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The evolutionary dynamics of evolvability in a gene network model

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Abstract

Evolvability, the ability of populations to adapt, has recently emerged as a major unifying concept in biology. Although the study of evolvability offers new insights into many important biological questions, the conceptual bases of evolvability, and the mechanisms of its evolution, remain controversial. We used simulated evolution of a model of gene network dynamics to test the contentious hypothesis that natural selection can favour high evolvability, in particular in sexual populations. Our results conclusively demonstrate that fluctuating natural selection can increase the capacity of model gene networks to adapt to new environments. Detailed studies of the evolutionary dynamics of these networks establish a broad range of validity for this result and quantify the evolutionary forces responsible for changes in evolvability. Analysis of the genotype–phenotype map of these networks also reveals mechanisms connecting evolvability, genetic architecture and robustness. Our results suggest that the evolution of evolvability can have a pervasive influence on many aspects of organisms.

Introduction

Evolvability describes the ability of populations to adapt through natural selection, encompassing timescales ranging from changes between generations to major evolutionary innovations (Pigliucci, 2007, 2008). Although variation within a population drives its response to selection, that variation ultimately derives from the variability, or spectrum of potential variants, of individual genotypes (Wagner & Altenberg, 1996). Evolvability connects genotypes to the appearance of adaptive variants, implying that the capacity to adapt is a quantifiable trait with mechanistic and evolutionary explanations. Many recent studies have invoked evolvability to address long-standing questions on mutation rates and the prevalence of recombination (Otto & Barton, 1997; Radman *et al.*, 1999; Tenaillon *et al.*, 2001; Bedau & Packard, 2003; Pepper, 2003; Earl & Deem, 2004; Goddard *et al.*, 2005; Andre & Godelle, 2006). Evolvability is an increasingly popular conceptual tool for understanding the evolution of genetic architecture, or the molecular and developmental processes that link genotypes and phenotypes (Burch & Chao, 2000; Plotkin & Dushoff, 2003; Dichtel-Danjoy & Felix, 2004; Kirschner & Gerhart, 2005; Masel, 2005, 2006; Meyers *et al.*, 2005; Tanay *et al.*, 2005; Wagner, 2005; Bloom *et al.*, 2006; Quayle & Bullock, 2006; Cowperthwaite & Meyers, 2007; Jones *et al.*, 2007; Kashtan *et al.*, 2007; Crombach & Hogeweg, 2008; Draghi & Wagner, 2008; Lehner, 2008). Evolvability also connects evolutionary biology to important applied questions: the directed evolution of proteins (Aharoni *et al.*, 2005; Khersonsky *et al.*, 2006; O'Loughlin *et al.*, 2006) and adaptive change in microbes, invasive species and other dynamic populations (Plotkin & Dushoff, 2003; Gilchrist & Lee, 2007; Blount *et al.*, 2008; Le Rouzic & Carlborg, 2008).

Despite these enthusiastic applications to diverse questions, the concept of evolvability remains controversial. Although some of these disagreements stem from the plurality of definitions in use (Pigliucci, 2008), many objections focus on the contentious claim that natural selection can act to increase evolvability. Several authors have questioned whether natural selection can favour a trait that confers a future benefit (Kirschner & Gerhart, 1998, 2005; Poole *et al.*, 2003; Earl & Deem, 2004;

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Sniegowski & Murphy, 2006). Others maintain that only group-level selection could act to increase evolvability (Lynch, 2007) or that individual-level selection can favour evolvability only when recombination is rare or absent (Sniegowski & Murphy, 2006). Theoretical rebuttals have been made to each of these arguments, as discussed in Draghi & Wagner (2008). However, these controversies seem to be sustained by a lack of concrete examples illustrating how evolvability varies and is shaped by natural selection.

Attempts to remedy this deficit must address the notorious complexity of the relationships between genotypes and phenotypes. One solution is to use a simple model to generate an ensemble of genotype–phenotype (GP) maps and quantify the variation in evolvability within this ensemble. Evolutionary simulations of populations based on these models can then reveal how selection differentiates among alternative developmental possibilities, and uncover how epistasis, pleiotropy and other aspects of the GP map determine evolvability. To implement this approach, we adapted a simple model of a network of genes to examine how the GP map shapes evolvability and its evolution.

Gene network models are both a useful set of tools for probing the evolution of complex systems and a promising step toward an evolutionary understanding of the empirical networks increasingly generated by molecular, genomic and developmental biology (e.g. Jeong *et al.*, 2000; Alon, 2003; de Silva & Stumpf, 2005). Although a number of simple transcriptional network models have been applied to evolutionary questions (e.g. Kauffman, 1993; Quayle & Bullock, 2006; Aldana *et al.*, 2007), we chose to implement a type of model that has led to significant recent insights into the evolution of mutational robustness (Wagner, 1996; Siegal & Bergman, 2002; Bergman & Siegal, 2003; Azevedo *et al.*, 2006; Siegal *et al.*, 2007). Robustness and evolvability are both variational properties deriving from the GP map (Wagner, 2005); so, this prior work establishes that selection can shape variability in this model. This connection also suggests that this model can help answer the open question of the nature of the relationship between evolvability and robustness (Schuster *et al.*, 1994; Kawecki 2000; de Visser *et al.*, 2003; Bloom *et al.*, 2006; Lenski *et al.*, 2006; Aldana *et al.*, 2007; Wagner, 2008).

Previous work on the evolution of mutation rates (Kimura, 1967; Sniegowski *et al.*, 2000; Earl & Deem, 2004) and genetic architectures (Jones *et al.*, 2007; Kashtan *et al.*, 2007; Crombach & Hogeweg, 2008; Draghi & Wagner, 2008) supports the intuition that evolution in changing environments could increase evolvability. Here, we evolve populations of these model networks in constant and varying environments and show that fluctuating selection does promote GP maps with higher evolvability. In contrast to some theoretical predictions (e.g. Sniegowski & Murphy, 2006), we find that recombination does not prevent this evolution of evolvability. By tracking the dynamics of mutants within evolving populations, we link these increases in evolvability to phenotypic adaptation. Finally, we show that a simple projection of the complex GP map provides insight into the genetic mechanisms underlying evolvability in this model, and connects evolvability to mutational robustness. These results suggest that evolvability can readily evolve through changes in the GP relationship, even in the presence of recombination, and demonstrate how to extract an intuitive basis for evolvability from a complex network of epistatic interactions.

Methods

Gene network model

Although our model is inspired by those used in other studies (Wagner, 1996; Siegal & Bergman, 2002; Bergman & Siegal, 2003; Azevedo *et al.*, 2006; Siegal *et al.*, 2007), our implementation is unique. Networks were modelled as a set of K genes, where each gene can potentially regulate itself and every other gene. The direct regulatory influence of gene j on gene i has weight w_{ij} in the interval [−1, 1]. The set of these weights, along with the description of which regulatory connections are present, completely describes a genotype. These weights represent regulatory regions associated with a gene, and they are the basic unit of heredity in our model; we therefore use the term 'site' to refer to the part of a regulator determining a single connection, and the term 'allele' for the presence or absence, sign and weight of a regulatory influence. There are therefore K^2 sites in a genome of K genes. Gene expression is either active or inactive; active genes have the state '1', whereas inactive genes have the state '0'. This is a departure from some previous versions of this model, and reflects the intuition that inactive genes should not influence regulation. At the beginning of each network's development, each gene is active. At a discrete time point t, each active gene can stimulate or repress the expression of other genes. The state of a gene at time $t + 1$ is therefore computed from the sum of its regulatory weights from active genes. If this sum is greater than zero, that gene will be active at $t + 1$; if the sum is zero or negative, the gene will be inactive. Following Wagner (1996), we refer to the vector of gene states at time t as $S(t)$, and the successive value is defined by:

$$S(t+1)_i = \begin{cases} 1, & \text{if } \sum\limits_{0 \le j < K} w_{ij} S(t)_j > 0 \\ 0, & \text{if } \sum\limits_{0 \le j < K} w_{ij} S(t)_j \le 0 \end{cases} \qquad (1)$$

Because $S(t + 1)$ is determined solely from $S(t)$, $S(t)$ fully describes the system's state, ensuring that within 2^K time points the system will reach a previously visited state. After returning to a previous state, the system has

either arrived at a static equilibrium, $S(\infty)$, or will continue to cycle perpetually among two or more states. If a network reaches a static equilibrium we classified it as stable, and can then compare its equilibrium phenotype, $S(\infty)$, to a defined optimal phenotype. If d represents the number of equilibrium gene states that differ from the optimum, or the phenotypic distance, and s the fitness cost of each mismatch between the phenotype and the optimum, then expected relative fitness was assigned as:

$$w_d = \frac{1}{(1+s)^d} \qquad (2)$$

This fitness function models multiplicative interactions among traits, ensuring that the selective benefit of a unit decrease in d will be independent of d. This independence simplifies comparisons among populations adapting to different optima. Furthermore, the strength of selection is easily tuned through the parameter s. Following Wagner (1996), we classified cycling networks as less fit than all stable networks by assigning them a phenotypic distance, d, of $K + 1$. As stability does not depend on the environment, we can refer to genotypes as stable or unstable.

Network evolution

To test if a periodically changing environment will favour increased evolvability, we simulated the evolution of populations of gene networks. These populations consisted of N individuals that reproduce in discrete generations. In each generation, networks dynamics are computed to determine their phenotypes, following eqn 1. Equation 2 is then used to assign a fitness to each 'adult' phenotype; these fitnesses directly influence fecundity. To create an individual of the next generation, one parent was first stochastically selected with a probability proportional to its assigned relative fitness. In asexual reproduction an offspring was cloned, with possible mutations, from this selected parent. In simulations with sexual reproduction, a second, distinct parent was chosen in the same way, and the value of each network connection in the offspring was inherited independently from one of the two parental genomes. Therefore, both sexual and asexual populations are haploid. This model of sexual reproduction simulates the absence of any linkage among the sites determining regulatory influences; although unrealistic, this extreme model provides the greatest contrast to asexual reproduction.

In either mode of reproduction, two types of mutation could occur. These types alter the two properties of each site: the presence/absence of a connection and the weight if that connection exists. The weight of each extant gene interaction could mutate with rate μ and by an amount uniformly chosen from the interval $[-m, m)$, and weights were confined to the interval $[-1, 1]$ by reflecting boundaries. These choices ensure that, at mutational equilibrium, all allele values in the interval are equally likely. As alleles values are initially uniformly distributed, mutation and drift alone will not systematically alter allele states, and the action of natural selection becomes easier to detect. By changing m, we can alter the degree to which mutations are contingent on the prior allele state, and consequently explore a broad class of models of mutation. A separate class of mutation alters network architecture: an extant connection may be deleted with rate μ_A, and an absent connection may be added with the same rate. Deleted connections have their weights set to zero, whereas new connections have weights drawn from the interval $[-1, 1)$. By equalizing the rates of insertion and deletion, we maximized the ability of mutation to produce diverse network topologies.

In a random initial network, each possible connection was present with probability 0.5, and connection weights were drawn uniformly. This procedure produced a high diversity of networks, and ensured that the number of connections in a network began near mutational equilibrium. However, random generation of networks produces a biased distribution of phenotypes. To prevent this inequality from influencing the differences between evolved and initial genotypes, we often analysed ensembles where an equal number of simulations were seeded with each of the 2^K phenotypes. Once a random network was generated, it was cloned to initiate the simulation with an identical population of N individuals.

Measuring evolvability

To produce a robust measure of the potential to adapt, we devised the following algorithm. First, a genotype was expanded into a population of identical clones, then evolved in a specified environment for T generations. To quantify adaptation, we compared the phenotypes in the evolved population with the ancestral phenotype. We then averaged the progress toward the phenotypic optimum across the population to produce a measure of adaptation based on the Hamming distance, or number of mismatched expression states, between phenotypes. For example, if the initial clone had three mismatches with the optimum, the potential evolved Hamming distance would be three. If, after T generations, half of the individuals had a single mismatch with the optimum, and half had two mismatches, the mean evolved distance would be 1.5. These evolution trials were replicated, and performed across all environments where adaptation is possible. In the case of $K = 4$, any stable genotype has one of 16 possible phenotypes, and so can adapt to any of the other 15. The evolved distances for these trials were summed, then normalized by dividing by the sum of the initial phenotypic distances across all trials. This calculation produced values between 0 and 1, where 1 measures a genotype that will result in a completely adapted population in any environment within T generations. To

illustrate, consider combining the example trial described above, where the population evolved 1.5 units out of a possible 3, with a different trial where the population begins with one mismatch, then half of the individuals evolve to perfectly match the target. The summed evolved distance of two would then be normalized by division by four, producing an evolvability measure of 0.5 for this example. We refer to this evolvability after T generations as E_T.

We found, as illustrated in Fig. S1, that the choice of T can change the rank order of E_T within a set of genotypes. To find the most informative observation period, we examined the correlation between the ordinal rank of the epoch and E_T for a range of values of T. Spearman rank correlations for 40 values of T are plotted in Fig. S2. The weakest correlation, between E_{200} and epoch number, is 0.3944; however, this value is higher than any of 10 000 correlations between epoch number and randomly shuffled values of E_{200}, suggesting that all of the correlations are significant. To further investigate the evolution of evolvability, we focused on E_{25}, the maximally correlated measure ($T = 25$ also yields the maximal correlation as calculated by Kendall's τ, an alternative metric of rank-order correlation).

Results

Evolvability in fluctuating environments

Figure 1 plots our evolvability measure, E_{25}, for sets of evolutionary simulations in three scenarios: asexual and sexual reproduction in fluctuating environments and asexual reproduction in a stable environment. E_{25} is measured for a random, stable genotype at the beginning of each epoch; population sizes and mutation rates for these assays are always identical to those of the evolving population. A point on this plot is the mean of 160 evolvability scores, each representing a separate population. Each evolvability score is composed of 30 replicate trials for each of the 15 alternative environments, yielding 450 measurements for each genotype. As above, $K = 4$, $N = 1000$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$, $s = 1$, $P = 100$ and 10 populations were initiated from each possible phenotype. Filled circles correspond to asexual populations, whereas filled triangles show the results of evolving sexual populations in the same scenario. Note that in sexual populations, model networks experienced obligate recombination during both evolution and E_{25} measurements, and yet the pattern of evolvability over time is very similar to the asexual case.

To confirm that a changing environment was necessary for evolvability to increase, we performed control simulations with the same parameters in static environments. Populations began with the optimal phenotype for the unchanging environment and therefore evolved under stabilizing selection. The open circles in Fig. 1 show these results for asexual reproduction: here E_{25} is seen to decline slightly but insignificantly over time (linear regression, $F_{1,1278}$, $P = 0.1314$). These results demonstrate that the directional selection experienced by populations in changing environments is necessary for evolvability to evolve.

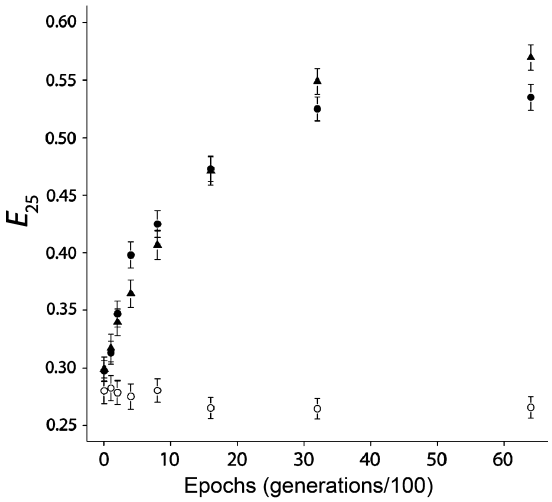

Fig. 1 E_{25} increases with epoch number in changing environments. Filled symbols are populations in which the optimal phenotype changes every 100 generations; the filled circles represent clonal reproduction, whereas the filled triangles represent populations with recombination. The open circles represent control populations that experience stabilizing selection for the entire 64 epochs. Each point is the mean of 160 simulations, consisting of sets of 10 populations starting from each of the 16 possible phenotypes. Bars are standard errors. Parameters are $K = 4$, $N = 1000$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$ and $s = 1$.

E_{25} was tuned to capture the increase in evolvability seen in populations with the specific parameters given above. In populations of different sizes, with different mutation rates, evolvability over greater or lesser periods than 25 generations might be more relevant, and E_{25} might underestimate evolvability. It is therefore difficult to quantitatively compare values of E_{25} across simulations with different parameter values. Instead, we have demonstrated that the fundamental result, that evolution in changing environments increases evolvability, is valid over a wide range of parameters. Appendix 1 presents simulations with different population sizes, rates of environmental change, mutation rates and network sizes. In summary, these results confirm that evolvability increases over a significant range for each parameter of the model.

Recombination and the evolution of evolvability

Based upon models of mutator alleles (Sniegowski *et al.*, 2000; de Visser, 2002), some authors have argued that recombination will generally suppress the evolution of evolvability (Sniegowski & Murphy, 2006). If

recombination can prevent the evolution of evolvability in our model, this result would indicate that the determinants of evolvability, like mutator alleles, could be separated from any adaptations they might cause. By contrast, recombination is not expected to oppose evolvability if the determinants of evolvability are epistatically linked to any beneficial mutations they promote, such that multiple alleles are required for the beneficial effect to be expressed (Draghi & Wagner, 2008). Further examination of the effects of recombination in this model can both test the hypothesis in Sniegowski & Murphy (2006) and describe the genetics of evolvability in model networks.

Although Fig. 1 shows that recombination does not necessarily prevent the evolution of evolvability, simulations with weaker selection can provide a more critical test. In Fig. 1, as in the majority of our data, each beneficial mutation doubles the fitness ascribed to a genotype. This strong selection allows beneficial mutants to fix quickly, potentially masking the effects of recombination. We performed additional simulations, in which beneficial mutations are 10-fold less strongly selected, to more stringently test the effects of recombination on evolvability.

To promote adaptation through these weakened beneficial mutations, we performed these simulations with $K = 4$, $N = 10\,000$ and $P = 100$, and used test populations of 10 000 individuals to test evolvability; other parameters were the same as those used in the section 'Evolvability in fluctuating environments'. The evolution of E_T in these populations is plotted in Fig. 2. In contrast to the results plotted in Fig. 1, asexual populations achieved a higher level of E_T than sexual populations when $s = 0.1$. Nonetheless, linear regressions confirmed that E_T significantly increased over time for both modes of reproduction, at both values of T (E_{25}, asexual: $F_{1,510} = 92.51$, $P < 2.2 \times 10^{-16}$; E_{25}, sexual: $F_{1,510} = 49.16$, $P = 7.51 \times 10^{-12}$; E_{100}, asexual: $F_{1,510} = 181.8$, $P < 2.2 \times 10^{-16}$; E_{100}, sexual: $F_{1,510} = 62.12$, $P = 1.97 \times 10^{-14}$).

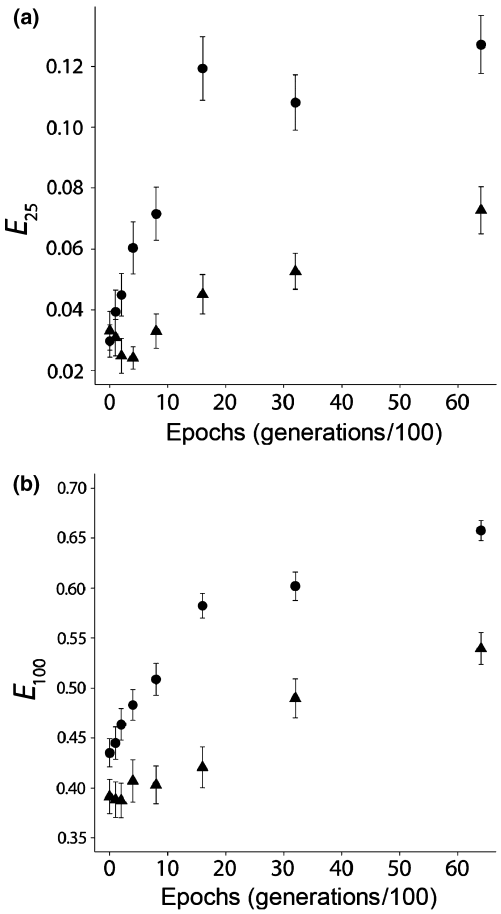

Fig. 2 The evolution of E_T in asexual and sexual populations with smaller selective coefficients, s. $N = 10\,000$, $P = 100$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$ and $s = 0.1$. T, the duration of the evolvability measurement, is 25 in (a) and 100 in (b). Circles indicate no recombination, triangles full recombination. Bars indicate standard errors.

The dynamics of evolvability in evolving populations

The results discussed above strongly imply that shifting directional selection causes evolvability to increase. To explain how evolvability is favoured in evolving populations, we conducted detailed studies of the dynamics of evolvability in evolving populations.

Without a genotypic proxy for evolvability, we measured E_{25} for each genotype of an evolving population. This was computationally feasible only for asexual populations, where most offspring are clonal and therefore inherit the parent's value of E_{25}. As in the section 'Evolvability in fluctuating environments', $K = 4$, $N = 1000$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$, $P = 100$, $s = 1$, and four populations, each starting from a different phenotype, were examined for 20 epochs each.

As illustrated in Fig. S3, evolution in these populations can be divided into two types of intervals: brief periods of rapid adaptation, and longer stretches of stasis in fitness. These dynamics are a consequence of the rarity, and uniformly large effect, of beneficial mutations. Based on this dichotomy, we analysed the change in E_{25} during the fixation of beneficial mutations and during periods of neutral evolution. With a complete record of the fitness of every individual, it was possible to use the variance in fitness within each generation as an indicator of the rate of adaptation. A useful heuristic was that those generations where the variance of the natural log of fitness was less than 0.02 were evolving neutrally, and generations above that cut-off were adapting.

The effect of adaptive evolution on evolvability was measured by comparing the mean E_{25} of individuals in the population before and after each period of

adaptation. These data were not normally distributed; so, a Wilcoxon signed-rank test was used to assess the paired differences. Selective sweeps were found to significantly increase E_{25} (two-tailed test, 76 pairs, $V = 1897$, $P = 0.0248$), confirming our expectation that directional selection increases evolvability. By contrast, neutral evolution significantly lowered E_{25} (two-tailed test, 81 pairs, $V = 882$, $P = 0.000249$). This agrees with the results from static environments in Fig. 1, and supports the conclusion that in our model nonadaptive evolution does not increase evolvability.

Our simulations also recorded the lineage of each organism, permitting analysis of the progenitors of beneficial mutants. Individuals that produced a beneficial, potentially adaptive mutant in the next generation had significantly higher values of E_{25} than their average contemporary (Fig. S4a; Wilcoxon signed-rank test, 628 pairs, two-tailed, $V = 143\,425$, $P < 2.2 \times 10^{-16}$), providing further evidence that E_{25} predicts evolutionary success and thus evolvability is a target of selection. These beneficial mutants had similar values of E_{25} to their parents (Fig. S4b; Pearson's $r = 0.96$); however, in the set of 628 mutants, we found no evidence that nonneutral mutations systematically changed E_{25} (Wilcoxon signed-rank test, two-tailed, $V = 95\,990$, $P = 0.5436$). These observations support a causal link between evolvability and beneficial mutations: the appearance of beneficial mutations increases with evolvability, but the substitution of a beneficial change does not, in itself, increase evolvability.

Evolvability at the network level

The preceding sections established that some genotypes are more likely to produce adaptive mutations, and that this evolvability can be passed down to the successful, mutant descendants of evolvable genotypes. We sought to explain these properties by correlating evolvability with structural characteristics of gene networks. Many studies have addressed the influence of mutation rate on evolvability (Tenaillon *et al.*, 2001; Bedau & Packard, 2003; Earl & Deem, 2004; Andre & Godelle, 2006); so, an obvious starting point is the contribution of network topology to mutation rate. Recall that extant connections change their weights at a rate μ, whereas topological mutations, where connections are inserted and deleted, occur at a lower rate μ_A. In the simulations examined above, μ is 100 times larger than μ_A. If we treat all mutations as equivalent and let c represent the number of extant connections in a network, the genomic mutation rate U is:

$$U(c) = \mu c + \mu_A K^2 = \mu\left(c + \frac{K^2}{100}\right) \qquad (3)$$

The overall mutation rate of a network can therefore be substantially modified by changes to the connectivity of its topology.

We compared c and E_{25} for a set of 10 000 randomly generated, stable networks. These networks were drawn from the same distribution as those used to initiate evolutionary simulations, except that phenotype frequencies were not equalized. Evolvability was assessed using the same parameters as in the section 'Evolvability in Fluctuating Environments'. $K = 4$, $N = 1000$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$ and $s = 1$. These data, plotted in Fig. S5, indicate that evolvability cannot be trivially reduced to the density of network connections and thus to the genomic mutation rate. After inspecting the plot, a third-order polynomial regression was performed. However, this regression explained only 1% of the variation in E_{25}, suggesting that c does not explain evolvability (overall $F_{3,9996} = 33.34$; $t_{\text{intercept}} = 5.828$, $P = 5.77 \times 10^{-9}$; x: $t = 1.842$, $P = 0.0656$; x^2: $t = -1.681$, $P = 0.0927$; x^3: $t = 1.096$, $P = 0.273$). In addition, high c values, which lead to higher mutation rates, were associated with lower evolvability. Finally, we measured c in evolving populations, with and without sex ($K = 4$, $N = 1000$, $P = 100$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$, $s = 1$ and 64 replicates). Linear regressions of c on time were not significant (asexual: $F_{1,574} = 1.357$, $P = 0.2445$; sexual: $F_{1,574} = 0.5381$, $P = 0.4635$), providing additional evidence that c does not determine evolvability.

After some experimentation, a simple network property was discovered that explained a substantial fraction of the variation in E_{25} among random networks. This property, which we refer to as the network excitation, is the sum of all the connection weights within a network. The relationship between E_{25} and network excitation is plotted in Fig. 3. The plotted data suggest that an asymmetrical curve might describe the apparent relationship; so, a third-order polynomial was chosen to regress this sum on E_{25}. This regression was highly significant for all four model parameters, and explained 22.6% of the variation in E_{25} (overall $F_{3,9996} = 972.7$; $t_{\text{intercept}} = 186.53$, x: $t = 38.66$, x^2: $t = -27.28$, x^3: $t = -14.44$; all $P < 2 \times 10^{-16}$). This curve has a maximum at about 1.8, suggesting that evolvability is generally the highest when positive regulatory connections somewhat outweigh negative influences. Below, we demonstrate that this result provides an intuitive explanation for evolvability and its evolution.

To understand how the sum of connection weights influences evolvability, we examined how genotypes map to phenotypes within our model. Recall that a gene without net positive regulation will be inactive, and that each gene is initially active. If all genes are connected to each other by exclusively negative interactions, each gene will begin in the active state, immediately be repressed into inactivation, then will maintain that inactive state indefinitely. Clearly, though, it is not necessary that all connections are negative, merely that negative regulation predominates for each gene. We therefore expected that this inactive phenotype, labelled

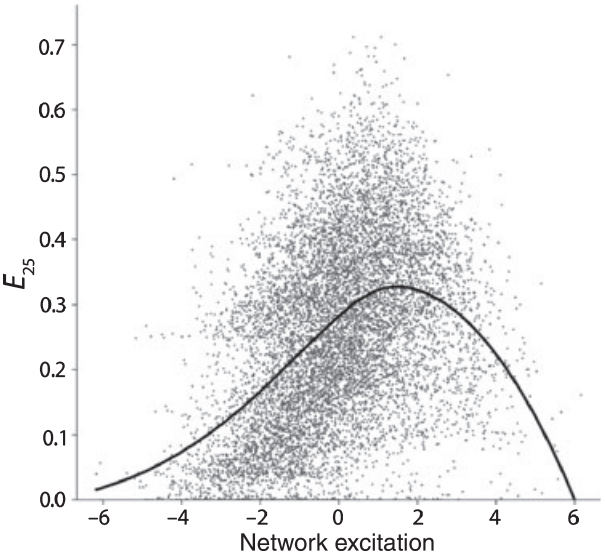

Fig. 3 The sum of the connections of a network, or network excitation, plotted against E_{25} for 10 000 randomly generated, stable networks. The curve illustrates a third-order polynomial regression. Reproduction is asexual, and $K = 4$, $N = 1000$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$ and $s = 1$.

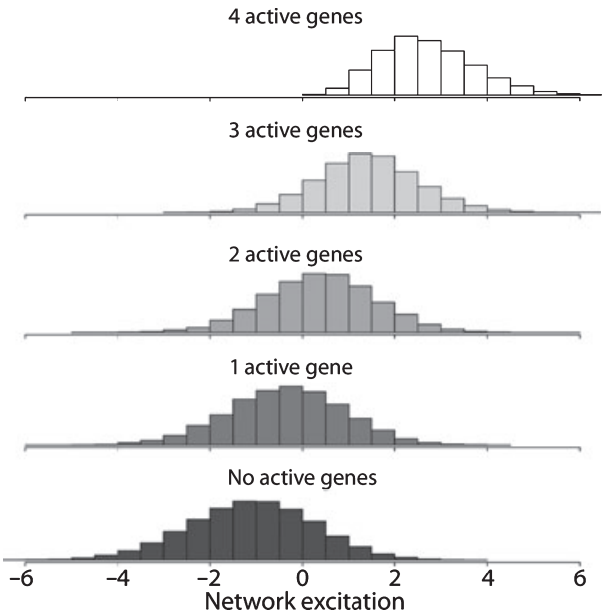

Fig. 4 Range of network excitations associated with networks of each class of phenotype. The 16 possible expression patterns in four-gene networks are reduced to five categories based upon the number of active genes in each phenotype. Network excitation is the sum of the positive and negative regulatory weights within a network. Histograms reflect the distribution of excitations among randomly generated networks with the specified phenotype.

as '0000' in the case of $K = 4$, might be associated with networks with mostly negative interactions. Similarly, networks with many positive interactions could results in the '1111' phenotype. Finally, a gene that is repressed and stimulated equally will be inactive. This asymmetry suggests that phenotypes with some active and some inactive genes, such as '1001', may have more positive than negative interactions on average. This reasoning suggested an association between the balance of regulatory weights and the number of active genes in the phenotype, and predicted that intermediate phenotypes will often have a positive balance of weights.

To confirm these predictions we produced a much larger set of 100 000 random networks, which was not filtered to equalize the representation of each phenotype. Networks with stable phenotypes were then grouped into five classes, corresponding to 0, 1, 2, 3 or 4 active genes. We then computed the sum of the regulatory weights for each network – network excitation. Figure 4 shows the distributions of network excitations associated with the five classes of phenotypes. These data reveal that the GP map is highly degenerate: each phenotype can be constructed from many genotypes with a range of network excitations. However, the hypothesized connection is evident: network excitation clearly correlated with the number of active genes in the phenotype. The data in Fig. 3 suggest that genotypes with network excitations between about 1 and 2 often have high evolvabilities; intriguingly, Fig. 4 reveals that this same range contains many networks corresponding to each phenotypic class.

These results suggest an explanation for the connection between network excitation and evolvability: networks with optimal excitation may be near, with respect to mutation, genotypes that map to a variety of other phenotypes. Evolvable genotypes might then be said to exist at a confluence of the genotype distributions for each phenotype. As evolvable genotypes are mutationally close to alternative phenotypes, they can rapidly adapt through a small number of changes. Thus, network excitation may change little during the adaptation of evolvable genotypes, and therefore high evolvability may be inherited and maintained. By contrast, networks with suboptimal excitations may not be able to produce alterative phenotypes with single mutations, and may require neutral changes before beneficial mutations are accessible. Adaptation in these populations will often be slow, and will tend to result in descendants that have evolved, through drift and selection, to be closer to the optimal excitation.

This hypothesis suggests a partial explanation of why networks vary in evolvability, and how evolution in changing environments leads to more evolvable genotypes. To test this model of evolvability and its evolution, we derived several new predictions. First, we note that the distributions of excitations for phenotypes '0000' and '1111' extend far from the predicted optimum: E_{25} should have strong, monotonic and opposite relationships with network excitation for these extreme

phenotypes. Second, network excitations in populations in changing environments should typically evolve to occupy a narrower range of values when compared with populations under stabilizing selection. Third, we expected that the probability that a mutation changes the phenotype, or the volatility of a genotype, might increase during evolution in a changing environment. Specifically, we define volatility as the fraction of mutations that produce an altered, but stable, phenotype, and expect that volatility will increase with evolvability. This relationship is expected if the variety of phenotypes in a mutant spectra and the frequency of neutral mutations are negatively correlated.

We reanalysed the data in Fig. 3 with respect to phenotype to test this first prediction. As the excitations of networks with the '0000' phenotype are often lower than those typical of other phenotypes, we expect a positive relationship between excitation and E_{25} for this phenotype. These data, shown in Fig. 5a, indeed positively correlated with a Pearson R^2 of 28.5%. Similarly, genotypes that map to the '1111' phenotype should often have very high excitations, and excitation should therefore be negatively associated with E_{25}. Data for this phenotype are plotted in Fig. 5b, and the Pearson R^2 for this negative relationship is 42.7%.

To test the latter two predictions, we measured network excitation and volatility during evolution in changing and static environments. Eight hundred simulations were performed for each condition, with parameters as above ($K = 4$, $N = 1000$, $P = 100$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$, $s = 1$), and one randomly chosen network was measured every 100 generations. Networks were then grouped into the five basic phenotypic classes to determine how the mean network excitation associated with each class changed over time. Figure 6a confirms that the mean network excitation

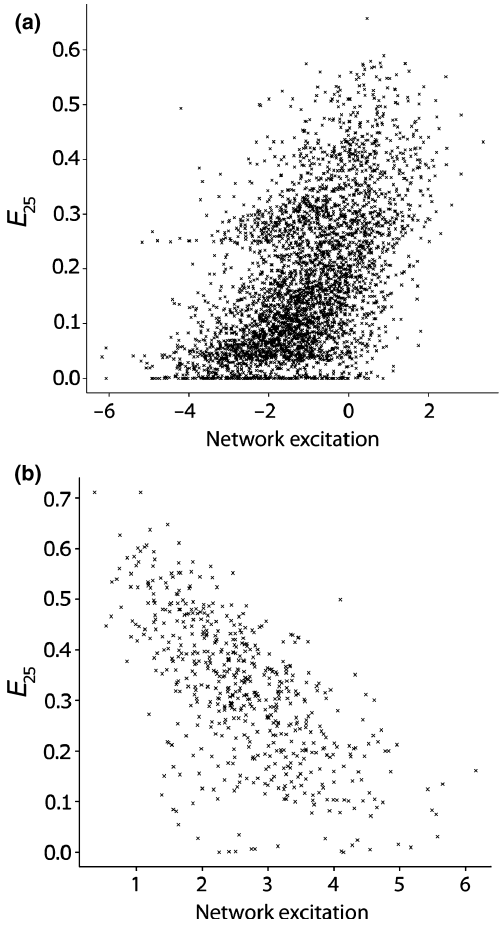

Fig. 5 Network excitation and E_{25} for genotypes mapping to specific phenotypes. (a) Genotypes corresponding to the '0000' phenotype, where all genes are inactive at equilibrium; (b) genotypes generating phenotype '1111', where all genes are active at equilibrium. Reproduction is asexual, and $K = 4$, $N = 1000$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$ and $s = 1$.

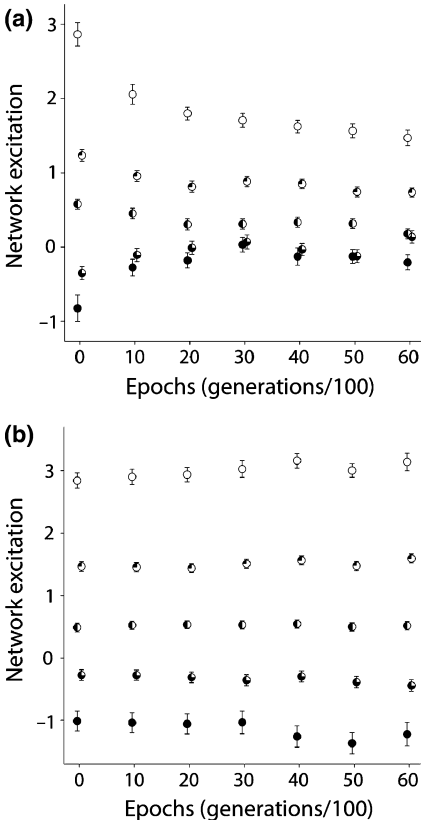

Fig. 6 Mean network excitations among phenotypic classes in evolving populations. Symbols represent mean network excitations for the five classes of phenotypes: the filled area in each symbol corresponds to the number of inactive genes in that phenotype. Reproduction is asexual, and $K = 4$, $N = 1000$, $P = 100$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$ and $s = 1$. Bars show standard errors. (a) Eight hundred replicates evolved in a changing environment; (b) 800 replicates evolved under stabilizing selection.

converged toward the predicted optimal range in populations evolving in a changing environment. By contrast, no convergence was seen when populations were subjected to stabilizing selection (Fig. 6b).

Figure 7a plots the volatility of sampled networks over time. Volatility is measured by producing a library of 10 000 genotypes with single-mutation differences from their parent genotype. These differences include changes in both connection weights and topology, in the same ratio as these mutations occur in our other evolutionary simulations. The fraction of this library that produces a different, but stable, phenotype from the parent is a measure of volatility. There is a clear increase in volatility with time in a changing environment, but not in static environments. This pattern suggests that the robustness to mutation, calculated as the fraction of mutations that do not alter the phenotype, might significantly decrease over time. Because the fraction of mutations that produce unstable phenotypes is not included in either measure, it is not strictly necessary for volatility and robustness to negatively covary. However, Fig. 7b shows

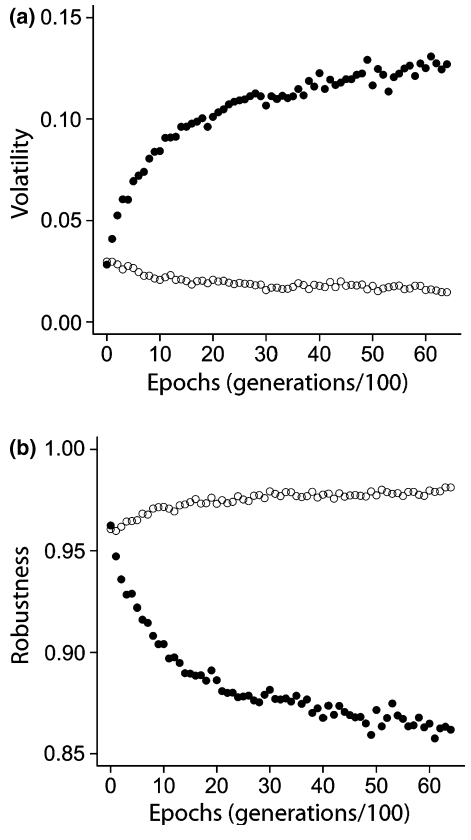

Fig. 7 Volatility of networks over time in changing and static environments. Data are drawn from sampled individuals averaged over 640 trials for each conditions; filled circles represent changing environments, open circles static environments. Reproduction is asexual, and $K = 4$, $N = 1000$, $P = 100$, $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$ and $s = 1$.

that robustness does decrease as volatility and evolvability increase. Network excitation therefore affects both the probability of a non-neutral mutation, and the distribution of those mutations among phenotypes.

Discussion

We have demonstrated that evolvability can evolve through changes in the GP relationship, and that this evolution of evolvability is a consequence of adaptation in a changing environment. This qualitative result holds over a range of population sizes, periods of change and mutation rates, although our method does not allow a fully quantitative analysis of the influence of these factors. Our results also demonstrate that recombination does not necessarily prevent natural selection from favouring an increase in evolvability. These findings illustrate the complex GP relationship inherent in a very simple model of gene interactions, and reveal surprising subtleties of the interplay between natural selection and an evolving GP map.

We have also shown that a projection of genotype space to a single dimension can help explain the mechanisms of evolvability in our model. Intriguingly, this quantity, which we call 'network excitation,' has a clear biological interpretation: it is the balance of positive and negative regulatory influences in a transcription factor network. This sum predicts the extent to which a gene's expression state is over-determined, and therefore the propensity for mutations of small effect to alter network dynamics. This discovery suggests that, although GP maps may be very complex, their connections with evolvability might be simple and intuitive.

The investigation of this model was designed to focus on the evolution of variability in the context of a complex GP relationship. Although our network model is obviously very simple in comparison with biological systems, its dynamics produce a nontrivial GP map with many of the features, such as widespread epistasis, degeneracy and pleiotropy, of real organisms. Our focus on changes in the GP map is complementary to the many studies that have explored how the evolution of mutation and recombination rates contributes to evolvability (e.g. Kimura, 1967; Otto & Barton, 1997; Sniegowski *et al.*, 2000; Earl & Deem, 2004). Unifying these approaches into a coherent picture of the factors contributing to evolvability is a major challenge for future research.

Our results complement several recent studies of evolvability in network models and establish a more detailed understanding of the population genetics of the evolution of evolvability. Two previous studies (Quayle & Bullock, 2006; Aldana *et al.*, 2007) examined evolvability with network models, but did not characterize the structural basis or evolution of the trait. Crombach & Hogeweg (2008) studied a much different network model, and found that over repeated adaptation to two

or three alternative environments, the average speed of adaptation increases. The authors link this evolution of evolvability to modifications in certain constitutively expressed genes, which they call 'evolutionary sensors'. Similarly, Kashtan *et al.* (2007) demonstrated that several models of circuits could adapt faster after evolving to a set of independently varying, modular goals. Our study reinforces these earlier results, and presents the first detailed exploration of the population genetics underlying the evolution of evolvability. By testing the effects of recombination, and analysing the evolutionary dynamics of the mutations that change evolvability, our results directly address controversial aspects of the concept of evolvability. Finally, our study highlights a simple, mechanistic connection linking a general aspect of network structure to both evolvability and robustness.

Our results achieve further relevance for biology by illustrating a clear and useful way to think about evolvability. Much of the discussion on evolvability has focused on special cases with an intuitive relation to selectable variation: mutator alleles (Sniegowski *et al.*, 2000; Earl & Deem, 2004; Sniegowski & Murphy, 2006) or Hsp90 (Rutherford & Lindquist, 1998; Wagner *et al.*, 1999), for example. These instances may be individually important, but they do not sum to a general and convincing foundation for a theory of evolvability. By contrast, our model suggests that the potential for evolvability to evolve derives from basic properties of the GP map: epistatic interactions among loci, pleiotropy allowing a single mutation to potentially change several traits and the existence of clusters of similar genotypes that generate the same phenotype. A unique aspect of our results is that each site appears to contribute to evolvability, and consequently has the potential to be shaped by its evolution. As the GP map is degenerate as well as epistatic, this potential can be realized by neutral changes at many sites. This degeneracy is ubiquitous in other simple models and at many levels of biological organization (Wagner, 2005), and creates the opportunity for variability to evolve (Ancel & Fontana, 2000; Plotkin & Dushoff, 2003; Meyers *et al.*, 2005). Our results therefore suggest that the significance of evolvability is not limited to a few specialized traits, suggest as mutator alleles, but may encompass all loci with degeneracy and epistatic interactions.

One significant consequence of the genome-wide basis of evolvability in this model is that recombination cannot easily separate beneficial changes from the determinants of evolvability. A simple explanation for this empirical result is that the fitness benefit of each adaptive change depends on the state of several other loci. Consequently, beneficial mutants cannot fix independent of their genetic background. This contrasts sharply with the dynamics of mutator alleles, as referenced above: mutator alleles do not interact epistatically with beneficial changes they contribute to, and so can be easily separated from fixing alleles. This clear difference between the behaviour of mutator allele models, and the results presented here and in an earlier paper (Draghi & Wagner, 2008), illustrate the importance of understanding the role of the GP map in evolvability.

The demonstration that evolvability can have a broad genetic basis also has major implications for understanding evolutionary patterns. For example, Wagner (2008) contends that phenotypes are less ephemeral, over evolutionary time, than their corresponding genotypes, and are therefore a more appropriate focus for the evolution of variability. However, our analysis of the quantity we called 'network excitation' suggests that properties of the genotype may evolve and persist at much longer timescales than phenotypes. Our results show that network excitation, an aggregate property of many genes, is on average shaped in a systematic manner to reflect hundreds of generations of evolutionary dynamics. Network excitation provides a concrete example of the difference between genotype and genetic architecture, and highlights the evolutionary relevance of gene networks.

The current results, along with previous simulation studies (Meyers *et al.*, 2005; Draghi & Wagner, 2008), demonstrate that random environmental change favours specific genotypes with less restricted patterns of variability. Isotropic variation at the phenotypic level is often a default expectation, but as Salazar-Ciudad (2006, 2007) and others have pointed out, genotypes that can produce such unbiased variation must be very rare. By examining thousands of gene networks, those networks that can produce beneficial mutations in many environments were seen to be exceptional. However, these evolved forms are only remarkable in comparison with their ancestors – without a sample space of possible GP maps, this derived evolvability is hidden. Although it is obvious that evolvability is a relative measure, this study reveals the benefits of studying evolvability using a model with a defined space of possible GP maps.

The results presented in Fig. 7 support the idea that evolvability negatively correlated with mutational robustness, or the tolerance of the phenotype to changes in the genotype. Although several studies have shown that evolution can increase robustness in gene network models (Wagner, 1996; Siegal & Bergman, 2002; Bergman & Siegal, 2003; Azevedo *et al.*, 2006; Siegal *et al.*, 2007), our study is the first to demonstrate that the opposite can also occur. This result coincides with the intuitive idea that robustness and evolvability are fundamentally in opposition, but also contradicts a number of recent studies suggesting that robustness can promote evolvability (de Visser *et al.*, 2003; Bloom *et al.*, 2006; Lenski *et al.*, 2006; Aldana *et al.*, 2007; McBride *et al.* 2008; Wagner, 2008). As basic questions surrounding robustness and evolvability have begun to be resolved, these evolutionary influences on variability must be modelled and observed acting in concert. Several recent

attempts to unify robustness and evolvability have illustrated the complexity of this question, and its significance for evolutionary understanding (Meyers *et al.*, 2005; Wagner, 2008). Important extensions of the current study include exploring the evolvability–robustness relationship over a wide range of population parameters and evolutionary scenarios to appreciate how these traits co-evolve.

Summary

Our results illustrate how the regulatory interactions among genes could evolve to substantially alter evolvability and robustness. Evolvability is shown to generally increase when the environment occasionally changes, and this pattern does not depend on group-level selection, the absence of recombination or special traits such as mutator alleles. The key to this evolution of evolvability lies in the GP map, which evolves to increase the mutational accessibility of alternative phenotypes. These results explicate how evolutionary forces can shape evolvability, and suggest that selection on evolvability may shape gene networks in nature.

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References

Aharoni, A., Gaidukov, L., Khersonsky, O., Gould, S.M., Roodveldt, C. & Tawfik, D.S. 2005. The 'evolvability' of promiscuous protein functions. *Nat. Genet.* **37**: 73–76.

Aldana, M., Balleza, E., Kauffman, S. & Resendiz, O. 2007. Robustness and evolvability in genetic regulatory networks. *J. Theor. Biol.* **245**: 433–448.

Alon, U. 2003. Biological networks: the tinkerer as an engineer. *Science* **301**: 1866–1867.

Ancel, L.W. & Fontana, W. 2000. Plasticity, evolvability, and modularity in RNA. *J. Exp. Zool.* **288**: 242–283.

Andre, J.B. & Godelle, B. 2006. The evolution of mutation rate in finite asexual populations. *Genetics* **172**: 611–626.

Azevedo, R.B.R., Lohaus, R., Srinivasan, S., Dang, K.K. & Burch, C.L. 2006. Sexual reproduction selects for robustness and negative epistasis in artificial gene networks. *Nature* **440**: 87–90.

Bedau, M.A. & Packard, N.H. 2003. Evolution of evolvability via adaptation of mutation rates. *Biosystems* **69**: 143–162.

Bergman, A. & Siegal, M.L. 2003. Evolutionary capacitance as a general feature of complex gene networks. *Nature* **424**: 549–552.

Bloom, J.D., Labthavikul, S.T., Otey, C.R. & Arnold, F.H. 2006. Protein stability promotes evolvability. *Proc. Natl Acad. Sci. USA* **103**: 5869–5874.

Blount, Z.D., Borland, C.Z. & Lenski, R.E. 2008. Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **105**: 7899–7906.

Burch, C.L. & Chao, L. 2000. Evolvability of an RNA virus is determined by its mutational neighbourhood. *Nature* **406**: 625–628.

Cowperthwaite, M.C. & Meyers, L.A. 2007. How mutational networks shape evolution: lessons from RNA models. *Annu. Rev. Ecol. Evol. Syst.* **38**: 203–230.

Crombach, A. & Hogeweg, P. 2008. Evolution of evolvability in gene regulatory networks. *Plos Comput. Biol.* **4**: e1000112.

Dichtel-Danjoy, M.L. & Felix, M.A. 2004. Phenotypic neighborhood and micro-evolvability. *Trends Genet.* **20**: 268–276.

Draghi, J. & Wagner, G.R. 2008. Evolution of evolvability in a developmental model. *Evolution* **62**: 301–315.

Earl, D.J. & Deem, M.W. 2004. Evolvability is a selectable trait. *Proc. Natl Acad. Sci. USA* **101**: 11531–11536.

Gilchrist, G.W. & Lee, C.E. 2007. All stressed out and nowhere to go: does evolvability limit adaptation in invasive species? *Genetica* **129**: 127–132.

Goddard, M.R., Charles, H., Godfray, J. & Burt, A. 2005. Sex increases the efficacy of natural selection in experimental yeast populations. *Nature* **434**: 636–640.

Jeong, H., Tombor, B., Albert, R., Oltvai, Z.N. & Barabasi, A.L. 2000. The large-scale organization of metabolic networks. *Nature* **407**: 651–654.

Jones, A.G., Arnold, S.J. & Burger, R. 2007. The mutation matrix and the evolution of evolvability. *Evolution* **61**: 727–745.

Kashtan, N., Noor, E. & Alon, U. 2007. Varying environments can speed up evolution. *Proc. Natl Acad. Sci. USA* **104**: 13711–13716.

Kauffman, S.A. 1993. *The Origins of Order: Self Organization and Selection in Evolution*. Oxford University Press, New York.

Kawecki, T.J. 2000. The evolution of genetic canalization under fluctuating selection. *Evolution* **54**: 1–12.

Khersonsky, O., Roodveldt, C. & Tawfik, D.S. 2006. Enzyme promiscuity: evolutionary and mechanistic aspects. *Curr. Opin. Chem. Biol.* **10**: 498–508.

Kimura, M. 1967. On evolutionary adjustment of spontaneous mutation rates. *Genet. Res.* **9**: 23.

Kirschner, M. & Gerhart, J. 1998. Evolvability. *Proc. Natl Acad. Sci. USA* **95**: 8420–8427.

Kirschner, M. & Gerhart, J. 2005. *The Plausibility of Life Resolving Darwin's Dilemma*. Yale University Press, New Haven, CT.

Le Rouzic, A. & Carlborg, O. 2008. Evolutionary potential of hidden genetic variation. *Trends Ecol. Evol.* **23**: 33–37.

Lehner, B. 2008. Selection to minimise noise in living systems and its implications for the evolution of gene expression. *Mol. Syst. Biol.* **4**: 170.

Lenski, R.E., Barrick, J.E. & Ofria, C. 2006. Balancing robustness and evolvability. *Plos Biol.* **4**: 2190–2192.

Lynch, M. 2007. Colloquium papers: the frailty of adaptive hypotheses for the origins of organismal complexity. *Proc. Natl Acad. Sci. USA* **104**(Suppl. 1): 8597–8604.

Masel, J. 2005. Evolutionary capacitance may be favored by natural selection. *Genetics* **170**: 1359–1371.

Masel, J. 2006. Cryptic genetic variation is enriched for potential adaptations. *Genetics* **172**: 1985–1991.

Meyers, L.A., Ancel, F.D. & Lachmann, M. 2005. Evolution of genetic potential. *Plos Comput. Biol.* **1**: 236–243.

McBride, R.C., Ogbunugafor, C.B. & Turner, P.E. 2008. Robustness promotes evolvability of thermotolerance in an RNA viurs. *BMC Evol. Biol.* **8**: 231.

O'Loughlin, T.L., Patrick, W.M. & Matsumura, I. 2006. Natural history as a predictor of protein evolvability. *Protein Eng. Des. Sel.* **19**: 439–442.
Otto, S.P. & Barton, N.H. 1997. The evolution of recombination: removing the limits to natural selection. *Genetics* **147**: 879–906.
Pepper, J.W. 2003. The evolution of evolvability in genetic linkage patterns. *Biosystems* **69**: 115–126.
Pigliucci, M. 2007. Do we need an extended evolutionary synthesis? *Evolution* **61**: 2743–2749.
Pigliucci, M. 2008. Opinion – is evolvability evolvable? *Nat. Rev. Genet.* **9**: 75–82.
Plotkin, J.B. & Dushoff, J. 2003. Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. *Proc. Natl Acad. Sci. USA* **100**: 7152–7157.
Poole, A.M., Phillips, M.J. & Penny, D. 2003. Prokaryote and eukaryote evolvability. *Biosystems* **69**: 163–185.
Quayle, A.P. & Bullock, S. 2006. Modelling the evolution of genetic regulatory networks. *J. Theor. Biol.* **238**: 737–753.
Radman, M., Matic, I. & Taddei, F. 1999. Evolution of evolvability. *Ann. NY Acad. Sci.* **870**: 146–155.
Rutherford, S.L. & Lindquist, S. 1998. Hsp90 as a capacitor for morphological evolution. *Nature* **396**: 336–342.
Salazar-Ciudad, I. 2006. Developmental constraints vs. variational properties: how pattern formation can help to understand evolution and development. *J. Exp. Zool. Part B* **306B**: 107–125.
Salazar-Ciudad, I. 2007. On the origins of morphological variation, canalization, robustness, and evolvability. *Integr. Comp. Biol.* **47**: 390–400.
Schuster, P., Fontana, W., Stadler, P.F. & Hofacker, I.L. 1994. From sequences to shapes and back: a case study in RNA secondary structures. *Proc. Biol. Sci.* **255**: 279–284.
Siegal, M.L. & Bergman, A. 2002. Waddington's canalization revisited: developmental stability and evolution. *Proc. Natl Acad. Sci. USA* **99**: 10528–10532.
Siegal, M.L., Promislow, D.E.L. & Bergman, A. 2007. Functional and evolutionary inference in gene networks: does topology matter? *Genetica* **129**: 83–103.
de Silva, E. & Stumpf, M.P.H. 2005. Complex networks and simple models in biology. *J. R. Soc. Interface* **2**: 419–430.
Sniegowski, P.D. & Murphy, H.A. 2006. Evolvability. *Curr. Biol.* **16**: R831–R834.
Sniegowski, P.D., Gerrish, P.J., Johnson, T. & Shaver, A. 2000. The evolution of mutation rates: separating causes from consequences. *Bioessays* **22**: 1057–1066.
Tanay, A., Regev, A. & Shamir, R. 2005. Conservation and evolvability in regulatory networks: the evolution of ribosomal regulation in yeast. *Proc. Natl Acad. Sci. USA* **102**: 7203–7208.
Tenaillon, O., Taddei, F., Radman, M. & Matic, I. 2001. Second-order selection in bacterial evolution: selection acting on mutation and recombination rates in the course of adaptation. *Res. Microbiol.* **152**: 11–16.
de Visser, J.A.G.M. 2002. The fate of microbial mutators. *Microbiol. SGM* **148**: 1247–1252.
de Visser, J.A.G.M., Hermisson, J., Wagner, G.P., Meyers, L.A., Bagheri, H.C., Blanchard, J.L., Chao, L., Cheverud, J.M., Elena, S.F., Fontana, W., Gibson, G., Hansen, T.F., Krakauer, D., Lewontin, R.C., Ofria, C., Rice, S.H., von Dassow, G., Wagner, A. & Whitlock, M.C. 2003. Perspective: evolution and detection of genetic robustness. *Evolution* **57**: 1959–1972.
Wagner, A. 1996. Does evolutionary plasticity evolve? *Evolution* **50**: 1008–1023.
Wagner, A. 2005. *Robustness and Evolvability in Living Systems*. Princeton University Press, Princeton, NJ.
Wagner, A. 2008. Robustness and evolvability: a paradox resolved. *Proc. Biol. Sci.* **275**: 91–100.
Wagner, G.P. & Altenberg, L. 1996. Perspective: complex adaptations and the evolution of evolvability. *Evolution* **50**: 967–976.
Wagner, G.P., Chiu, C.H. & Hansen, T.F. 1999. Is Hsp90 a regulator of evolvability? *J. Exp. Zool.* **285**: 116–118.

Appendix 1 – exploring variation in model parameters

We first addressed the influence of population size and the period of environmental change. Table 1 shows the mean increase in E_{25} after 30 epochs of evolution and the significance of this difference. Because distributions of E_{25} are typically skewed, we apply the nonparametric Wilcoxon signed-rank test to the pairs of measurements. These results confirm that evolvability increases in populations of at least 200 individuals, and suggest that larger populations generally enhance this increase. Evolvability also evolves for a range of period lengths, although we cannot judge the interaction of period and population size. Kawecki (2000) suggests that very small period lengths can lead to the evolution of canalization, and consequently a decrease in evolvability. However, we observed a significant increase in evolvability when $P = 30$, suggesting that our results are robust to changes in period.

Table 2 shows the robustness of our result to changes in mutation rates. Evolvability significantly increases with the exception of two cases with very high mutation rates. Evolvability also evolves when network topology cannot evolve, i.e. $\mu_A = 0$. Table 3 establishes that our main result is not sensitive to changes in m, the size of mutational changes to connection weights.

Finally, we confirm that evolvability can also evolve in larger networks. As K increases, computational require-

Table 1 The influence of population size and epoch length on the evolution of evolvability.

Epoch period (P)	Population size (N)			
	100	200	1000	10 000
30	0.02 (ns)	0.077 (++)	0.151 (+++)	0.248 (+++)
100	0.059 (+)	0.142 (+++)	0.205 (+++)	0.294 (+++)
300	0.015 (ns)	0.107 (++)	0.202 (+++)	0.213 (+++)
1000	0.025 (ns)	0.051 (+)	0.079 (+)	0.106 (+++)

Simulations were performed with 80 replicates for $\mu = 0.00317$, $\mu_A = 3.17 \times 10^{-5}$, $m = 0.2$, $s = 1$ and N and period as indicated. Numbers indicate the mean increase in E_{25} from the initial genotype to the end of epoch 30. Symbols indicate level of significance of a Wilcoxon signed-rank test: ns, $P \geq 0.01$; +, $0.01 \geq P > 10^{-4}$; ++, $10^{-4} \geq P > 10^{-8}$; +++, $P \leq 10^{-8}$.

Table 2 The influence of mutation rates on the evolution of evolvability.

	Weight mutation rate (μ)		
	7.92×10^{-4}	3.17×10^{-3}	1.27×10^{-2}
Topology mutation rate (μ_A)			
0	0.125 (++)	0.218 (+++)	0.214 (++)
3.17×10^{-5}	0.185 (+++)	0.205 (+++)	0.233 (+++)
3.17×10^{-4}	0.117 (++)	0.145 (+++)	0.149 (+++)
3.17×10^{-3}	0.053 (+)	0.028 (ns)	0.025 (ns)

Weight mutations change an existing connection by a maximum of m; topology mutations insert and delete connections. Simulations were performed with 80 replicates for $m = 0.2$, $N = 1000$, $P = 100$ and $s = 1$. Numbers indicate the mean increase in E_{25} from the initial genotype to the end of epoch 30. Symbols indicate level of significance of a Wilcoxon signed-rank test: NS, $P \geq 0.01$; +, $0.01 \geq P > 10^{-4}$; ++, $10^{-4} \geq P > 10^{-8}$; +++, $P \leq 10^{-8}$.

Table 3 The evolution of evolvability is robust to changes in μ and the size of weight mutations, m.

	Weight mutation rate (μ)		
	1.58×10^{-3}	3.17×10^{-3}	6.34×10^{-3}
Mutation size (m)			
0.1	0.123 (+++)	0.154 (+++)	0.203 (+++)
0.2	0.19 (+++)	0.205 (+++)	0.23 (+++)
0.5	0.22 (+++)	0.255 (+++)	0.186 (+++)

Simulations were performed with 80 replicates for $\mu_A = 0.0000317$, $N = 1000$, $P = 100$ and $s = 1$. +++ indicates a value of $P \leq 10^{-8}$ for a Wilcoxon signed-rank test, whereas the number in each cell is the mean increase in E_{25} from the initial genotype to the end of epoch 30.

ments quickly become prohibitive – network complexity increases geometrically with K, and the number of environments to assay for evolvability increases exponentially. We therefore only examined networks with 12 and 20 nodes for a single set of parameters. We also limit the number of iterations during a network's development to 30 for 12 nodes and 100 for 20 nodes. In theory, 12-node networks could take 4096 iterations to converge, and 20-node networks might require over one million. However, raising these limits increased the fraction of genotypes with stable phenotypes by negligible amounts, suggesting that these limits are a very close approximation. To facilitate comparisons across network sizes, we maintained a consistent per genome mutation rate. For $K = 12$, $N = 1000$, $\mu = 3.526 \times 10^{-4}$, $\mu_A = 3.526 \times 10^{-6}$, $P = 100$, $m = 0.2$, $s = 1$ and reproduction was asexual. For $K = 20$, $\mu = 1.27 \times 10^{-4}$, $\mu_A = 1.27 \times 10^{-6}$ and other parameters were the same.

For $K = 12$, E_{25} increases over 60 epochs from an average of 0.075–0.170, which was highly significant (Wilcoxon signed-rank test, two-tailed, 60 replicates, $V = 97$, $P = 1.765 \times 10^{-9}$). For $K = 20$, E_{25} increases over the same period from an average of 0.052–0.110, which was also significant (Wilcoxon signed-rank test, two-tailed, 60 replicates, $V = 100$, $P = 2.022 \times 10^{-9}$). Note that the E_{25} of the initial, random genotypes declines as K increases, perhaps reflecting the decrease in the per site mutation rate with increasing number of sites. Despite this decrease in initial E_{25}, the increase in evolvability remains strongly significant.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1 E_T over a range of T for five genotypes.

Figure S2 Spearman's rank correlations of E_T and epoch number.

Figure S3 The dynamics of adaptation in a typical population.

Figure S4 The relationship between E_{25} and beneficial mutations in evolving populations.

Figure S5 The number of existing connections, c, in a network is plotted against E_{25} for a set of 1000 randomly generated, stable networks.

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