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SELECTION BIASES THE PREVALENCE AND TYPE OF EPISTASIS ALONG ADAPTIVE TRAJECTORIES

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The contribution to an organism's phenotype from one genetic locus may depend upon the status of other loci. Such epistatic interactions among loci are now recognized as fundamental to shaping the process of adaptation in evolving populations. Although little is known about the structure of epistasis in most organisms, recent experiments with bacterial populations have concluded that antagonistic interactions abound and tend to deaccelerate the pace of adaptation over time. Here, we use the NK model of fitness landscapes to examine how natural selection biases the mutations that substitute during evolution based on their epistatic interactions. We find that, even when beneficial mutations are rare, these biases are strong and change substantially throughout the course of adaptation. In particular, epistasis is less prevalent than the neutral expectation early in adaptation and much more prevalent later, with a concomitant shift from predominantly antagonistic interactions early in adaptation to synergistic and sign epistasis later in adaptation. We observe the same patterns when reanalyzing data from a recent microbial evolution experiment. These results show that when the order of substitutions is not known, standard methods of analysis may suggest that epistasis retards adaptation when in fact it accelerates it.

KEY WORDS: **Adaptation, epistasis, models/simulations, molecular evolution, population genetics.**

Two sites in a genome interact epistatically when the contribution to a trait at one site depends on the state of the other site. Although epistasis has been a significant theme in topics such as the evolution of sex and robustness to mutation, its role in the dynamics of evolving populations has only begun to be explored. Recent experimental evolution studies of microbes (Blount et al. 2008; Chou et al. 2011; Khan et al. 2011; Woods et al. 2011) and biomolecules (Reetz and Sanchis 2008; Bloom and Arnold 2009; Hayden et al. 2011; Salverda et al. 2011) have revealed that epistasis is widespread and consequential for adaptation. These studies, combined with experiments that reconstruct ancestral genotypes (Weinreich et al. 2006; Bridgham et al. 2009; Lozovsky et al. 2009; Bloom et al. 2010; Lunzer et al. 2010; Novais et al. 2010; Martínez et al. 2011) or examine numerous combinations of adaptive mutations (Remold and Lenski 2004; Trindade et al. 2009; Kvitek and Sherlock 2011; Rokyta et al. 2011; Szendro et al. 2013), have amply demonstrated that molecular evolution cannot be explained or predicted without understanding how gene interactions shape adaptive possibilities.

These kinds of evolution experiments may seem especially informative because they quantify the epistatic interactions among the rare, beneficial changes that drive adaptation. However, these data reveal the interactions only among the sets of mutations that happened to substitute in the population, which may provide a different picture of epistasis than would sets of mutations

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that did not co-occur (Szendro et al. 2013). These potential biases highlight the limitations of our current understanding of how epistasis influences adaptation. Measuring epistatic interactions among alleles may help us to account for the outcome of a single experiment, but how can we use these data to predict the behavior of replicate experiments, or to predict adaptation in a larger population or in one with a different mutation rate? If selection and other evolutionary forces were blind to epistasis—that is, if interactions among sites did not systematically influence the likelihood that they would substitute in an evolving population—then the genetic changes we see in evolution experiments would perfectly mirror the epistatic properties of the underlying adaptive landscapes. If, however, evolution is biased toward the fixation of groups of alleles with specific patterns of interactions, then evolution experiments present a complex problem: if epistasis shapes evolution, and evolution distorts the appearance of epistasis, then how can we use evolution experiments to infer the underlying fitness landscape? This ambiguity complicates even qualitative inferences such as whether interactions among genes can be said to have slowed or hastened adaptation. To resolve this ambiguity, researchers must first understand how evolution biases the combinations of sites that substitute in an adapting population. Only then can researchers hope to correct for these biases, which will depend upon the size, mutation rate, and other characteristics of the population, to infer the underlying fitness landscape from experimental data.

Many theoretical studies of epistasis and patterns of asexual adaptation have focused on questions of the existence and accessibility of multiple fitness peaks (Kauffman 1993; Whitlock et al. 1995; Weinreich and Chao 2005; Cowperthwaite et al. 2006; Weissman et al. 2009; Carneiro and Hartl 2010; Dawid et al. 2010; Franke et al. 2011; Østman et al. 2012). Although such work has clarified the broad-scale picture of how epistasis shapes adaptation, its usefulness in predicting microevolutionary dynamics is limited. In contrast, our interest here is how experiments on adapting populations can be used to infer the properties of an organism's underlying fitness landscape and how epistasis shapes those experimental outcomes.

We use a computational model to clarify the evolutionary effects of two contradictory roles of epistasis: epistatic interactions can undermine the benefits of previously adaptive genetic substitutions, but they can also produce new paths to higher fitness (Draghi et al. 2011; Wagner 2011). The first of these effects would tend to retard adaptation, and the latter effect would accelerate it. Our results show that natural selection biases the prevalence and type of epistatic interactions among the mutations that substitute, even when mutations are too rare to interact directly as coexisting polymorphisms. Here we work to describe how selection biases the epistasis among mutations that substitute in an adapting population and to understand why these biases arise.

Methods

MATHEMATICAL FITNESS LANDSCAPES

Invented by Stuart Kauffman to describe rugged fitness landscapes (Kauffman and Levin 1987; Kauffman and Weinberger 1989; Kauffman 1993), the NK model produces complex but computationally tractable genotype-fitness maps using only the parameters N, K, and A. The parameter N defines the number of sites, each of which can assume any of A alleles. These sites can be seen as representing nucleotides or amino acids within a single gene, or as representing separate genes with multiple alleles. The fitness of a genotype is calculated in two steps: first, the fitness contribution of each site is determined by reference to a table of precalculated values; second, these fitness contributions are multiplied together and the Nth root of this product is taken as fitness. When K is 0, the fitness contribution of a site depends only on its own allele, and not on the state of other sites; the lookup table for each site therefore contains only A possible fitness contributions. When $K > 0$, the fitness contribution of a site depends on its own allele as well as the alleles at K other sites, yielding a lookup table with A^{K+1} entries. By specifying that each of the entries in the lookup tables for all N sites are drawn independently from a broad distribution (in our case the uniform distribution), the NK model ensures that the fitness effect of a substitution depends strongly and randomly on some fraction of the genetic background, determined by K. K is constant across sites and genotypes for a particular landscape, and the K sites upon which each locus depend are drawn uniformly from the $N - 1$ possibilities.

Gene interactions described by the NK model are directional: the fitness contribution of site i may depend on the state of site j without implying that the contribution of j also depends on i. We can therefore categorize relationships between sites: i is *downstream* of j if the fitness contribution of i depends on j, and j is conversely *upstream* of i in this example. Although this direction of influence is significant for some of our results later, we note a subtle confusion between epistasis as defined in the NK model, and epistasis as defined by an experimentalist. This confusion stems from the fact that an experimentalist measures the fitness effect of a substitution, although the NK model considers contributions of sites to a total measure of fitness. Therefore, the fitness effect of a substitution at site i will show epistasis with site j not only if i is downstream of j, but also if it is upstream of j. Similarly, the fitness effect of a substitution at i will be epistatic with j if both i and j are upstream of some mutual site k, even if neither i nor j directly influence one another—we refer to this specifically as an *indirect* interaction. To minimize this potential confusion, we follow the empirical definition and use the term "epistasis" to refer to any case in which the fitness effect of a substitution at a site depends on the state of some other site. This usage makes the common assumption that independent

fitness effects are multiplicative. Choosing a multiplicative scale for fitness has the advantage that the fixation probability of an allele is independent of the absolute fitness of the genetic background. Therefore, if two mutations are not epistatic, then the fixation of the first does not affect the fixation probability of the second.

We can easily calculate the probability of each form of epistasis for two sites i and j chosen at random. Later we calculate p_u, the probability that i is upstream of j, p_d, the probability that i is downstream of j, and p_i, the probability of an indirect interaction. These random expectations are used to establish baseline frequencies of epistasis for random mutations.

$$p_u = \frac{K}{N-1}, \tag{1}$$

$$p_d = \frac{K}{N-1}, \tag{2}$$

$$p_i = 1 - \left(1 - \frac{K}{N-1}\frac{K-1}{N-2}\right)^{N-2}. \tag{3}$$

In addition to NK landscapes, we also explored epistasis and evolutionary dynamics using RNA folding landscapes, as well as experimental bacterial data, described later.

EVOLUTIONARY SIMULATIONS

To investigate epistasis among genetic substitutions, we employed two types of simulations: adaptive walks, which model simplified fixation dynamics in essentially monomorphic populations, and individual-based Monte Carlo simulations of populations. In an adaptive walk the population is represented by a single genotype, and a mutation to any of the $A-1$ alternative alleles at any site is a candidate substitution. For simplicity, we assume that the probability of fixation for a mutation is directly proportional to its selective coefficient, $s_i = \frac{w_i}{w} - 1$, where w is the fitness of the currently fixed genotype and w_i the fitness of the mutant i. Evolution is then a Markov process with transition probabilities defined by

$$P_i = \frac{s_i}{\sum\limits_{i \in M} s_i}, \tag{4}$$

where M_i is the set of all adaptive, one-mutant neighbors of the current genotype (Gillespie 1984; Orr 2002). This approximation is accurate for small selective coefficients in large populations and avoids the need to add an explicit population-size parameter to this simple model. These walks therefore substitute one mutation at a time, strictly increasing in fitness until a local maximum is reached.

We used the Wright–Fisher to describe evolution in polymorphic populations: an asexual population of fixed size n reproduces with discrete generations and selection on fertility. Mutations occur at Poisson frequencies according to the per-genome rate μ. Because our goal was to investigate evolutionary dynamics when $n\mu = \theta$ is near or greater than 1, we required a method of detecting substitutions that does not depend on independent, well-demarcated fixation events. We therefore traced the line of descent from the most common genotype in the final population back to the initial generation and record changes along this lineage.

To compare our results with empirical data, we performed two types of regressions on the fitness effects of the first few substitutions in evolutionary simulations. Following recent experimental examples (Chou et al. 2011; Khan et al. 2011), we examined the first five substitutions in a simulation and measured the fitness effect of each of these substitutions with all combinations of the allele states at the other four sites. The fitness of each of the 16 genetic backgrounds is taken as the independent variable, and the fitness effect of the focal substitution as the dependent variable; a separate regression was performed for each of the five sites, although the resulting five correlation coefficients are not independent. These analyses were contrasted with a second type of regression, in which the ranks of epistatic deviations of each successive substitution was compared to their order of substitution. If we consider the first five substitutions, then there are four epistatic deviations:

$$e_{12} = W_{12} - W_1 W_2, \tag{5}$$

$$e_{123} = W_{123} - W_1 W_2 W_3, \tag{6}$$

$$e_{1234} = W_{1234} - W_1 W_2 W_3 W_4, \tag{7}$$

$$e_{12345} = W_{12345} - W_1 W_2 W_3 W_4 W_5, \tag{8}$$

where W_{12}, for example, represents the fitness of the genotype with the first two substitutions, divided by the fitness of the ancestor. Because these regressions are based on four points, we use this analysis only to classify epistasis along a walk; for example, we highlight the significance of walks in which $e_{12345} > e_{1234} > e_{123} > e_{12}$ as examples where epistasis consistently leads to greater than expected fitness.

In both cases, we ignore simulations with little epistasis among the first five substitutions because such data sets had insufficient ranges of values to allow meaningful correlations to be calculated; our filtering removed about 20% of simulations. Because we remove walks without regard to the direction of epistatic effects, this removal should not bias any results.

New landscapes were generated for each replicate simulation. In both types of populations, a simulation began from a randomly drawn genotype with fitness in a certain range, usually close to the 50th percentile (i.e., within 0.002 of the desired starting fitness). Individual replicates varied considerably in the number of adaptive steps and their fitness effects; on average, the walks

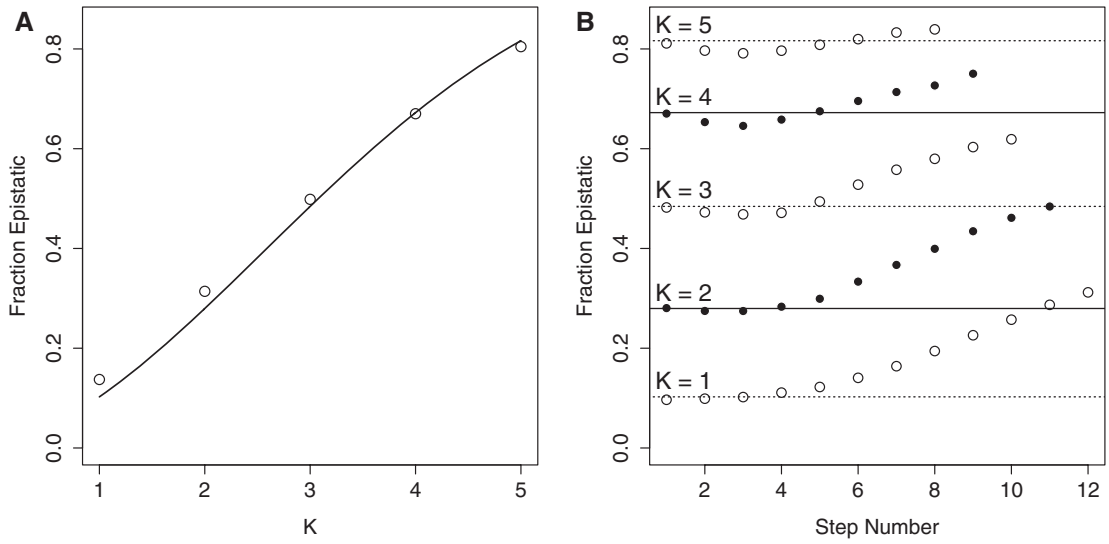

Figure 1. **(A) The overall amount of epistasis along an adaptive walk in an NK landscape roughly agrees with its random expectation. The dots show the frequency of epistatic interactions between substitution *i* and its immediate successor *j*, averaged across all steps in the adaptive walk. The solid line depicts the predicted incidence of epistasis, if sites are chosen to substitute randomly (see supplement). $N = 20$ and $A = 2$. Standard errors are less than 0.001. (B) The frequency of epistasis between subsequent substitutions is depressed compared to the random expectations, early in adaptation, and augmented late in adaptation. Dots show the frequencies of epistasis between substitution *i* and its immediate successor *j*, indexed by the position of substitution *i* along the adaptive walk. Lines indicate the corresponding random expectation. Standard errors are less than 0.01 for all plotted means.**

on landscapes with higher K are significantly shorter, but reach the same level of fitness. Mean and example values of fitness and walk length are shown in Figures S1 and S2.

RNA FITNESS LANDSCAPES

We also performed Wright–Fisher simulations of evolving RNA populations using the Vienna RNA folding package, version 1.8.5, with default folding parameters. RNA sequences of 72 bases in length constituted the genotypes, and the predicted minimum free-energy structures determined the phenotypes. Fitness of an RNA genotype was calculated as $(1 + s)^{-d}$, where d denotes the tree-edit distance between the RNA's phenotype and a defined optimal phenotype. Here s quantifies the strength of selection and is equivalent to the multiplicative selective coefficient associated with a mutation that changes d by a single unit; $s = 0.01$ in the results shown. The tree-edit distance algorithm, included in the Vienna package, determines the minimum number of steps from a group of edit operations that are needed to transform one structure into another. The initial genotype was drawn randomly, and the optimum phenotype, used to impose directional selection, was also created by randomly drawing genotypes and discarding those whose minimum free-energy structure is the trivial, unfolded state. This optimum was also required to be 40 units from the phenotype of the initial genotype so that the pressure to adapt was strong and uniform across replicates. Simulations were run for 50,000 generations.

Substitutions in RNA simulations were determined by tracing a line of descent, as described earlier for NK simulations. Because our goal was to study adaptation among beneficial mutations, we filtered the resulting records of substitutions by ignoring adjacent pairs on the line of descent when both members of the pair were neutral or deleterious on the background in which they originally fixed. In practice, less than 1% of pairs were excluded by this rule, so our results are not sensitive to this criterion.

DATA ARCHIVING

Simulation results, C code, and R scripts have been archived at the Dryad doi:10.5061/dryad.f468j.

Results

PREVALENCE OF EPISTASIS ALONG AN ADAPTIVE WALK

To understand how selection shapes epistasis among the mutations that substitute, we first simulated adaptive walks in which beneficial mutations substitute sequentially, according to the simple infinite-population expectation of their fixation probabilities; we refer to these simulations as adaptive walks (see Methods). Østman et al. suggested that epistatic interactions should not influence substitution patterns when rare beneficial mutations fix independently (Østman et al. 2012). Our results initially appear to confirm this expectation. Figure 1A shows that sites that fix

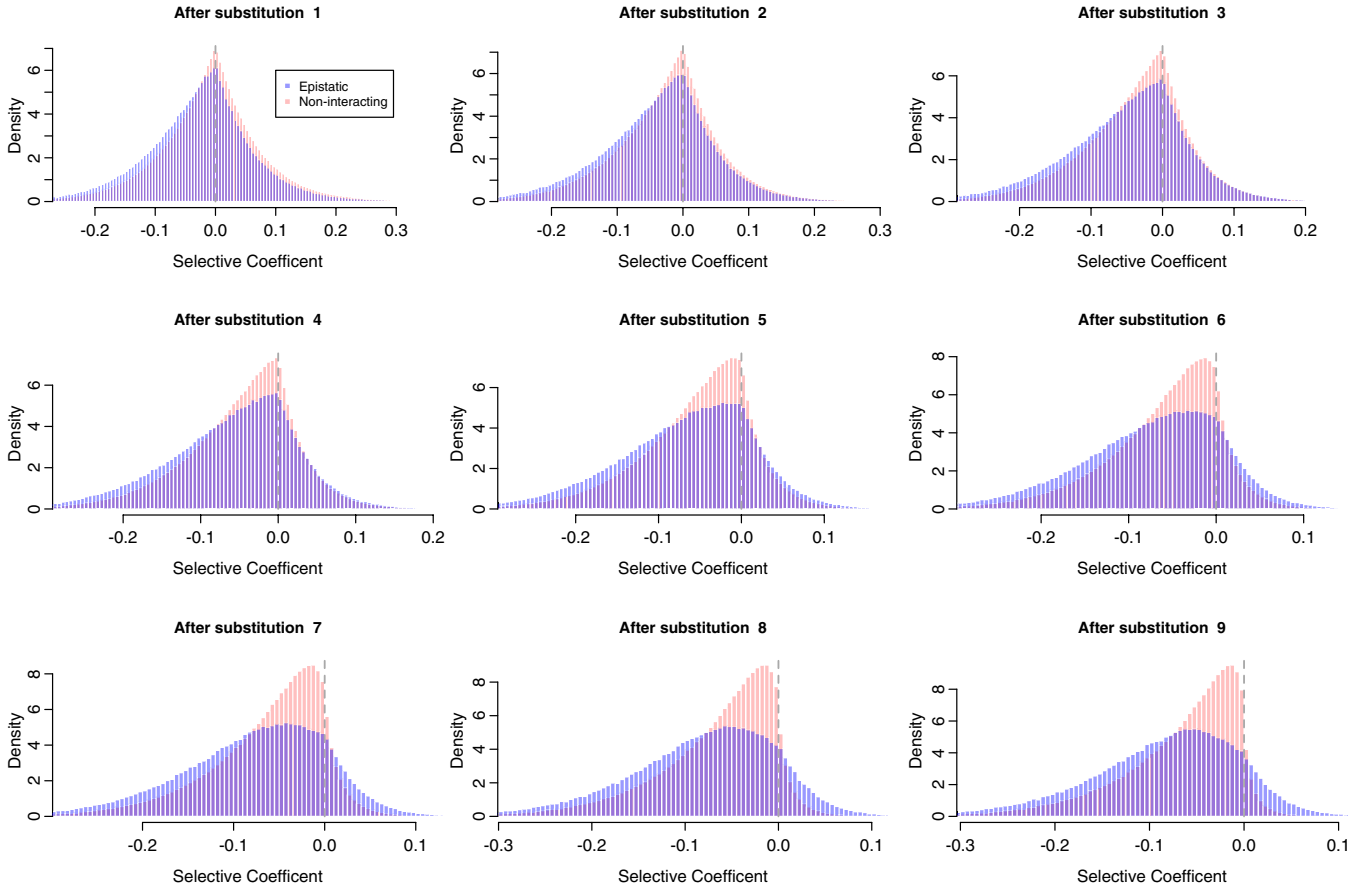

Figure 2. **Mutations at sites that interact with recent substitutions are less favorable early in evolution and more favorable late, in comparison to noninteracting sites. Histograms depict the frequencies of the selective coefficients of mutations across many replicate adaptive walks with parameters $N = 20$, $K = 1$, and $A = 2$. After an adaptive substitution occurs at site i, all $(A - 1)(N - 1)$ other possible mutations are inspected and classified by whether their fitness effects depend on site i epistatically, or are independent of it. As the adaptive walk proceeds, the selection coefficients of available mutations shift toward negative values in general. At the same time, among the adaptive mutations available late in evolution, the great majority of them interact with a recent substitution. Thus, epistasis tends to retard the substitution of interacting sites early in evolution, and promote such substitutions late in evolution.**

sequentially are almost as likely to interact epistatically as are pairs of randomly chosen sites. However, this concordance disappears when epistasis is examined along the sequence of steps comprising an adaptive walk. Figure 1B shows that epistasis is in fact suppressed early in adaptation, and enriched among later steps, compared to a random (neutral) walk. Thus, selection biases the amount of epistasis among the mutations that fix along an adaptive walk, and it does so in a complex manner. Because walks vary substantially in length (Fig. S1), the strong positive biases seen after a number of steps occur infrequently and are therefore approximately balanced by the smaller negative biases characteristic of early steps. These opposing effects therefore produce an apparent agreement with the random expectation for the overall prevalence of epistasis when observations are coarsely averaged across entire walks, but in fact these results demonstrate that epistasis can shape patterns of substitutions even when mutations fix independently, one after another.

Why does selection suppress apparent epistasis early in walks and promote it later? To address this, we studied how mutations at sites along an adaptive walk influence the fitness effects of the sites with which they interact. In particular, in the simple case of $K = 1$, Figure 2 shows the distribution of fitness effects of mutations at those sites that do and do not interact with the site that has just substituted along a walk. For a site i that changes early in the walk, mutations at its interacting sites are less likely to be beneficial. In other words, adaptive substitutions early in the walk partly undermine the benefits that would be conferred by mutations at their partner sites. Therefore, after an early substitution at one site, its epistatic partners are less likely to substitute than they would have otherwise—and so the early steps in an adaptive walks exhibit a deficit of epistasis compared to the neutral expectation.

This bias against epistasis early in a walk is caused by the dependency of selected substitutions on the backgrounds in which they were selectively favored. When a site forms part of the

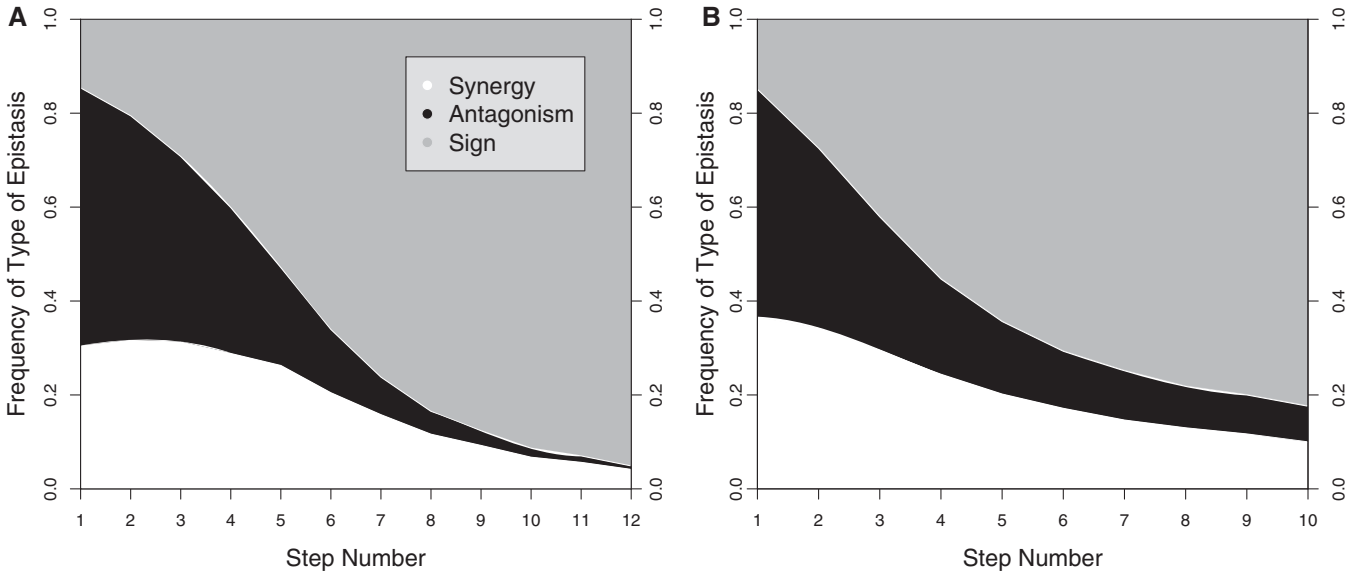

Figure 3. Prevalence of synergy, antagonism, and sign epistasis among pairs of consecutive substitutions (i, j) along adaptive walks on NK landscapes. Such walks are characterized by an abundance of antagonism early in adaptation, and an abundance of sign epistasis late in adaptation. (A) $K = 1$, (B) $K = 5$. $N = 20$ and $A = 2$.

relevant genetic background for a beneficial substitution, it is statistically likely that changes at such a site would partially undermine the beneficial effect of this fixed adaptive substitution. Figure S3 demonstrates this regression to the mean effect: selective coefficients of mutations at a site j are suppressed when j interacts with a site i that has just fixed an adaptive mutation, and this suppression is greater when the beneficial effect of the substitution at i is larger.

This argument leads to the opposite pattern late in adaptive walks, when most mutations are deleterious. Consider the case of a high-fitness genotype in which only one locus may mutate to a beneficial allele. When this one beneficial change fixes, it may then alter the fitness effects of its epistatic partners so that a potential mutation at another site now becomes beneficial. Because this now-beneficial change is the only possible route to further adaptation, it too will surely fix; this fixation may epistatically perturb the fitness effects of yet another locus, leading to still further adaptation. Thus, when the vast majority of possible genetic changes are deleterious, epistatic interactions become the only avenue for adaptation, and so interacting pairs of substitutions are frequently observed late in adaptation.

FORM OF EPISTASIS ALONG AN ADAPTIVE WALK

Aside from biasing the amount of epistasis along a walk, selection also biases the type of epistasis between successive substitutions. We find that the predominant sign of epistasis, as well as its prevalence, depends on the position of the substitutions along an adaptive walk. Figure 3 shows that early substitutions tend to show antagonism with one another, whereas later substitutions typically exhibit sign epistasis, defined as pairs of mutations where at least one member has a beneficial fitness effect on one background and a deleterious effect on the other. Synergy between beneficial mutations is present but less common than antagonism at early steps and less common than sign epistasis at later steps. The shift from antagonistic toward synergistic/sign epistasis helps to explain why epistasis is suppressed early in an adaptive walk and augmented later in the walk.

Selection also biases the directionality of interactions between successive substitutions along an adaptive walk (see Methods). Figure 4A shows that interactions with i upstream of j are more frequent than the converse along the entire adaptive walk. When $K > 1$, another type of interaction is possible: i and j might not influence each other directly, but both might influence a third site. Epistasis of this type is expected to be very common when K is a substantial fraction of N, and results for $K = 5$ show that the prevalence of this type of interaction also changes along adaptive walks (Fig. 4B). Thus, evolution biases the types and directions of interactions among substitutions along an adaptive walk.

Choosing the K sites which influence each of the N loci defines a network of directed interactions. Although the number of sites that influence a locus (its in-degree) is fixed at K, the number of sites that a locus influences (its out-degree) is variable and approximately Poisson distributed. Figure S4 shows that this variation in epistasis affects the rate of substitution. The out-degree of substituted sites is initially slightly higher than expected, then declines with substitution number. Because loci with a high out-degree influence more sites, they may have stronger effects on fitness when mutated. In the backgrounds of the

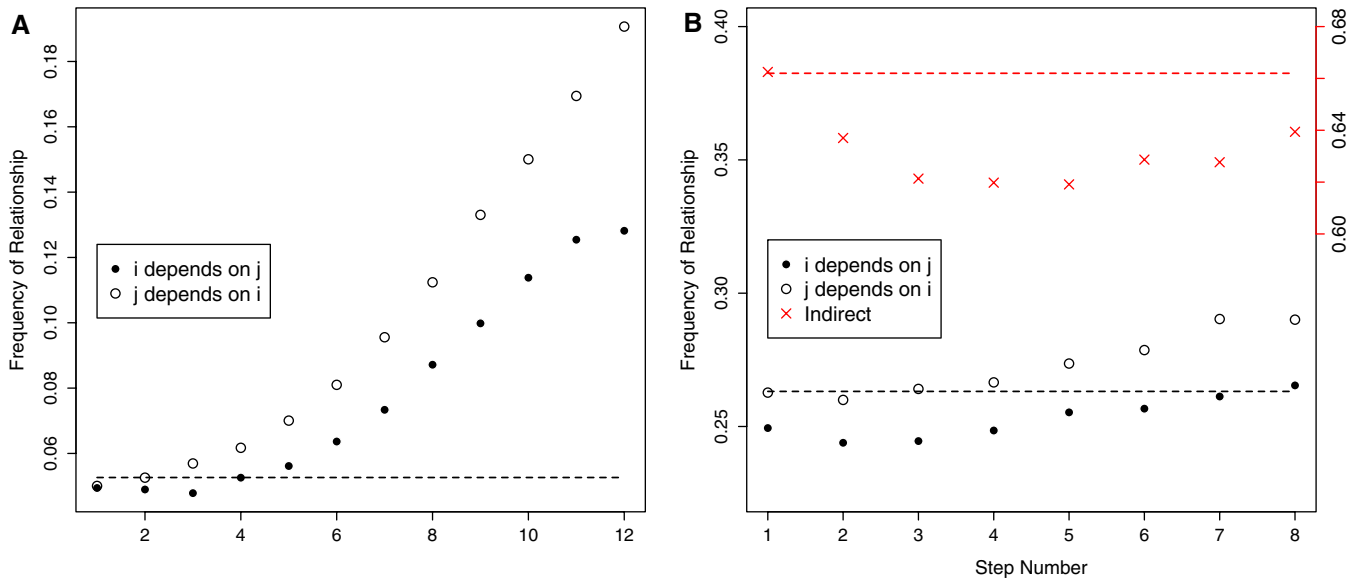

Figure 4. Observed frequency of directional epistatic interactions between substitution *i* and its immediate successor *j* along adaptive walks on NK landscapes. (A) $K = 1$ and (B) $K = 5$. A single pair may be counted as more than one type of epistasis, as in the case of reciprocal interactions. The dashed lines depict the predicted incidences if substitutions are chosen randomly (Eqs. (1)– (3)). For $K > 1$, another class of epistasis is possible: both *i* and *j* may jointly influence a third site, which we refer to as an indirect interaction. $N = 20$ and $A = 2$. Standard errors are less than 0.01 for all plotted means.

starting genotypes, site out-degree does correlate with the mean size of a beneficial mutation: each increment in degree corresponds to about a 0.01 increase in mean selective coefficient (slope $= 0.0097 \pm 0.0001$, $R^2 = 0.03$, $P < 2 \times 10^{-16}$). However, there is no relationship with the probability that a mutation is beneficial (logistic regression, $P = 0.72$). The greater size of selective coefficients for high out-degree loci probably explains their greater tendency to substitute early, as well as the influence of the directionality of epistasis shown in Figure 4.

ROBUSTNESS OF RESULTS AND COMPARISON TO DATA

The NK model has several features that could amplify the biases in epistasis introduced by natural selection; for example, alternative fitness contributions of a locus are drawn independently, each locus contributes equally to fitness, and conditionally neutral changes are very rare. To assess whether these model assumptions might be responsible for our qualitative results, we explored variants of the NK model as well as a completely different class of genotype–phenotype maps.

We first examined how the patterns shown in Figure 4 change as the number of alleles per site and the starting fitnesses were varied. Figure S5 shows that deviations from the expected prevalence of epistasis are qualitatively similar, and quantitatively greater, when the number of alleles per site, A, is increased. This suggests that the 20 sites in the genetic sequence of our model can be interpreted flexibly; by changing A and K, we can represent nucleotides or amino acids in a single protein, or multiple genes with many possible alleles. Figure S6 confirms that the basic pattern of our results is also robust to changes in the fitness of the starting genotype.

We also examined if either the ratio K:N, or the absolute magnitude of K predicts the nature of the epistatic pattern by varying N as well as K. Fig. S7 shows that the ratio of K:N does not predict a consistent pattern of epistasis across variation in N. The value of K is similar for $N = 20$ and $N = 40$, but differences are apparent at higher step numbers. Larger genomes permit more adaptive steps, suggesting that the pattern of epistasis among adaptive substitutions will depend on both N and K.

Although we have focused on evolutionary dynamics in the simplified, strong-selection–weak-mutation regime, we can use individual-based simulations to explore epistasis in polymorphic populations with larger values of the population-scaled mutation rate, θ. Figure S8 show patterns for the prevalence of epistasis among adaptive substitutions which differ from the neutral expectations, even when θ is one or greater 1. Although the deviations are smaller in these more complex populations, these results at high θ confirm the major patterns found in adaptive walks at low θ: there is a deficit of epistasis early in evolution, and a surplus of epistasis later in adaptation, with a predominance of interactions in which the effect of each substitution depends on the preceding substitution along the line of descent.

We also considered a very different set of fitness landscapes—computationally predicted RNA folding—to assess the generality of our principal findings. RNA sequences do not have predetermined epistatic interactions between sites; instead,

interactions emerge from the folding topology and change with genotype. However, we can still measure the average frequency of epistasis between substitutions on an evolutionary line of descent; such data show that the prevalence of epistasis does vary systematically along a series of substitutions, although the trend is toward decreased epistasis (Fig. S11A). The type of epistasis can also be quantified, with the addition of a fourth type; if mutation i is entirely neutral on its evolved background but has a fitness effect when combined with mutation j, we call this "neutral epistasis." The opposite case, in which i has a fitness effect on its evolved background but is neutral in the background containing j, is classified identically. This additional category of neutral epistasis is needed because many mutations in RNA do not change the minimum-free-energy structure, and are therefore truly neutral in that context. Figure S11B shows that, as in the NK model, early antagonism gives way to a high prevalence of sign (and neutral) epistasis later in adaptation. Although these results are qualitatively different in the direction of the trend of epistatic prevalence along a walk compared to those obtained in the NK model, they further illustrate that evolution at $\theta < 1$ can indeed bias the epistatic properties of fixed mutations, and it does so differentially at different stages of adaptation.

IMPLICATIONS FOR INFERENCES FROM EXPERIMENTAL DATA

Two recent studies on experimental populations of bacteria have inferred that antagonistic epistasis among beneficial mutations is common and ultimately explains a trend of diminishing fitness gains over time (Chou et al. 2011; Khan et al. 2011). These studies relied in part on regression analyses of the fitness effects of observed substitutions in the presence and absence of the other beneficial substitutions observed in the experiment. Both studies found a trend toward smaller beneficial effects when substitutions were assayed in backgrounds of higher fitness and so concluded that antagonistic epistasis decelerates adaptation. However, we demonstrate later that in the NK model, such regressions are not a reliable indicator of the effect of epistasis on the speed or extent of adaptation. Our results suggest that a common statistical artifact—regression to the mean—confounds the interpretations of such regressions and that analyses of the role of epistasis in adaptation may be meaningful only when the actual ordered sequence of substitutions is known.

We performed the same kinds of regressions as Chou et al. (2011) and Khan et al. (2011) on adaptive walks simulated on NK landscapes. Specifically, we computed rank regression coefficients of background fitness versus fitness effect for the first five substitutions in such adaptive walks (see Methods). The distribution of average regression coefficients in Figure 1A shows a bias toward negative values similar to those seen in bacterial experiments (Chou et al. 2011; Khan et al. 2011), suggesting that epistasis becomes more negative with each substitution and decelerates the pace of adaptation. However, this interpretation is contradicted by our results earlier. Figure 3 clearly shows that, on average, epistasis becomes more positive with each substitution. Figures 1, 2, and 4 also support this view: epistasis is initially disruptive to the large fitness gains of early adaptive changes, but then facilitates later adaptive steps. Finally, Figure 1B shows that a genotype along the line of descent is typically more fit than would be predicted from the fitness effects of its component mutations in the ancestor, and that this synergistic effect increases along adaptive walks.

Our results imply that regression analysis of fitness effects on different genetic backgrounds (e.g., those performed by Khan et al. (2011) and Chou et al. (2011)) may be misleading. To clarify this issue, we examined the mean regression coefficients for those adaptive walks that were unequivocally accelerated by epistasis—namely, those walks in which the effect of each subsequent mutation was greater than expected under multiplicativity. Even when restricted to these completely synergistic walks, the regression analyses of the type shown in Figure 1A. are most often negative and so would erroneously suggest increasing antagonism (Fig. S12). Furthermore, we also performed random (neutral) walks, in which substitutions along the line of descent are equally likely to exceed or fall short of their expected multiplicative fitness, given the fitness effect in the ancestor; even in these walks, regression coefficients of the type studied by Khan et al. (2011) and Chou et al. (2011) tend be negative (Fig. S13). These data confirm that regressions of fitness effects against the fitnesses of genetic backgrounds cannot be reliably used to infer whether epistasis has slowed or accelerated epistasis, at least in the NK model.

The tendency of these regressions toward negative slopes may be caused by the well-known confound of "regression to the mean." In these regressions, the dependent variable, fitness effect of substitution i on a genetic background, is mathematically interrelated with the independent variable, the fitness of that same background. If the fitness of the background genotype is very poor, then the epistatic effects of its alleles are statistically likely to be unusually poor. Any change that perturbs these epistatic effects is likely to improve their fitness contributions. Therefore, a substitution in a very unfit background is likely to show a large beneficial effect simply by perturbing the fitness effects of interacting sites. A similar argument can be made to explain why fitness effects are often small or even deleterious in highly fit backgrounds and, by extension, why negative correlations are a likely consequence of the interdependence between the variables in this regression.

To assess the relevance of this potential confound for experimental results, we re-examined data from the microbial evolution experiment of Khan et al. (2011), in which the actual order of

substitutions that occurred is known. As shown in Figure S14, we used their fitness measurements to calculate epistasis along the path of adaptive change. Two methods of calculating expected fitness, which differ in their choice of reference genotype, both yield the same qualitative result: epistasis is initially negative, then becomes positive during the later stages of adaptation. Although this pattern represents only a single instance of an empirical evolutionary trajectory, its similarity to the patterns expected under our analysis of a broad class of mathematical fitness landscapes (Fig. 3) is striking. This reanalysis suggests that epistasis may in fact be accelerating late adaptation in these experimental populations, in contrast to the original interpretation of the data (Khan et al. 2011; Kryazhimskiy et al. 2011).

Discussion

To solve the dual problem of epistasis in evolution experiments—that evolution experiments are both shaped by epistasis, and provide a biased sampling of the interactions among the genes that evolve—requires a substantial expansion of population-genetic theory for arbitrary fitness landscapes. Because the term "epistasis" encompasses all scenarios in which fitness effects of alleles do not combine independently, no entirely general model of epistasis has been proposed, let alone analyzed. Instead, exploration of a few "toy" models has led to appreciation of the subtle and significant ways that epistasis complicates our understanding of evolution.

Here we have used the NK model to contravene the intuitive notion (e.g., (Østman et al. 2012)) that sites substituting one after another will be selected without regard to their epistatic interactions. To summarize, we have shown that, even when mutations are rare, evolution selects among possible substitutions based upon the number and direction of connections to other loci, and that these selective biases change substantially along the course of adaptation. In the NK landscapes, epistasis is less prevalent than the random expectation early in adaptation and much more prevalent later, with a concomitant shift from predominantly antagonistic interactions early in adaptation to synergistic and sign epistasis later in adaptation. In addition, sites with more epistatic influences on other loci are more likely to substitute early than late in adaptive evolution.

The basic intuition behind our results is simple. Early on, large-effect mutations tend to act antagonistically, which suppresses the frequency of epistasis among subsequent substitutions. Later on, the only way to achieve further fitness gains is by fortunate sign epistasis, and this effect tends to augment the appearance of epistasis as the population approaches a fitness peak. These results suggest that even the most basic evolutionary process acting in the context of a simple fitness landscape can produce a complex expectation for epistasis. Experimentalists must account for this baseline action of natural selection on epistasis among substitutions if they hope to infer the properties of the fitness landscape from experimental or natural evolutionary outcomes.

Our results agree with the general empirical finding that the sets of mutations that occur in a given evolution experiment tend to show less epistasis than sets of mutations that did not fix in the same population (Szendro et al. 2013). This empirical trend suggests that current experiments have examined epistasis only in the initial phase of adaptation, when beneficial mutations are relatively common. As a result, it will be difficult to assess what level of epistasis (i.e., what value of K) best fits empirical fitness landscapes. To do so will require studies that quantify epistasis for a broad set of mutations—identified using both random mutagenesis and experimental evolution—in the same organism and environment. Our results also highlight the importance of replication in evolution experiments: examining beneficial mutations that occurred across several replicate populations may reveal greater epistasis. In particular, an experiment that finds significant epistasis but infers only a single adaptive peak (Chou et al. 2011; Khan et al. 2011) might well identify multiple peaks if replicate populations are examined.

We have focused on evolution by sequential fixation of beneficial substitutions to demonstrate that this seemingly simple case conceals several layers of complexity. However, our results suggest patterns, such as a decrease in observed epistasis among early substitutions and an increase in epistasis among later ones, that extend to polymorphic populations as well. By focusing on the differences between the distribution of epistasis among all sites, and the specific sequence of substitutions, our approach highlights the potential for misleading inferences from evolution experiments when the order of substitutions is unknown. Specifically, it may be difficult to reliably determine how epistasis varies with fitness using only the fitnesses of an ancestral, derived, and possible intermediate genotypes. Regression analyses that ignore substitution order might suggest that epistasis is decelerating adaptation (Chou et al. 2011; Khan et al. 2011; Fig. 5), whereas in fact epistasis has had an accelerating effect on the trajectory of fitnesses along the actual path of adaptation. Indeed, our reanalysis of data from Khan et al. (2011) supports this possibility. Such discrepancies illustrate the importance of measuring the order in which substitutions occur, in future experimental studies, to understand how epistasis has shaped a population's trajectory.

While the NK model has the advantages of a tunable level of epistasis and an extensive history of prior work, it certainly does not capture the full range of possibilities of interactions among genes. Our results with larger numbers of alleles in the NK model, with a computational model of RNA folding, and with data from evolving microbial populations, suggest that the patterns we have identified in simple cases may be even more pronounced in models that better approximate biological complexity. However, the

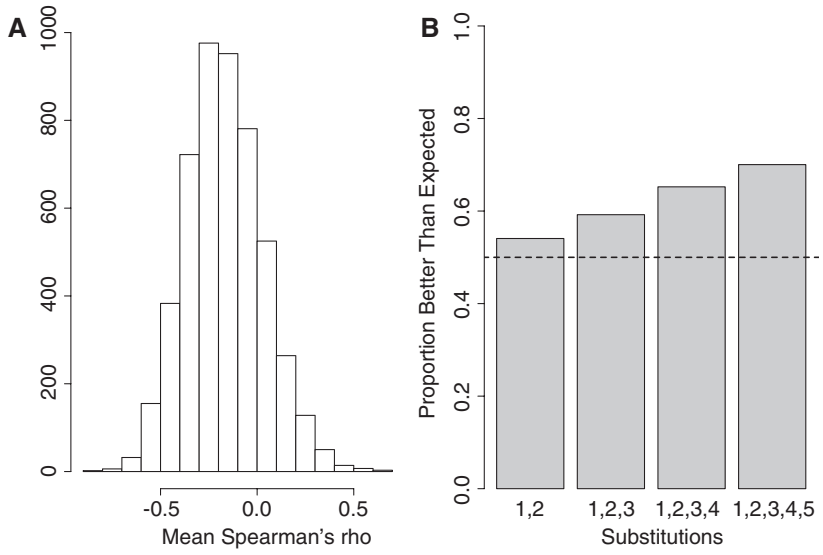

Figure 5. **Two views of epistasis among the first five substitutions in an adaptive walk. (A) Spearman regression coefficients for the relationships between the fitness effect of a substitution and the fitness of the genetic background in which that substitution is made (Chou et al. 2011; Khan et al. 2011). Each substitution is tested against the 16 genetic backgrounds comprising all combinations of the other four substitutions. (B) Proportion of combinations of substitutions along the line of descent which exceed the fitness expected from the fitness effects of the individual mutations in the ancestor. Expectations are computed according to equations (5)–(8). Standard errors are less than 1%. In both figures, replicates are filtered to remove adaptive walks with too little epistasis among substitutions (see Methods)—data shown in panel (B) are additionally filtered to remove cases where $W_{12} = W_1 W_2 / W$. The analysis on the left panel would suggest that epistasis is primarily antagonistic, whereas in fact the right panel shows that synergistic interactions dominate along the walk. $N = 20$, $K = 5$, and $A = 2$.**

most important conclusion from our study is that new methods of data-gathering, such as cheap whole-genome sequencing and high-throughput fitness assays, will not suffice to answer basic questions about the role of genotype-phenotype maps in evolution. Pioneering studies have provided compelling examples of the ubiquity of epistasis, but they also serve to exemplify the substantial gap that separates data from hypotheses in experimental evolution. To definitively link gene interactions to the rate or predictability of adaptation will require a significant expansion of the theory of population genetics, and a vital first step is serious engagement with "toy" models of interacting loci.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1: Lengths of adaptive walks.
Figure S2: Fitness changes over the course of adaptive walks.
Figure S3: Relation of the beneficial fitness effect of substitution *i* to the change in the fitness effect conferred by a mutation at interacting site *j*.
Figure S4: Mean out-degree (number of loci which depend epistatically on the focal site) of substituted sites.
Figure S5: Observed frequency of directional epistatic interactions between substitution *i* and its immediate successor *j* for adaptive walks on *NK* landscapes with different values of *A*, the number of alleles per locus.
Figure S6: Observed frequency of directional epistatic interactions between substitution *i* and its immediate successor *j* for adaptive walks on *NK* landscapes for different values of the fitness percentile of the starting genotype.
Figure S7: Frequency of epistasis between substitution *i* and its immediate successor *j* in adaptive walks, divided by the neutral expectation (Eqs. 1–3).
Figure S8: Observed frequency of directional epistatic interactions between substitution *i* and its immediate successor *j* for individual-based simulations.
Figure S9: Observed density of substitutions over time for individualbased simulations.
Figure S10: Mean (red) and five example fitness trajectories for individual-based simulations.
Figure S11: Epistasis in individual-based evolution in a model of RNA folding.
Figure S12: Spearman rank regression coefficients for the relationships between the fitness effect of a substitution and the fitness of the genetic background in which that substitution is made for adaptive walks which exceed multiplicative expectations at each step.
Figure S13: Two views of epistasis among the first five substitutions on random (neutral) walks; contrast with the corresponding data from adaptive (selected) walks in Fig. 5.
Figure S14: Epistasis deviation is initially negative, then becomes positive along the sequence of the first five substitutions observed in a microbial evolution experiment (Khan et al. 2011).