying the existence of multiple evolutionary trajectories (consistent with prediction (a) of OptiEvo theory).

Weinreich et al. [11] studied the resistance adaptation of bacterial β-lactamase to an antibiotic cefotaxime. Five point mutations were known to be responsible for increasing resistance, and all $2^5 = 32$ possible mutants were synthesized and their fitness measured. Amongst all of the $5! = 120$ mutational trajectories available from single-point mutations, only 18 are selectively accessible, meaning that the fitness value always increased following

every mutation along these trajectories. Only one single global fitness maximum was observed. The authors suggested that the inaccessible genotypes are the result of sign epistasis. We emphasize that this is not equivalent to the existence of reciprocal sign epistasis, which is only a sub-class of sign epistasis. In fact, since the optimal genotype is accessible from every genotype via single-point mutations, reciprocal sign epistasis does not exist because it will create local fitness traps. The results in this study agree with predictions (a) and (c) of OptiEvo theory.

Bridgham et al. [12] investigated the evolution of molecular recognition between the steroid hormone aldosterone and its partner the mineralocorticoid receptor. Using ancestral gene resurrection, the authors found that the ancestral hormone (which binds to an ancestral receptor) was structurally preadapted for activation by its current receptor before evolving into the current hormone millions of years later. A follow-up study [13] showed that, in addition to the conserved mutations that allowed for the evolution of hormone specificity, five "restrictive" mutations with little fitness effect were also required in the process of evolution. These observations may be an indication of connected level sets (prediction (b) of OptiEvo theory). In addition, although complicated epistatic interactions constrained the evolutionary pathways, the evolution process did not go through fitness valleys, indicating that reciprocal sign epistasis does not exist (prediction (c) of OptiEvo theory).

Poelwijk et al. [14] studied the adaptation of repressor-operator binding in the *Escherichia coli lac* system using published mutation data. The fitness landscape does not contain local peaks (consistent with prediction (c) of OptiEvo theory) as a result of network compensation [15]. In contrast, reciprocal sign epistasis was observed in the affinity landscape, suggesting that affinity was not the target of optimization in the evolutionary process.

B.2 Laboratory evolution studies involving replicate populations

A long-term laboratory evolution experiment has been carried out on twelve identical populations of *Escherichia coli* in identical glucose-limited environments for over 30,000 cell gen-

erations [16–19]. The populations achieved similar fitness values [16–18]. By contrast, the evolution of cell size [17], catabolic abilities [18, 20], global gene-expression profiles [21] shows more pronounced inter-population differences. Sequencing of random genes also showed that "evolution in these 12 *E. coli* populations was often parallel at the level of genes, but only rarely were the substitutions identical at the base pair level" [19]. A recent study sequenced, at the whole-genome scale, the preserved samples for one of these populations during its evolution [22]. The outcome revealed substantial intra-population genetic diversity. More importantly, transient polymorphisms that later disappeared were discovered, which "probably represent alternative beneficial alleles" (i.e., different pathways leading to the same fitness peak). Some of these evolution experiments are reviewed in [23].

A recent outcome from the *E. coli* evolution experiment was the appearance of a citrate-using strain (Cit+) [24]. The inability to metabolize citrate under oxic conditions has long been considered a defining characteristic of *E. coli*, hence the spontaneous appearance of this variant is rather surprising, especially considering the fact that it occurred after 30,000 generations when most single point mutations should have been fully explored by the large bacterial populations. Experimental results suggest that the Cit+ phenotye resulted from some potentiating genetic changes. Based on prediction (a) of OptiEvo theory, Cit- may reside on a level set of the fitness landscape; hence, it could be easily lost in genetic drift, which may explain why it took so long for Cit+ to appear. In this case, the potentiating mutations can be either epistatic mutations or mutations physically required for the Cit+ phenotype, as proposed by the authors of the paper [24]. However, we do not expect reciprocal sign epistasis to be the cause for the late arrival of Cit+. Based on prediction (d) of OptiEvo theory, it is also possible that the genome for Cit- strains, while being optimal for glucose utilization, may not fully satisfy the sufficient flexibility condition (hence there is a false local trap) when citrate utilization is in the genome's fitness function. Note that the fitness landscape will not contain local peaks if every genotype→fitness mapping can be realized. False local traps exist only because some of the important mappings are inaccessible via allowable genetic changes. With OptiEvo theory, the role of the potentiating genetic transformations

is therefore to enhance the evolvability of the genome and consequently eliminate the false local traps. In this regard, insertions may be the most likely potentiating genetic change, because they increase the number of a genome's operating variables, which can enhance evolvability. The genome sequence experiments planned by the Lenski group [24] will help reveal the underlying cause for the evolution of the Cit+ strain.

Korona et al. [25] studied the evolution of 18 identical *Comanonas* sp. populations on a physically unstructured (mass-action liquid) and a structured (agar surface) environment. After 1,000 cell generations, the replicate populations in the mass-action environment converged to similar fitness values (consistent with prediction (a) of OptiEvo theory), while the populations in the structured environment showed more divergent fitness. The latter observation was considered to be a result of spatial heterogeneity in the concentrations of carbon, oxygen, moisture, and metabolites in the structured environment [25], hence not contradictory to the predictions of OptiEvo theory.

Travisano et al. [26] performed two evolution experiments on *E. coli*, one in a novel nutrient environment, and the second in a novel thermal environment. In the first experiment, the replicate populations were propagated for 2,000 generations in glucose-limited medium. The resultant populations had very similar fitness values. These populations were then propagated for another 1,000 generations in maltose medium. The derived fitness values were again similar in the new medium. In contrast, the deviations in cell size were much larger after evolution. The second experiment was similar, except that the temperature was the variable in the evolution process. Again, the derived fitness values showed convergence, while cell size varied significantly. All the observations are consistent with prediction (a) of OptiEvo theory.

Multiple replicate populations from *Ralstonia* sp. strain TFD41 were propagated for 1,000 generations with 2,4,-dichlorophenoxyacetic acid as the carbon source [27, 28]. Both parallel and divergent genetic changes were observed, while all populations improved their competitive fitness by approximately 40% relative to their common ancestor (agreeing with prediction (a) of OptiEvo theory).

Compared with the direct landscape studies, laboratory evolution experiments on an organism's population provide more global, hence more realistic evaluation of the fitness landscapes. However, working with bacterial/virus populations can increase the uncertainties in the measurements due to population heterogeneity, environmental fluctuations, and many other factors. As a result, the measured fitness values are often not as precise and reproducible as in the direct landscape studies where the mutant genotype is fully controllable. Despite these uncertainties, the fact that fitness convergence is almost always observed when evolution occurs in these very high-dimensional genome spaces strongly indicates the existence of trap-free landscapes (prediction (a) of OptiEvo theory). The observed multiple genotypes with similar fitness values implies the existence of connected level sets (prediction (b) of OptiEvo theory), although this finding is not conclusive without sequence and fitness information of all intermediate genotypes.

B.3 Reverse evolution experiments

Lenski [29] performed a reverse evolution experiment where ancestral *E. coli* populations sensitive to the T4 virus were evolved to produce T4-resistant mutants. These mutants were then evolved in the absence of T4. After 400 generations, the T4-resistant strains reverted to the same fitness value of the T4-sensitive strain, but their resistance to T4 was largely retained, indicating that they have different genetic compositions as a result of compensatory reverse evolution.

Burch and Chao [30] studied the evolution of the RNA virus $\phi 6$ where a single deleterious mutation was introduced to the ancestral clone. The virus was then evolved at different population sizes. It was found that the ancestral fitness level was recovered at larger population sizes (due to genetic reversion) and partially recovered at lower population sizes (due to compensatory mutations). The incomplete fitness recovery may result from the combination of the small number of generations and the low population size.

Crill et al. [31] studied the adaptation of the bacteriophage ϕX174 to alternating bac-

terial hosts, *Escherichia* and *Salmonella*. It was found that fitness recovery was achieved quickly after switching hosts regardless of previous history. Again, the experiments were not performed for very long and some incomplete fitness recovery was also observed.

Maisnier-Patin et al. [32] studied the evolution of 81 streptomycin-resistant strains of *Salmonella typhimurium* in the absence of the antibiotic. Complete fitness recovery was observed in many of the clones after 200 generations. 77 out of 81 clones involved compensatory mutations instead of reversions. Maisnier-Patin et al. [33] also studied the evolution of *Salmonella typhimurium* with mutations in the ribosome protein L19. After 300 generations, the fitness cost conferred by the L19 mutations was fully compensated for by additional mutations in L19 itself, as well as reflected in other proteins.

A common observation in these reverse evolution experiments is that compensatory mutations occur much more frequently than reversions [34, 35]. The fact that complete fitness recovery can be achieved through multiple compensatory mutations strongly implies that fitness landscapes do not contain multiple local optima while there exist multiple evolutionary trajectories leading towards the global fitness maximum value (consistent with predictions (a) and (c) of OptiEvo theory). Again, this coexistence of fitness convergence and genotypic diversity suggests the presence of connected level sets, although there is insufficient sequence data to fully confirm this conclusion.

In many duplicate population experiments and reverse evolution experiments, partial fitness recovery was observed and was considered as evidence supporting the model of rugged landscapes. For example, Burch and Chao [36] found that two populations of RNA bacteriophage $\phi 6$, while derived from a single ancestral phage, repeatedly evolved at different rates toward different fitness maxima, one higher and one lower than the initial fitness value. The authors considered the results as evidence for the constraint of evolvability by the distribution of mutational neighbors. We suggest that these experiments often were not performed for a sufficient number of generations to allow for complete fitness recovery (see discussion in the main text). In fact, fitness convergence was observed much more often in long-term evolution experiments than in short-term ones. Also, as discussed in the main text, we cannot

exclude the possibility that local fitness optima do exist in some cases, which (according to prediction (d) of OptiEvo theory) may occur in experiments involving gene deletion/loss [37] and for genomes of small size (such as for viruses [38]).

B.4 Experiment on the Connectedness of Optimal Genotypes

Whibley et al. [39] studied the flower color variation in natural populations and species of *Antirrhinum*. Using principle component analysis, they were able to define a 3D genotypic space controlling flower color. The analysis results indicate that *Antirrhinum* species with diverse floral phenotypes formed a U-shaped cloud within the genotypic space, which the authors proposed as an evolutionary path that allows flower color to evolve while circumventing valley genotypes. In addition, a 2D slice through the U-shaped 3D cloud produces two separated fitness peaks. According to prediction (b) of OptiEvo theory, these high-fitness genotypes may be evolutionarily connected, and traditionally plotted rugged landscapes may be simply an artifact of low-dimensional visualization of fitness spaces [40].

B.5 Laboratory evolution studies with higher organisms

In addition to microorganisms, *Drosophila* and *Caenorhabditis elegans* have also been employed in laboratory evolution studies, especially with reverse evolution experiments [41]. Unlike micoorganisms, fitness in these organisms is difficult to define. Hence, laboratory observations are carried out mostly on phoenotypic properties.

In one experiment [42, 43], replicate populations of *Drosophila melanogaster* were first selected for increased late-life reproduction for over five years, leading to differentiated pheontypes such as fecundity, longevity, glycogen content, and stress resistance. These populations were then returned to the original culturing environment selecting for early fertility. After only 22 generations, some characters such as starvation resistance and early fecundity converged to ancestral values. After over 100 generations, all assayed characters returned to their ancestral states.

In a related work [44], reverse evolution was performed on 25 diverged populations of *Drosophila melanogaster* for 50 generations. The results were similar to the previous studies, with some phenotypes returning to ancestral values while some did not. Hybrid populations were also employed in the study, showing insignificant difference in convergence. The results suggest that reverse evolution is historically contingent and is not caused by insufficient genetic variation. In a more comprehensive experiment [45], it was found that phenotypes that are directly related to fitness tend to converge more quickly, while characteristics less related to fitness converged more slowly or did not converge.

A recent study monitored the changes in the frequency of SNPs in both arms of the third chromosome of *Drosophila melanogaster* in 50 generations of reverse evolution [46]. An estimated 50% return of allele frequency was observed. Since the reversibility in viruses is $\sim 20\%$, and viruses mostly use new mutations to create diversity, the authors concluded that *Drosophila melanogaster* reverse evolution results more from standing variation (which is more repeatable) than new mutations.

A laboratory evolution study was also carried out on the nematode *C. elegans* [47]. In this work, *C. elegans* was first propagated as single individuals to allow for accumulation of mutations. These individuals were then maintained in large population sizes under competitive conditions. Full fitness recovery (measured by survival to maturity and progeny production) was achieved in fewer than 80 generations in some lines, driven at least partially by compensatory mutations.

In the above studies, the convergence of phenotypes seems consistent with predictions (a) and (c) of OptiEvo theory, while the less convergent cases seem to indicate false local traps. However, one must be cautious in drawing conclusions, because phenotype-genotype landscapes of higher organisms may not correspond well to genotypic fitness landscapes.

B.6 Affinity Maturation

Affinity maturation of antibodies in higher organisms is also an example of evolution. Affinity maturation can be understood in terms of a population, the initial antibody repertoire, that undergoes multiple rounds of selection and mutation to generate optimal antibodies in response to an antigen [48].

Shih et al. [49] created two lines of transgenic mice containing point mutations at the heavy chain loci that conferred high and low affinity anti-(4-hydroxy-3-nitrophenyl)acetyl(NP) antibodies. The two lines of mice were challenged with chicken-γ-globulin conjugated NP (NP-CGG) and their immune responses were analyzed. It was found that both lines produced similar numbers of germinal centers as the wild-type. Furthermore, their primary antibody responses were similar in both lines. These data suggest that antibodies have the same intrinsic ability to respond to an antigen and converge to similar fitness values via different evolutionary pathways (prediction (a) of OptiEvo theory).

De Genst et al. [50] analyzed the crystal structure of two heavy-chain antibodies(cAb-Lys2 and D2-L19) to hen egg white lysozyme recovered from different dromedaries to elucidate the mechanism of binding. Both antibodies used the same D gene segment but had differing rearranged V and J segments. However, both had high-affinity binding to the same epitope on the lysozyme. Moreover, the conformation of binding was identical for both antibodies. Such an example of convergent antibody evolution from different starting points is strong evidence for a trap-free fitness landscape.

Nickerson et al. [51] compared the immunochemical properties of a human and a mouse antibody in response to a range of blood group A substances. The heavy chains of the two antibodies had only 43% amino acid identity while the light chains had 57% amino acid identity. However, both antibodies displayed similar specificities to tested blood group A substances as quantified by the concentration required for inhibition and free energy of binding. This appears to be an example of fitness convergence, although a thorough analysis of the binding site structure was not done.

Various factors can affect the applicability of OptiEvo theory to affinity evolution. For example, the phenomenon of original antigenic sin [52, 53] has been cited as evidence of trapping [54, 55], although its biological basis is obscure [55], and this phenomenon may represent preferential induction of memory cells over naïve B-cells for antigen binding. Also, ruggedness in the antibody-antigen affinity landscape does not imply ruggedness in the fitness landscape. Affinity does tend to increase in the course of maturation (hence "affinity maturation"). However, affinity ceilings *in vivo* occur beyond which there is little selective pressure [56, 57], yet mutations of phenotypic benefit continue to occur. Furthermore, the selection step in the process may not be solely based on affinity [58, 59], and high-affinity antibodies are sometimes less effective *in vivo* at neutralizing an antigen than lower-affinity antibodies [60, 61].

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