DOI: 10.1534/genetics.110.125997

Epistasis Increases the Rate of Conditionally Neutral Substitution in an Adapting Population

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Manuscript received December 16, 2010
Accepted for publication January 24, 2011

ABSTRACT

Kimura observed that the rate of neutral substitution should equal the neutral mutation rate. This classic result is central to our understanding of molecular evolution, and it continues to influence phylogenetics, genomics, and the interpretation of evolution experiments. By demonstrating that neutral mutations substitute at a rate independent of population size and selection at linked sites, Kimura provided an influential justification for the idea of a molecular clock and emphasized the importance of genetic drift in shaping molecular evolution. But when epistasis among sites is common, as numerous empirical studies suggest, do neutral mutations substitute according to Kimura's expectation? Here we study simulated, asexual populations of RNA molecules, and we observe that conditionally neutral mutations—*i.e.*, mutations that do not alter the fitness of the individual in which they arise, but that may alter the fitness effects of subsequent mutations—substitute much more often than expected while a population is adapting. We quantify these effects using a simple population-genetic model that elucidates how the substitution rate at conditionally neutral sites depends on the population size, mutation rate, strength of selection, and prevalence of epistasis. We discuss the implications of these results for our understanding of the molecular clock, and for the interpretation of molecular variation in laboratory and natural populations.

KIMURA'S observation that the rate of substitution at a neutral site should equal the neutral mutation rate is one of the most elegant and widely applied results in population genetics (Kimura 1968; Kimura and Ota 1971; Bromham and Penny 2003; Hughes 2008; Nei *et al.* 2010). This theory performs well for sites in a genome that can be classified as unconditionally neutral: that is, sites at which the fitness effects of mutations are negligible in any environment, and in combination with any genetic background. But what does neutral theory predict about the fate of a mutation that is known to be neutral only in the genetic background in which it arose? Such mutations may interact epistatically with subsequent mutations at other loci and are thus called conditionally neutral. In light of recent studies supporting a constructive role for such epistatic neutral variation in adaptive evolution (Schuster and Fontana 1999; Depristo *et al.* 2005; Koelle *et al.* 2006; Amitai *et al.* 2007; Cowperthwaite and Meyers 2007; Wagner 2008a; Bloom and Arnold 2009; Draghi *et al.* 2010), we ask whether Kimura's foundational result extends to conditionally neutral mutations.

To understand the generality of Kimura's result, it is helpful to consider an informal derivation. Imagine an idealized population of N haploid individuals, one of which will eventually be the ancestor of the future population. If unconditionally neutral mutations occur at rate μ per replication, then on average $N\mu$ mutations will arise in the population each generation. Because these mutations can never affect fitness, they cannot affect the eventual fate of the lineages in which they arise. Therefore, each unconditionally neutral mutation will arise in the eventual common ancestor with probability 1/N; otherwise, it will be lost. The average rate of neutral substitution, k, therefore, equals the rate of (unconditionally) neutral mutation times the fixation probability of each mutant:

$$k = N\mu\frac{1}{N} = \mu. \quad (1)$$

The reasoning behind Equation 1 is compelling, and many studies have argued that this result holds for sexual and asexual species, for neutral mutations linked to positively or negatively selected sites, and for populations of varying sizes (Kimura and Ota 1971; Birky and Walsh 1988; Gillespie 2000; Bromham and Penny 2003). As a result, the rate of substitution at neutral sites is now viewed as one of the most robust and well-understood features of molecular evolution. Extensions

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.125997/DC1.

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to the neutral theory have mainly focused on the apparent overdispersion of neutral substitutions (Gillespie 1986, 1993; Takahata 1987; Bastolla *et al.* 1999, 2002, 2003; Cutler 2000; Wilke 2004; Bloom *et al.* 2007; Raval 2007). With the exception of a few studies that predict small deviations in models with lethal mutations and stabilizing selection (Bastolla *et al.* 1999; Bloom *et al.* 2007), most work has confirmed or, more often, tacitly assumed that Equation 1 accurately describes the mean substitution rate. These studies have largely ignored the impact of conditionally neutral mutations: mutations that are neutral on the genetic background in which they arise, but that may alter the fitness effects of subsequent mutations. If neutral mutations have epistatic interactions of this sort, then it is unclear whether Kimura's equation describes their substitution rate.

A diverse array of recent computational and empirical studies has demonstrated the importance of neutral mutations with epistatic effects (reviewed in Wagner 2008a). Evolutionary simulations with RNA folding algorithms (Huynen 1996; Huynen *et al.* 1996; Fontana and Schuster 1998; Ancel and Fontana 2000; Wagner 2008b) and model gene networks (Bergman and Siegal 2003; Ciliberti *et al.* 2007) indicate that neutral changes may often be prerequisites for adaptive substitutions and that the interactions between neutral and adaptive changes can lead to complex dynamics of phenotypic evolution; theoretical developments have generalized and expanded these results (van Nimwegen and Crutchfield 2000; Lenski *et al.* 2006; Wagner 2008a,b; Weissman *et al.* 2009; Draghi *et al.* 2010). Additional evidence comes from laboratory evolution experiments with proteins, in which apparently neutral mutations permit future adaptations by changing thermodynamic stability, codon usage, or promiscuous protein–ligand interactions (Depristo *et al.* 2005; Bloom *et al.* 2006; Amitai *et al.* 2007; Cambray and Mazel 2008; Bloom and Arnold 2009). The epistastic effects of nearly neutral mutations can even explain the evolution of consequential innovations, such as adaptive expansion into a new niche (Blount *et al.* 2008), the sudden escape of a pathogen from population immunity (Koelle *et al.* 2006; van Nimwegen 2006; Kryazhimskiy *et al.* 2011) or susceptibility to a drug (Bloom *et al.* 2010; Kryazhimskiy *et al.* 2011).

If some neutral mutations can facilitate future adaptation through epistatic interactions, selection might drive these neutral mutations to fixation by hitchhiking—that is, by linkage to subsequent beneficial mutations. However, other neutral mutations will impede future adaptive changes, and fixation of these neutral mutations would be disfavored by selection. In each case, the effects of a mutation on an individual's evolvability—that is, its capacity for adaptation—causes its probability of fixation to be larger or smaller than that of an unconditionally neutral mutation. Naively, one might expect that conditionally neutral mutations would be no more likely to enhance evolvability than to diminish it. Consequently, the effects of evolvability on the fixation of these mutations might average out, and Equation 1 might accurately describe the substitution rate of epistatic neutral mutations. Here we show that this naive expectation is incorrect. Instead, "neutral epistasis" in an asexual, adapting population causes a significant elevation of the substitution rate at conditionally neutral sites, compared to Kimura's classical expectation for unconditionally neutral sites. We first demonstrate these departures from the conventional substitution rate in simulated populations of replicating RNA molecules, and we confirm that the substitution rate is caused by the epistatic effects of neutral mutations. We then explore a simple population-genetic model that quantifies how epistasis, population size and mutation rate, and selection coefficients jointly determine the substitution rate at conditionally neutral sites in adapting populations. Finally, we discuss the implications of these results for the molecular clock and for the inference of evolutionary processes in natural and laboratory populations of nonrecombining organisms and chromosomes.

METHODS

Measuring substitution rates: Following Gillespie and others (Gillespie 1993), we distinguish between two types of substitution events. An origination event is the first appearance of a genotype which will later be ancestral to everyone in the population. Inspecting a genealogy, we say that such mutant genotypes were "destined" to fix; when they do fix, we mark a fixation event. Each fixation event corresponds to an earlier origination event. Because of this correspondence, the mean rate of origination events and the mean rate of fixation events will eventually converge, and such convergence defines a steady-state population with respect to substitution. We illustrate transient and steady-state substitution dynamics in supporting information, Figure S1. Linkage and changes in mutation rate may cause the rate of one process to temporarily exceed the other, in which case we may choose to inspect whichever process is more informative.

We quantified substitutions using origination events. Specifically, we choose an individual at random from the final generation and trace its ancestry back to the initial generation. This lineage eventually passes through the most recent common ancestor (MRCA) of the final population. Each mutation encountered before the MRCA represents an origination event. If neutral mutations were unconditionally neutral, we would expect them to arise on this lineage at their mutation rate. We therefore measured the neutral mutation rate of each parent along the lineage as our null expectation of the neutral substitution rate. Using the origination process

instead of the fixation process allows us to calculate a null expectation that is robust to variation in neutral mutation rates and to selective bottlenecks.

In our simulations we did not directly determine which genotype is the MRCA of the final population. For the RNA results, we tracked a lineage back from a single individual: the population size, N, and the rate of beneficial substitution determine how quickly this lineage converges to the MRCA. As a consequence, we may have missed some potential substitutions that arose late in each simulation and erroneously classified some polymorphic variants as neutral substitutions. However, such events could only cause the measured neutral substitution rate to tend toward the null expectation (*i.e.,* the neutral mutation rate). We therefore expect that our RNA results on the neutral substitution rate may be somewhat conservative when t is close to the end of the simulation (30,000 generations). In the simple population-genetic model we tracked substitutions in real time, and we ran each simulation until the first origination event after the nominal ending time, T. We could then be assured that all mutations arising before T had been fixed or lost and that the origination events measured in these T generations were an unbiased estimate of the substitution rate. In both cases, the measured substitution rates correspond to those expected with infinite sites; substitutions that occur at the same base in the RNA model would each be counted.

Simulation methods: All simulations used the Wright–Fisher model to evolve populations of discrete, asexual, and haploid individuals. RNA simulations used the Vienna RNA folding package (version 1.6.1) with default folding parameters. These simulations considered RNA sequences of 72 bases in length and computed the minimum-free-energy structure to determine the phenotype. Fitness was then calculated as a function of the tree edit distance, d, between an organism's phenotype and a defined optimal phenotype. 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. Fitness is related to structural distance as $(1 + s)^{-d}$. Here s quantifies the strength of selection; it is equivalent to the multiplicative selective coefficient associated with a mutation that changes d by a single unit. The initial genotype was drawn randomly, and its phenotype was defined as the optimum for the initial period of stabilizing selection. The second 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. The second optimum was also required to be 40 units from the first optimum, so that the pressure to adapt in the new environment was strong and uniform across replicates. Populations were initially clonal, and each replicate began from an independently drawn genotype and later adapted to an independently drawn optimum. Substitutions were measured as described above.

We also performed simulations of the simple population-genetic model. Selection and reproduction are modeled identically in the two kinds of simulations. Again, the parameter s quantifies the strength of selection by specifying the multiplicative fitness advantage associated with each beneficial mutation.

In both the abstract and RNA models, mutations occur as a Bernoulli process: either no mutations occur, or a single mutation arises with probability U. Although this simplification is biologically unrealistic, it enables us to unequivocally assign a fitness effect to each mutation. Because a neutral mutation cannot arise at the same time as a beneficial mutation, unconditionally neutral mutations may actually substitute at a slightly lower rate than their mutation rate. This downward deviation from Kimura's prediction was found to be negligible.

In a separate series of RNA simulations, we measured the evolvability and robustness of the population as a whole, as well as the effects of neutral mutations on these properties. The most straightforward method, which is to measure these properties in every individual and every neutral mutant, is seriously distorted by the presence of deleterious variation in the evolving populations: individuals harboring deleterious mutations may appear to be evolvable, but most often can produce only mutants as fit as existing genotypes. We therefore chose to measure robustness and evolvability only in those individuals whose fitness exceeded the mean of their population; we also assayed the effects of neutral mutations only when those mutations arose from such individuals. In practice, due to the large disadvantage (at least 10%) of deleterious variants, the distribution of fitnesses in a population is left skewed, and this heuristic typically excludes much less than half of the individuals in a typical population.

RESULTS

Neutral substitutions in simulated RNA populations: Kimura's classic result and its subsequent elaborations (*e.g.*, Charlesworth *et al.* 1993; Gillespie 2000) apply to unconditionally neutral sites. Although unconditionally neutral sites are a convenient theoretical concept, empirical measurements of fitness effects could never suffice to identify such sites in practice (Wagner 2005). Even in the simplest genome, there are far too many possible genetic backgrounds to feasibly test the fitness consequences of a mutation at a given site on all backgrounds, as well as in a range of possible environments.

In practice, we must use an empirically tractable definition of neutrality. We classify a mutation as neutral if it has no effect on fitness in the genetic background and environment in which it arises. Two considerations motivated us to study substitutions at sites satisfying this

broad definition of neutrality. First, the initial fitness effect of a mutation strongly determines its eventual fate, especially in large populations with little recombination. Second, this broad definition can be easily applied in evolution experiments. The use of whole-genome sequencing in experimental evolution has made neutral theory testable in new ways, and several studies have attempted to combine experimental fitness measurements with ideas from neutral theory (*e.g.*, Wichman *et al.* 2005; Barrick *et al.* 2009). By considering a broad definition of neutrality, which includes conditionally neutral sites, we examine the dynamics of the types of mutations that an experimentalist would, in practice, typically classify as neutral.

To study substitutions of such (conditionally) neutral mutations, we simulated an asexual population of 72-base RNA molecules evolving according to the Wright–Fisher model (see methods). We used the Vienna RNA software package to assign a structure to each sequence and to measure differences between structures. We assigned fitness to each sequence according to its corresponding structure's similarity to a chosen optimal structure (see methods). Populations were subject to stabilizing selection for T_1 generations; the optimal phenotype was then substantially altered, and the populations adapted to this novel environment for T_2 generations. To precisely compare our substitutions to Kimura's expectation, we measured substitutions by recording when they initially arise as mutations (see methods).

As Figure 1 shows, the rate of (conditionally) neutral substitutions in an evolving population of RNA molecules departs significantly from Kimura's expectation. In particular, the substitution rate spikes sharply when the environment (*i.e.,* the target phenotype) shifts, coincident with a period of rapid adaptation. For a population of size $N = 5000$, the neutral substitution rate is about twice as large as the neutral mutation rate shortly after the environmental shift, and it remains elevated for thousands of subsequent generations. Furthermore, populations of different sizes exhibit distinct rates of neutral substitution, again in contrast to Kimura's expectation for nonepistatic neutral mutations.

The first clue to the mechanism causing this high rate of neutral substitution lies in the dynamics of adaptation in simulated RNA populations. While the ensemble mean fitness graphed in Figure 1 increases steadily, adaptation in the individual populations is much less predictable. A population of replicating RNA molecules in this regime exhibits punctuated evolution: periods of constant fitness are disrupted by the sudden appearance and fixation of a beneficial substitution (Fontana and Schuster 1998; Ancel and Fontana 2000). In our study, the ensemble mean population fitness rapidly increased immediately following the environmental shift (see Figure 1), because many beneficial mutations were available at the time of the shift. Moreover, the ensemble mean population fitness continued to grow, at a slower pace, throughout

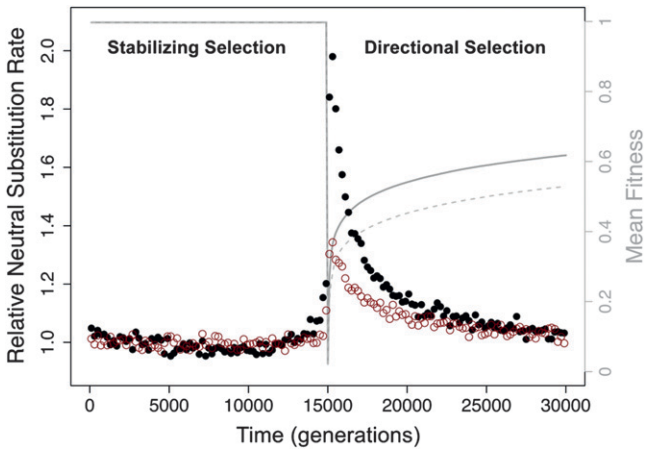

Figure 1.—The observed rate of substitution at (conditionally) neutral sites depends on the population size and the rate of adaptation in simulated RNA populations. Each point reflects neutral substitutions originating within a 100-generation bin, averaged over at least 20,000 replicates. Points are calculated as the observed number of substitutions minus the expected number, divided by the expected number; this is an estimate of origination rate relative to Kimura's expectation for unconditionally neutral mutations. Solid circles correspond to $N = 5000$, while open circles correspond to $N = 800$; for both, the genomic mutation rate, U, is equal to 0.004 and $s = 0.1$. The lines depict ensemble means of mean fitnesses within each replicate; the solid line corresponds to $N = 5000$; and the dashed line corresponds to $N = 800$.

the subsequent 15,000 generations—reflecting the rare discoveries of beneficial mutations within the ensemble of replicates. Since beneficial mutations were strongly selected and quite rare in this period, we expect that those neutral mutations with positive epistatic consequences (*i.e.,* those neutral mutations that increased a genotype's chance of receiving a subsequent beneficial mutation) were effectively selected and fixed through hitchhiking (*i.e.,* by linkage to a beneficial mutation).

In light of the considerations above, it is important to note that the epistatic consequences of neutral mutations were, indeed, mostly positive in our simulations. In particular, we observed that neutral mutations tended to increase evolvability. We define the evolvability of a genotype as the fraction of its point mutations that improve fitness. Although we observed many neutral mutations that decreased evolvability, the average effect was positive in all generations after the environmental shift (Figure 2a). Analysis of the median effects confirmed that neutral mutations were more likely to increase evolvability than to decrease it. As many studies have shown, mutations that increase evolvability are favored by selection, especially in nonrecombining populations adapting under strong selective pressures (Kashtan and Alon 2005; Meyers *et al.* 2005; Draghi and Wagner 2008, 2009). These results suggest that neutral mutations substitute more often than Kimura's expectation because of their biased effects on evolvability.

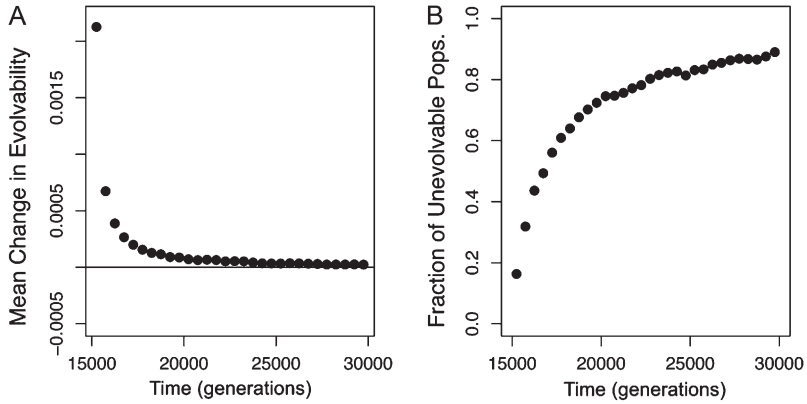

FIGURE 2.—Neutral mutations have a mean positive effect on evolvability in evolving RNA populations. (A) The mean change in evolvability caused by a neutral mutation. Each point represents mutations observed in 3000 replicate simulations, averaged into 500-generation bins. (B) The fraction of replicate populations without any available beneficial point mutations. Points represent averages from observations at 500-generation intervals, averaged across over 2000 replicates. $N = 5000$, $U = 0.004$, and $s = 0.1$, as above.

To understand why neutral mutations typically increased evolvability in our simulations, we quantified how often the populations became "stuck" on fitness plateaus, unable to further adapt. Figure 2b shows the fraction of replicate populations in which no individuals can produce any beneficial point mutation. This fraction is initially small at the time of the environmental shift but grows quickly to over 80%. This behavior is not caused by a large fraction of the replicate populations reaching a "local peak" on their fitness landscape and thereafter remaining stuck at that fitness indefinitely. Instead, we observed that most populations became temporarily stuck on fitness plateaus and then subsequently evolve the potential to find further beneficial mutations. In particular, we measured evolvability along lineages that survive until the end of the simulation. Such lineages passed through a genotype with zero evolvability 4.11 times, on average, during a 15,000 generation period of adaptation. Neutral mutations, as opposed to deleterious ones, were primarily responsible for potentiating adaptation in populations that had become stuck (in 20 replicates of the above simulations, 55 of the 57 observed substitutions that increased evolvability in unevolvable populations were neutral, as opposed to deleterious). This result supports the decision to measure evolvability only in genotypes with fitness exceeding the population mean (see METHODS). Thus, the most important consequence of neutral mutations was to facilitate adaptation in populations that had become temporarily stuck—a phenomenon that we explore in greater generality below.

A simple population-genetic model: In the RNA simulations described above, populations often reached unevolvable states, which were subsequently alleviated by epistatic neutral mutations. To explore this process more generally, we have devised a population-genetic model that links neutral mutations, beneficial mutations, and evolvability. In addition to the RNA simulations, our modeling approach was influenced by our previous work on robustness and evolvability (DRAGHI *et al.* 2010) and by Gillespie's pseudohitchhiking model (GILLESPIE 2000). We model a population of N haploid individuals evolving according to the Wright–Fisher model. We consider all sites in which mutations could be neutral, conditionally neutral, or beneficial, and define U as the total mutation rate of these sites. We assume two categories of genotypes. Some genotypes are evolvable: a proportion $p_B > 0$ of their mutations are beneficial, conferring a multiplicative fitness advantage $1 + s$. The other class of genotypes are unevolvable—*i.e.*, no beneficial mutations are (directly) available to them. When a beneficial mutation occurs in an evolvable genotype, the mutant genotype becomes unevolvable with probability α. Finally, a proportion p_{NE} of mutations in both categories of genotypes are neutrally epistatic, and these mutations switch an individual from one category to another (*i.e.,* from evolvable to unevolvable, or conversely) without changing its fitness.

Under this simple model, the epistatic effects of mutations are described by two parameters: the parameter α quantifies the chance that a beneficial mutation arrives at a fitness plateau, with no immediate potential for further adaptation; whereas p_{NE} quantifies the chance that a neutral mutation is epistatic and therefore alters an individual's evolvability. Crucially, the epistatic neutral mutations are not constrained to increase evolvability. In fact, if they arise in evolvable and unevolvable backgrounds equally, neutral mutations will have no mean effect on evolvability. For this section we focus on the substitution rate at the conditionally neutral sites—*i.e.,* the proportion p_{NE} of mutations that are neutral but that alter an individual's evolvability. The remaining fraction $1 - p_{NE} - p_B$ of uncategorized mutations are unconditionally neutral and substitute at a rate equal to their mutation rate.

Monte Carlo simulations of this simple model confirm that neutral epistasis is a sufficient mechanism with which to explain an elevated rate of neutral substitution. Figure 3 shows the neutral substitution rate measured in our simplified model, in simulations using mutation parameters similar to the RNA model. When neutral mutations have no epistatic effects (*i.e.,* when we set $p_{NE} = 0$), the neutral substitution rate is indistinguishable from the neutral mutation rate, as expected (see METHODS). However, when genotypes can be evolvable or unevolvable, and some neutral mutations switch between these categories (*i.e.,* $p_{NE} > 0$), then the neutral

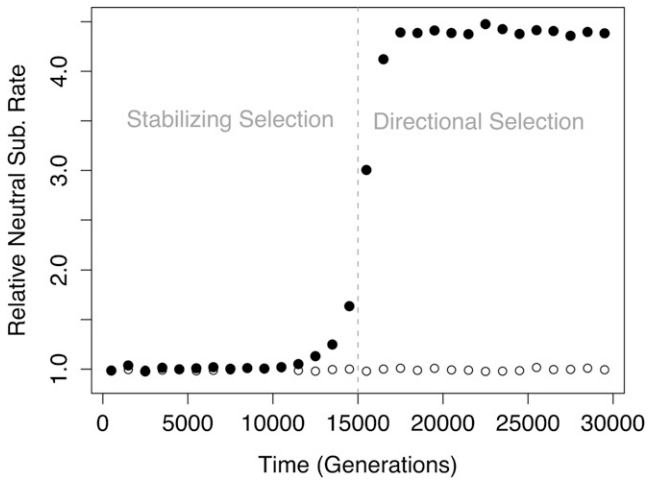

FIGURE 3.—The substitution rate of epistatic neutral mutations, relative to their mutation rate, is elevated under directional selection in our simple population-genetic model. The solid circles show the mean origination rate measured in simulations that allow neutral mutations to change an individual's evolvability ($p_{NE} = 0.01$). The open circles show control simulations in which neutral mutations have no effect on evolvability (*i.e.*, $p_{NE} = 0$; all genotypes are evolvable). In analogy to our RNA experiments, populations evolved under stabilizing selection for 15,000 generations (*i.e.*, p_B was set to zero for this period), and then under directional selection for another 15,000 generations. $N = 5000$, $U = 0.004$, $s = 0.1$, and $p_B = 0.001$.

substitution rate is significantly higher than Kimura's expectation.

We have derived an analytic expression that predicts the substitution rate at conditionally neutral sites in this simple population-genetic model (See APPENDIX: DERIVATION OF EQUATION 2). We first derived an expression for the expected waiting time until the first beneficial mutation that is destined to fix arises, starting from a population of N unevolvable individuals:

$$\mathbb{E}[T_{un}] = \sqrt{N}\frac{2^{2\theta_{NE}-1}}{\sqrt{2\theta_B \pi(s)}}\frac{\Gamma(\theta_{NE})^2}{\Gamma(2\theta_{NE})}. \quad (2)$$

Here $\theta_{NE} = NUp_{NE}$, $\theta_B = NUp_B$, and $\pi(s) = (1 - e^{-2s})/(1 - e^{-2Ns})$. Because an unevolvable population requires neutral changes in evolvability before it can adapt, we expect that one conditionally neutral mutation will hitchhike with the first beneficial substitution in an initially unevolvable population.

If, on the other hand, a population is composed of entirely evolvable genotypes, then the waiting time before the next beneficial substitution is geometric with mean: $\mathbb{E}[T_{ev}] = 1/\theta_B \pi(s)$. Because all individuals in such a population are already adaptable, we expect that no epistatic neutral mutations will fix during this interval; such mutations would make their genotypes unevolvable and therefore could not fix through hitchhiking.

We can use our expressions for T_{un} and T_{ev}, above, to calculate the expected time between epistatic neutral substitutions, denoted τ. To do so, however, we make two simplifying approximations. We assume that the fixation of beneficial mutations occurs immediately and that all epistatic neutral substitutions occur by hitchhiking, and not solely by neutral drift. (We expect these approximations to be accurate provided s is large and clonal interference is negligible.) These assumptions lead to a recurrence: $\mathbb{E}[\tau] = \alpha\mathbb{E}[T_{un}] + (1 - \alpha)(\mathbb{E}[T_{ev}] + \mathbb{E}[\tau])$, and thus the expected substitution rate at conditionally neutral sites is

$$k_{cond} \approx \frac{1}{\mathbb{E}[\tau]} = \frac{\alpha}{\alpha\mathbb{E}[T_{un}] + (1 - \alpha)\mathbb{E}[T_{ev}]}. \quad (3)$$

To quantify the magnitude of the changes in the neutral substitution rate caused by epistasis, we simulated this simple model for several combinations of parameters. Figure 4 shows the results of these simulations along with our analytical predictions (Equation 3). In the RNA simulations, we were limited by computation time to modeling strong selection (*i.e.*, $s = 0.1$) and evolution toward a distant optimum. Here, using our simple population-genetic model we generalize the RNA results to explore weaker selection and lower rates of beneficial mutation—that is, parameters that correspond to less dramatic environmental changes. We find that even modest selective coefficients can drive substantial increases in the neutral substitution rate: linkage to selected mutations conferring a 1% benefit can produce a twofold increase in the substitution rate of epistatic neutral mutations. Larger selective coefficients can increase the substitution rate of neutral mutations by 10-fold or more. Moreover, increasing the beneficial mutation rate of evolvable genotypes greatly enhances the elevation of the neutral substitution rate. This suggests that the magnitude of the change in evolvability caused by neutral mutations strongly affects their substitution rate. Finally, we find that increasing the rate of epistatically neutral mutations diminishes their per-capita fixation probability, although this change is small when $\theta_{NE} \ll 1$ (Figure 4).

Figure 4 also plots predictions derived from the analytical approximation above. In general, the predictions closely match the simulations, despite the approximations we have used. Note that our simple population-genetic model allows for clonal interference that is, competition between beneficial mutations in different lineages in an asexual population—while our analytical approximation assumes that only one beneficial mutation is segregating at any time. Clonal interference may explain the departures of the prediction from the simulated data, particularly when s and θ_B are large. This close agreement suggests that differences in evolvability, combined with hitchhiking on selected mutations, largely determines the dynamics of epistatic neutral mutations over a broad range of parameters.

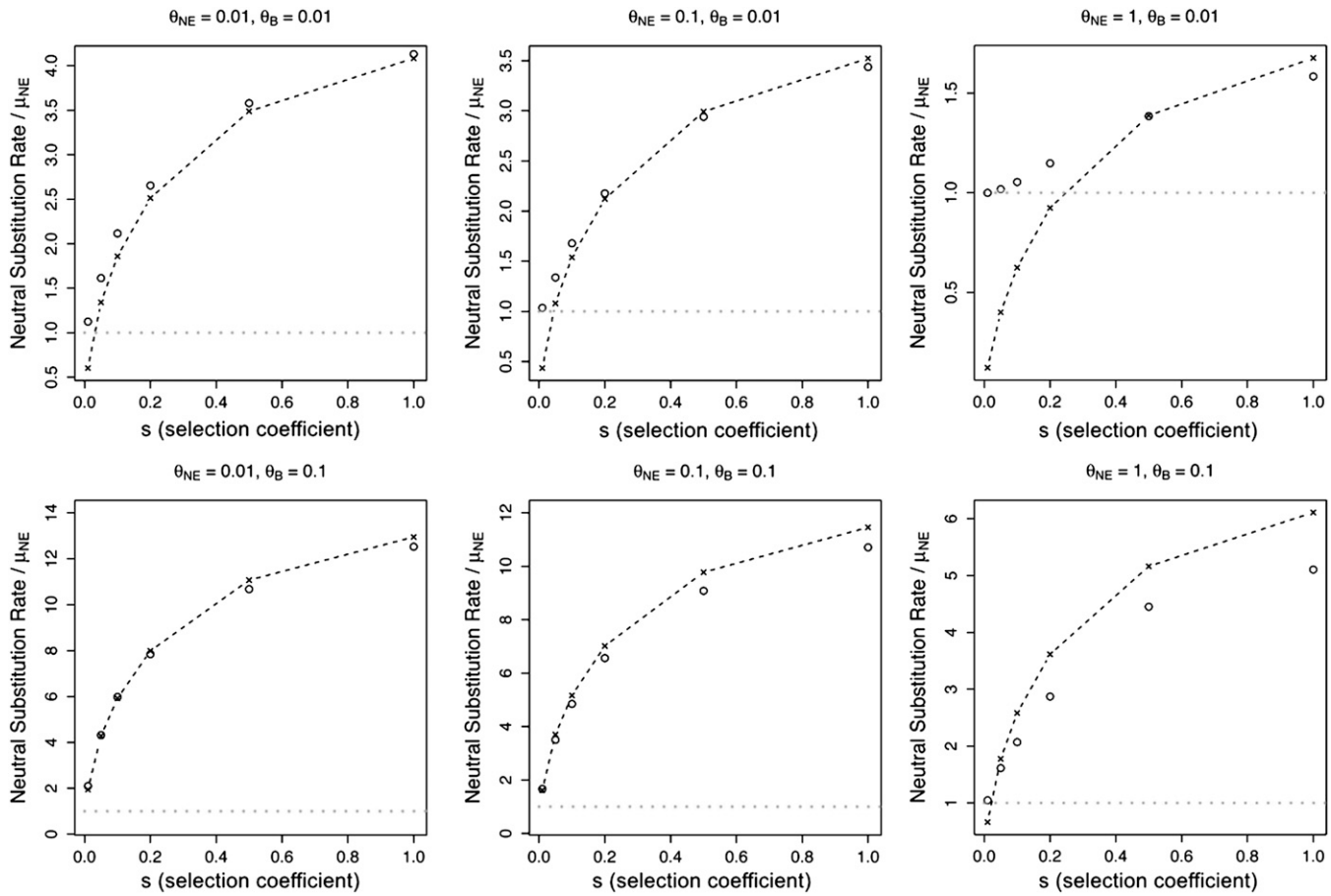

FIGURE 4.—Simulated and predicted rates of substitution of epistatic neutral mutations. Open circles show mean origination rates across 4000 replicate simulations of 100,000 generations each. The dashed lines and X's show analytical predictions calculated from equation 3. $N = 1000$ and $U = 0.01$ for all simulations; $s = 0.01$ is the smallest selection coefficient simulated.

Although we have ignored deleterious mutations in this simple model, they could interact with beneficial and neutral changes to influence our results in several ways. We explored three scenarios for the parameters used in Figure 3. First, we added a large fraction of deleterious mutations ($p_D = 0.5$) to the mutation probabilities for both evolvable and unevolvable backgrounds. Therefore, in an evolvable background, a fraction p_D mutations are neutral, p_{NE} are conditionally neutral, p_B are beneficial, and $(1 - p_D - p_{NE} - p_B)$ are unconditionally neutral; in an unevolvable genotype, p_D mutations are neutral, p_{NE} are conditionally neutral, none are beneficial, and $(1 - p_D - p_{NE})$ are unconditionally neutral.

We found that adding the possibility of deleterious mutations had little effect, regardless of whether these mutations have large ($s = 0.2$) or small ($s = 0.02$) fitness effects (see Figure S2). We also explored the possibility that evolvable genotypes might be less robust to mutation, and so have a higher probability of deleterious mutation. In this scenario, the rate of conditionally neutral substitution is lower than in the absence of deleterious mutations, but still much higher than Kimura's expectation (see Figure S3).

Finally, we modeled epistatic deleterious mutations. These mutations had a direct fitness cost, but like conditionally neutral mutation, would change unevolvable backgrounds into evolvable genotypes and vice versa. When such mutations are at least modestly deleterious, the rate of conditionally neutral substitution is still substantially elevated compared to Kimura's expectation (see Figure S4).

Neutral epistasis and stabilizing selection: In this section we discuss another distinct anomaly in neutral substitutions observed in our RNA simulations: the increase in substitutions prior to the environmental shift (Figure 1). This effect is quite striking in the large population we simulated: an increased origination rate of neutral mutations destined to fix is apparent for as many as 1000 generations prior to the environmental shift. This pattern may in part be caused by mutations that segregate neutrally in the first environment, but that become beneficial in the later selective environment and are then directly selected and brought to

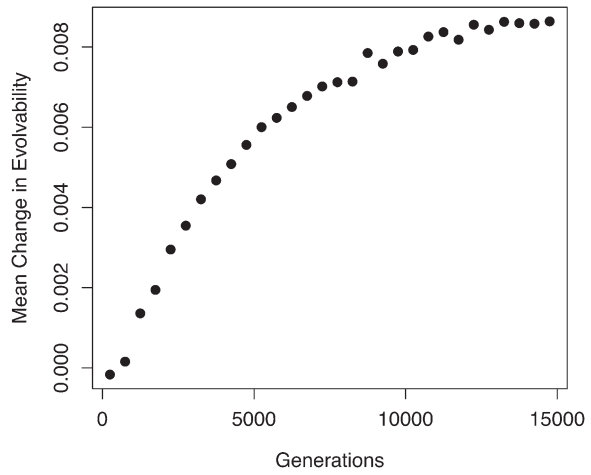

Figure 5.—Neutral mutations that arise in a population under stabilizing selection have a mean positive effect on evolvability in some future environment. Evolvability, on the *y*-axis, is measured relative to the target phenotype in the future environment. Mutant effects are averaged in 500-generation bins from the 15,000 generation preceding the environmental shift and averaged across 3000 replicate simulations. $N = 5000$, $U = 0.004$, and $s = 0.1$, as above.

fixation. Another possibility is that neutral mutations segregating prior to the environmental shift may increase evolvability after the shift, and thus sweep to fixation by hitchhiking on subsequent beneficial mutations. Figure 5 shows that this latter phenomenon does indeed occur: the neutral mutations that arise before the environmental shift tend to increase an individual's evolvability after the shift. But why should neutral mutations arising in population under stabilizing selection be biased toward increasing an individual's evolvability in some future, novel environment?

The key to understanding this unusual phenomenon is that populations under stabilizing selection evolve to reduce the negative effects of deleterious mutations. As predicted by theoretical models of neutral networks (van Nimwegen *et al.* 1999) and observed in other studies (*e.g.*, Plotkin *et al.* 2006; Draghi and Wagner 2009), our large RNA populations evolved high amounts of robustness while under stabilizing selection (Figure 6). Theory also predicts that when every genotype can produce beneficial mutations, robustness and evolvability will be negatively correlated (Draghi *et al.* 2010); indeed we find in our simulations that, under stabilizing selection, the effects of a neutral mutation on robustness in the current environment are negatively correlated with evolvability in the future, novel environment ($R^2 = -0.993$, Spearman rank correlation). When robustness in the population is high, the typical neutral mutation will tend to reduce robustness (in the current environment), which has the the consequence of increasing evolvability (in the future environment). Thus, the elevated neutral substitution rate observed before the environmental shift can be explained by the trade-off between robustness and evolvability in a large population under stabilizing selection (Figure 6).

DISCUSSION

We have demonstrated that Kimura's classic expectation for the substitution rate of strictly neutral mutations substantially underestimates the substitution rate of conditionally neutral mutations that are linked to selected sites, when populations are adapting in a mutation-limited regime. Although we initially used a computational model of RNA to illustrate this phenomenon, our results are not specific to that genotype–phenotype model, or to the relatively strong selection used in those simulations. In general, we have shown that whenever adaptation in a population is limited by the supply of beneficial mutations, genotypes will typically have lower evolvability than mutationally adjacent genotypes with the same fitness, and so conditionally neutral mutations will tend to increase evolvability and fix more often than their mutation rate. This behavior will occur in any genotype–phenotype-fitness landscape in which selectively equivalent genotypes are linked by mutation and differ in the phenotypes found in their mutational neighborhoods. There is ample evidence that such landscapes, in which neutral or nearly neutral mutations can potentiate adaptation, are common at the level of proteins (Depristo *et al.* 2005; Wagner 2005, 2008a; Bloom *et al.* 2006, 2010; Koelle *et al.* 2006; van Nimwegen 2006; Amitai *et al.* 2007; Cambray and Mazel 2008; Bloom and Arnold 2009; Kryazhimskiy *et al.* 2011) and at the level of gene networks and metabolism (Bergman and Siegal 2003; Ciliberti *et al.* 2007; Blount *et al.* 2008). The prevalence of epistasis linking neutral and adaptive changes, and the consequences we have documented for the resulting patterns of molecular evolution, should motivate further effort to measure the epistatic consequences of neutral mutations.

There is a long history of considering the waiting time to the arrival of a double mutant. The first approach (Gillespie 1984; Kimura 1985; Weinreich and Chao 2005) assumes that the first mutation is deleterious and the second is compensatory, under the so-called *strong mutation–weak mutation regime*: $s \gg 1/N \gg \mu$. By neglecting back mutation, and assuming that a sufficiently large population size and a sufficiently long time has elapsed since the first appearance of the mutation, they argue that the frequency of the deleterious mutation is well approximated by the mutation–selection balance—its frequency in the deterministic, infinite population approximation. Under these assumptions, they show that the waiting time for the arrival of the double mutant is $O(\mu^2)$. More recently, the problem has been considered in the context of carcinogenesis: Iwasa *et al.* (2004a,b) and Weissman *et al.* (2009) consider the

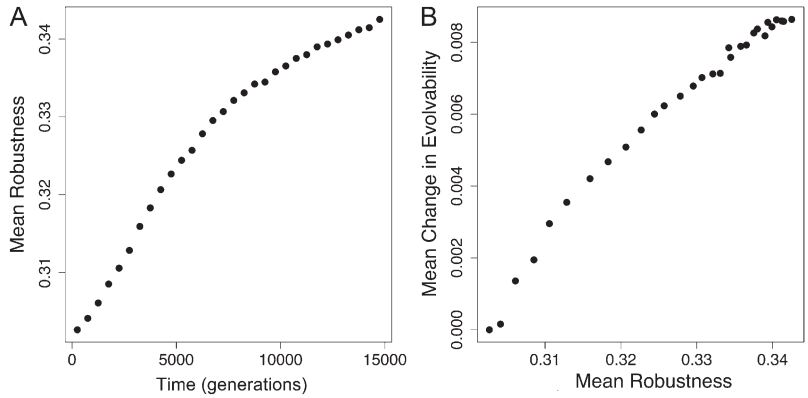

Figure 6.—Robustness increases during stabilizing selection, and so neutral mutations tend to increase an individual's evolvability in a future environment. (A) Mean robustness, measured as the fraction of mutations that are neutral in the current environment, for large RNA populations under stabilizing selection. Each point (solid circle) is a population mean, averaged across over 2000 replicate populations. (B) The *y*-axis measures the mean effect on evolvability, measured in the novel environment, of neutral mutations, plotted against the mean robustness of populations. Mutant effects are averaged in 500-generation bins from the 15,000 generation preceding the environmental shift, and averaged from 3000 replicate simulations. $N = 5000$, $U = 0.004$, and $s = 0.1$, as above.

waiting time to the arrival of the second mutant for a monomorphic, wild-type population. Those authors obtained approximations to the rate of production of double mutants, again neglecting back mutation and assuming small mutation rates:

$$\left(Up_{\mathrm{NE}}, Up_{\mathrm{B}} \ll \frac{1}{N}\right).$$

Under these assumptions they found that the rate is

$$O\left(\frac{\sqrt{2\theta_{\mathrm{B}}\pi(s)}\theta_{\mathrm{NE}}}{\sqrt{N}}\right).$$

In our regime of interest, Up_{NE}, $Up_{\mathrm{B}} = O(N^{-1})$, this expression gives the correct asymptotic order, $\left(O\left(\sqrt{N}\right)\right)$, but consistently underestimates the expected waiting time given in Equation 2. Assuming that the first mutation is neutral, the analysis of Iwasa *et al.* (2004a,b) was made rigorous and extended to our regime of interest in Durrett *et al.* (2009), where a waiting time distribution analogous to (4) is derived for a Moran model, again neglecting back mutation. Our analysis additionally justifies the neglect of back mutations.

Our analysis shows that Kimura's classic result does not hold for neutral mutations that have epistatic consequences. This result illustrates, and complicates, a familiar quandary: How can we assign causal mechanisms to observed molecular changes? If a neutral mutation allows a change at another site to be beneficial, then the substitution of that neutral mutation clearly causes the later substitution. This type of epistasis depends on the genotype–phenotype-fitness relationship, but not on the population size or mutation rate. Whether the beneficial mutation reciprocally explains the fixation of the prerequisite neutral change, however, depends critically on whether the adaptation arose when the first mutation was still polymorphic, and so depends on demography and mutation rates. The statement that "neutral changes facilitate adaptation" does not suffice to predict an elevated neutral substitution rate without an analysis of the population dynamics. Together with recent work on robustness and evolvability (Draghi *et al.* 2010), the analysis here demonstrates that standard population-genetic models combined with contemporary observations about epistasis can lead to surprising departures from traditional evolutionary theory.

These results seem to lead to a conflict in the definition of neutral mutations. Kimura's elegant formula for the substitution rate applies only to unconditionally neutral mutations. But the locations of unconditionally neutral sites, or whether any such sites exist at all, cannot be resolved by empirical measurements (Wagner 2005)—principally because it is impossible to test fitness effects in all genetic backgrounds. By contrast, the common, operational definition we use here is empirically convenient: a mutation is neutral if it does not alter fitness in the genome and environment in which it arises. Even though substitutions at such sites do not follow Kimura's simple expectation, we can nevertheless analyze and understand their behavior (Equation 3). Moreover, other results in population genetics suggest that it is important to study the behavior of such sites, even though the neutrality of such sites depends on the genetic background and may be transient. The fact that such mutations are neutral when they arise is extremely important—allowing them to reach high frequency and subsequently alter, via epistasis, the long-term evolutionary trajectory of a population (van Nimwegen and Crutchfield 2000; Weissman *et al.* 2009; Draghi *et al.* 2010). Unfortunately, Kimura's simple expression for the substitution rate may not be useful under this revised, operational definition of neutrality.

Although our analysis relies on complete linkage between sites, neutral mutations may play a role in the adaptation of sexual populations as well. However, the basis of our approach here is to classify mutations as conditionally neutral by assessing their effect with respect to a genotype's otherwise identical, asexual parent. This method allowed a precise comparison with Kimura's expectation for strictly neutral sites, but it is inapplicable to sexual populations, where parents and their offspring may have different genotypes even in the absence of recent mutations. Future research could

develop rigorous methods to measure the contribution of neutral variation to the rates of adaption and patterns of substitution in sexual organisms.

Kimura's result is often cited as the basis for the molecular clock, and so it is reasonable to ask if our results undermine that basis. On the one hand, we have seen that adaptive evolution may fix ostensibly neutral mutations through their epistatic interactions with beneficial changes. However, the foundation for the molecular clock has been the belief that periods of adaptive change of a gene are much more rare than periods of stabilizing selection. Whether or not this belief has a broad empirical basis is still an active controversy (Takahata 2007; Hahn 2008), which certainly cannot be resolved by theoretical studies such as this. However, our results can inform the design of methods to infer selection from molecular data and contribute to resolving empirical questions surrounding the molecular clock. Recently, theory and experiments have begun to challenge the notion that a constant rate of molecular evolution is indicative of neutral evolution (Wichman *et al.* 2005; Barrick *et al.* 2009; Kryazhimskiy *et al.* 2009). To understand how these studies, and experimental evolution in general, reveal mechanisms of molecular evolution acting in nature we must expand population genetics to accommodate the effects of epistasis, particularly those interactions that link neutral and beneficial changes, on the patterns of substitution.

We thank Sergey Kryazhimskiy and Michael Desai for many productive discussions about epistasis and two anonymous reviewers for helpful critiques. J.B.P. acknowledges funding from the Burroughs Wellcome Fund, the David and Lucile Packard Foundation, the James S. McDonnell Foundation, the Alfred P. Sloan Foundation, the Defense Advanced Research Projects Agency (HR0011-09-1-0055), and the U.S. National Institute of Allergy and Infectious Diseases (2U54AI057168).

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Communicating editor: H. G. Spencer

APPENDIX: DERIVATION OF EQUATION 2

In what follows, we derive the distribution for T_{un}, the waiting time to the arrival of the first beneficial mutation, assuming that the initial number of adaptable individuals is zero. In previous work (Draghi *et al.* 2010), we obtained the expected waiting time for arbitrary initial numbers. Here we present an alternate approach that both gives the complete distribution, and is more conceptually clear, though is only applicable for more limited initial conditions. In the interest of readability, we omit the details of convergence, which may be readily supplied, and adopt an informal tone. We remark that Durrett *et al.* (2009) obtained an equivalent expression for the waiting-time distribution starting from the Moran model with one-way mutation. Their arguments are similar, although ours are simplified by our reference to known results regarding the Galton–Watson process with immigration.

We will assume a Wright–Fisher model in a population of a fixed number of individuals, N, obtaining an asymptotic expression in the limit $N \to \infty$. Let X_n^N be the number of adaptable individuals in the nth generation, and let T_{un}^N be the time of arrival of the first beneficial mutation.

The transition probabilities for the number of adaptable individuals is binomially distributed,

$$\mathbb{P}\left\{ X_n^N = j | X_{n-1}^N = i \right\} = \binom{N}{j} p_i^j (1 - p_i)^{N-j},$$

where

$$p_i = Up_{\text{NE}}\left(1 - \frac{i}{N}\right) + (1 - Up_{\text{NE}})\left(\frac{i}{N}\right)$$

is the probability that a random individual chosen from the previous generation produces an adaptable offspring. We assume weak mutation—that is, we assume $\theta_{\text{NE}} = NUp_{\text{NE}}$ is constant.

Thus, if $i \ll N$, then

$$Np_i = \theta_{\text{NE}}\left(1 - \frac{i}{N}\right) + \left(1 - \frac{\theta_{\text{NE}}}{N}\right) i \to \theta_{\text{NE}} + i$$

as $N \to \infty$, and using the Poisson approximation to the binomial distribution (see *e.g.*, Feller 1957 or Durret 2005):

$$\lim_{N\to\infty} X_n^N = X_n,$$

where $X_n \sim \text{Poisson}(X_{n-1} + \theta_{\text{NE}})$ and convergence is in distribution (technically, in the limit we take a stopped process, to ensure $X_n^N \ll N$.) Equivalently, each adaptable individual in generation $n-1$ produces a Poisson(1)-distributed number of offspring, while a Poisson(θ_{NE})-distributed number of adaptable offspring are born to nonadaptable parents. Thus, for small i, we may approximate X_n^N by a critical Galton–Watson process with immigration (Athreya and Ney 1972), about which much is known.

More generally, we may consider the case when the adaptable types are weakly beneficial or weakly deleterious. This corresponds to replacing p_i with

$$\tilde{p}_i = Up_{\text{NE}}\left(1 - \frac{(1+s)i}{N - i + (1+s)i}\right) + (1 - Up_{\text{NE}})\left(\frac{(1+s)i}{N - i + (1+s)i}\right),$$

where $Ns = \sigma$, for σ constant. Proceeding as before, we find that

$$N\tilde{p}_i \to \theta_{\text{NE}} + i$$

as $N \to \infty$; *i.e.,* incorporating weak selection leaves our Galton–Watson approximation unchanged.

We begin with a heuristic argument that we hope provides some intuition for our exact result; intuitively, since each individual has probability $Up_{\text{B}} = \theta_{\text{B}}/N$ of acquiring a beneficial mutation, and such mutation has probability $\pi(s)$ of fixing, we would expect that the first beneficial mutation *that will eventually fix* arises when the total number of adaptable individuals who have ever lived is

$$O\left(\frac{1}{Up_{\text{B}}\pi(s)}\right).$$

Since each individual in the nth generation has on average one offspring, while migration is a Poisson process with mean $\theta_{\text{NE,}}$ we have that

$$\mathbb{E}[X_{n+1}|X_n] = X_n + \theta_{\text{NE}},$$

so

$$\mathbb{E}[X_{n+1}] = \mathbb{E}[X_n] + \theta_{\text{NE}}.$$

Iterating this relation, we get $\mathbb{E}[X_n] = \mathbb{E}[X_0] + n\theta_{\text{NE}}$. Hence,

$$\begin{aligned}\mathbb{E}\left[\sum_{k=0}^{n} X_k\right] &= \sum_{k=0}^{n} \mathbb{E}[X_k] \\ &= \sum_{k=1}^{n} k\theta_{\text{NE}} + (n+1)\mathbb{E}[X_0] \\ &= \frac{n(n+1)}{2}\theta_{\text{NE}} + (n+1)\mathbb{E}[X_0],\end{aligned}$$

which, for large values of n, is asymptotically equivalent to $n^2\theta_{\text{NE}}$. Setting

$$n^2\theta_{\text{NE}} \approx \frac{1}{Up_{\text{B}}\pi(s)},$$

we get

$$n \approx \frac{1}{\sqrt{\theta_{\text{NE}} Up_{\text{B}}\pi(s)}} = \frac{\sqrt{N}}{\sqrt{\theta_{\text{NE}}\theta_{\text{B}}\pi(s)}}.$$

As we show below, this underestimates the true waiting time, but is asymptotically of the correct order.

We now proceed with a derivation of the waiting-time distribution; to simplify the exposition, we omit some of the details of convergence. In each generation, each adaptable individual may independently mutate to a beneficial type with probability $Up_{\text{B}} = \theta_{\text{B}}/N$, which is destined to fix with probability $\pi(s)$. Thus, the waiting time to the first beneficial mutation that will fix is

$$\mathbb{P}\{T_{\text{un}}^N > n\} = \mathbb{E}\left[\prod_{k=0}^{n}\left(1 - \frac{\theta_B}{N}\pi(s)\right)^{X_k^N}\right] = \mathbb{E}\left[e^{-\left(\frac{\theta_B}{N}\pi(s)+\varepsilon^N\right)\sum_{k=0}^{n} X_k^N}\right],$$

where

$$\varepsilon^N = \ln\left(1 - \frac{\theta_B}{N}\pi(s)\right) - \frac{\theta_B}{N}\pi(s) = O(N^{-2}),$$

and the expectation is taken over all possible sample paths $\{X_k^N\}_{k=0}^n$.

As we have seen, $\mathbb{E}\left[\sum_{k=0}^n X_k\right] = O(n^2)$; the full distribution is asymptotically characterized via Theorem 5 in Pakes (1972)

$$\frac{\sum_{k=0}^n X_k}{n^2} \to Y,$$

where Y has Laplace–Stieltjes transform

$$\mathbb{E}\left[e^{-\lambda Y}\right] = \operatorname{sech}^{2\theta_{NE}}\left(\sqrt{\frac{\lambda}{2}}\right),$$

and convergence is in distribution. Motivated by our previous heuristic, we take $n = \sqrt{N}t$, so that

$$\mathbb{P}\{T_{\text{un}}^N > \sqrt{N}t\} = \mathbb{E}\left[e^{-(\theta_B\pi(s)+N\varepsilon^N)t^2\left(\sum_{k=0}^{\sqrt{N}t} X_k^N/Nt^2\right)}\right].$$

Thus, taking

$$T_{\text{un}} = \lim_{N\to\infty} \frac{T_{\text{un}}^N}{\sqrt{N}},$$

i.e., rescaling time by $\sqrt{N}$ and passing to the large population limit, we have

$$\mathbb{P}\{T_{\text{un}} > t\} = \mathbb{E}\left[e^{-\theta_B\pi(s)t^2 Y}\right] = \operatorname{sech}^{2\theta_{NE}}\left(\sqrt{\frac{\theta_B\pi(s)}{2}}\,t\right). \tag{4}$$

Finally, using Equation 2 in Gradshteĭn and Ryzhik (2000, Sect. 3.512), we have

$$\begin{aligned}\mathbb{E}[T_{\text{un}}] &= \int_0^\infty \mathbb{P}\{T_{\text{un}} > t\}\,dt \\ &= \int_0^\infty \operatorname{sech}^{2\theta_{NE}}\left(\sqrt{\frac{\theta_B\pi(s)}{2}}\,t\right)dt \\ &= \frac{1}{\sqrt{2\theta_B\pi(s)}}\mathrm{B}\left(\frac{1}{2},\theta_{NE}\right),\end{aligned}$$

which, using Gauss's duplication formula for the Gamma function, Γ (Abramowitz and Stegun 1965),

$$\Gamma(2z) = \frac{2^{2z-1}}{\sqrt{\pi}}\Gamma(z)\Gamma\left(z+\frac{1}{2}\right),$$

yields

$$\mathbb{E}[T_{\text{un}}] = \frac{2^{2\theta_{NE}-1}}{\sqrt{2\theta_B\pi(s)}}\frac{\Gamma(\theta_{NE})^2}{\Gamma(2\theta_{NE})}.$$

Using Stirling's approximation to estimate the Beta distribution, B, we see that

$$\mathbb{E}[T_{\mathrm{un}}] = \sqrt{\frac{\pi}{\theta_{\mathrm{NE}}\theta_{\mathrm{B}}\pi(s)}}\left(1 + O\left(\frac{1}{\theta_{\mathrm{NE}}}\right)\right),$$

which is, up to a constant, the result of our heuristic argument. We also note that,

$$\mathrm{B}\left(\frac{1}{2}, \theta_{\mathrm{NE}}\right) = \frac{1}{\theta_{\mathrm{NE}}} + 2\ln 2 + O(\theta_{\mathrm{NE}}),$$

so that

$$\mathbb{E}[T_{\mathrm{un}}] = \frac{1}{\sqrt{2\theta_{\mathrm{B}}\pi(s)}\theta_{\mathrm{NE}}} + \frac{2\ln 2}{\sqrt{2\theta_{\mathrm{B}}\pi(s)}} + O(\theta_{\mathrm{NE}}).$$

Thus, in the limit as $\theta_{\mathrm{NE}} \to 0$ we approximately recover the result in Iwasa *et al.* (2004a,b), but $\mathbb{E}[T_{\mathrm{un}}] > 1/\sqrt{2\theta_{\mathrm{B}}\pi(s)}\theta_{\mathrm{NE}}$ for all values of θ_{NE}.

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Epistasis Increases the Rate of Conditionally Neutral Substitution in an Adapting Population

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DOI: 10.1534/genetics.110.125997

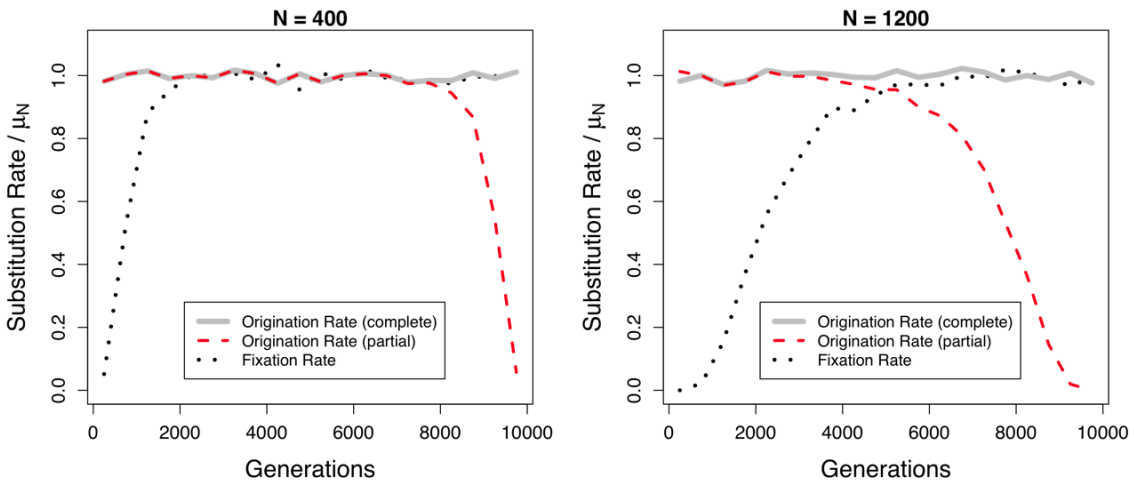

FIGURE S1.—Comparison of three measures of substitution rates in populations with strictly neutral mutations. The dashed, red line tracks the mean origination rate, or the rate at which mutations arise that will fix, in 10,000-generation simulations; the dotted line tracks the rate at which mutations fix in the population. Both rates converge at steady-state, but the origination rate is underestimated near the end of each simulation, and the substitution rate similarly underestimated near the beginning. Note that these transitory periods are proportional to N, as expected from coalescent theory. The error in the origination rate measurement can be fixed by simulating populations for longer than the measurement period; the solid line shows the results of measuring origination rates over 10,000 generations, but running the simulations until a mutation fixes that originated after 10,000 generations. This last method produces an accurate estimate of the substitution rate for the entirety of the observation period.

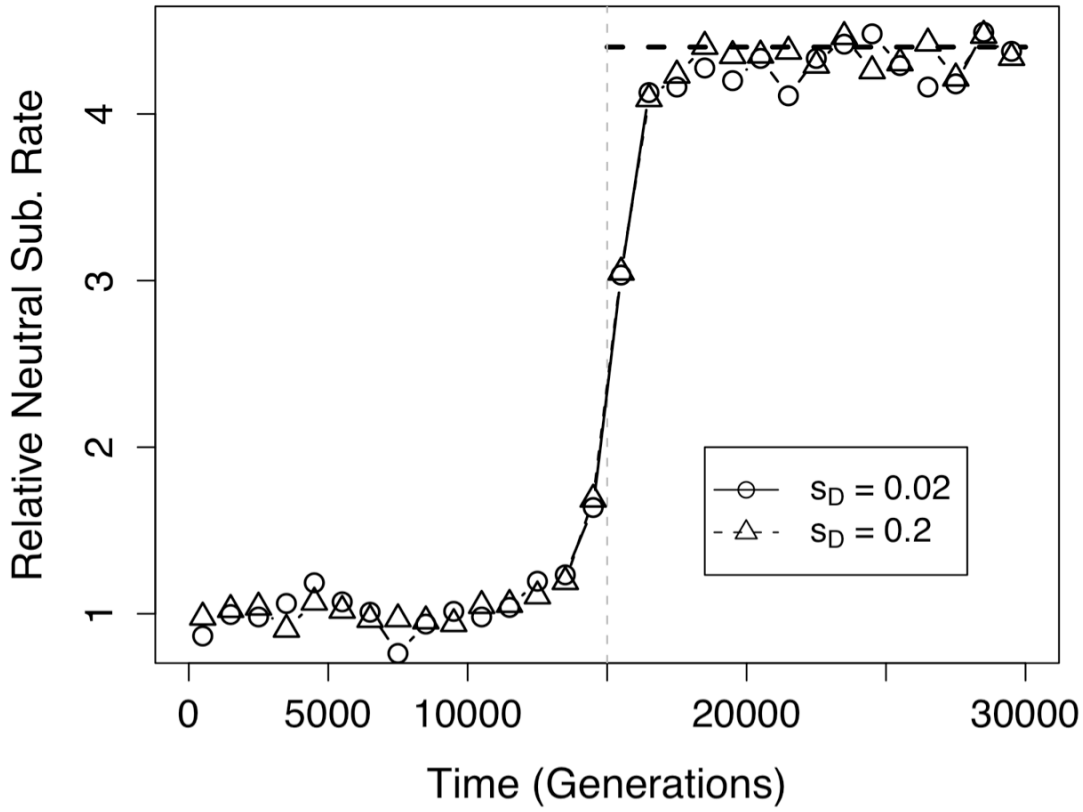

FIGURE S2.—Deleterious mutations have a negligible effect on the substitution rate of conditionally neutral mutations. For all genotypes, a fraction $p_D = 0.5$ of mutations are unconditionally deleterious with selective coefficient s_D. The dashed line shows the mean results from Fig. 3 for comparison. N = 5000, U = 0.004, s = 0.1 for beneficial mutations, and $p_B = 0.001$.

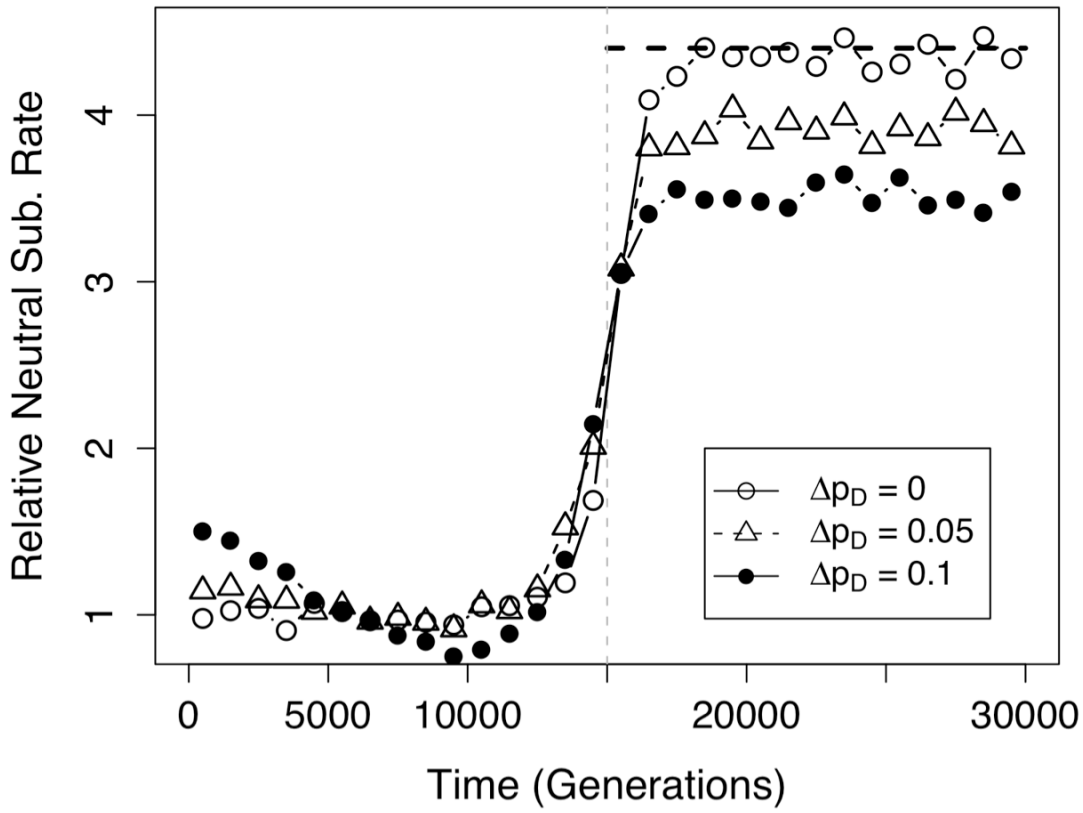

FIGURE S3.—The substitution rate of conditionally neutral mutations is substantially elevated, even when more evolvable genotypes experience a greater deleterious mutation rate. For all genotypes, a fraction $p_D = 0.5$ of mutations are unconditionally deleterious with selective coefficient $s_D = 0.2$. A separate fraction Δp_D of mutations are deleterious in evolvable backgrounds (genotypes for which $p_B = 0.001$) and neutral in unevolvable backgrounds ($p_B = 0$). When $\Delta p_D > 0$ conditionally neutral mutations are shaped by selection against deleterious mutations and do not behave neutrally even under stabilizing selection. However, conditionally neutral sites still substitute much more often than Kimura's expectation when the population is adapting. The dashed line shows the mean results from Fig. 3 for comparison. N = 5000, U = 0.004, s = 0.1 for beneficial mutations, and $p_B = 0.001$.

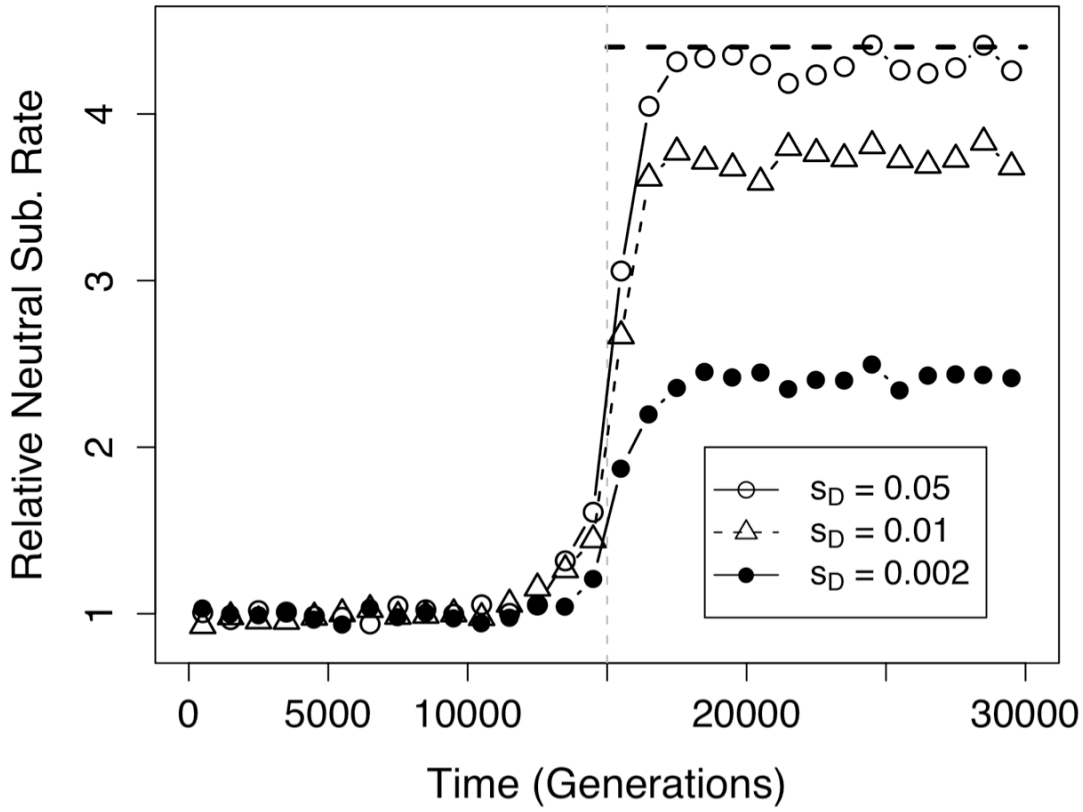

FIGURE S4.—Epistatic deleterious mutations reduce but do not eliminate the increase in the substitution rate of conditionally deleterious mutations in adapting populations. For all genotypes, a fraction $p_D = 0.1$ of mutations are unconditionally deleterious with the selective coefficients given in the figure. Conditionally neutral mutations occur at one-tenth this rate; $p_{NE} = 0.01$. Both kinds of epistatic mutations change evolvable genotypes to unevolvable ones and vice versa. The dashed line shows the mean results from Fig. 3 for comparison. N = 5000, U = 0.004, s = 0.1 for beneficial mutations, and $p_B = 0.001$.