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Phenotypic robustness can increase phenotypic variability after non-genetic perturbations in gene regulatory circuits

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Abstract

Non-genetic perturbations, such as environmental change or developmental noise, can induce novel phenotypes. If an induced phenotype appears recurrently and confers a fitness advantage, selection may promote its genetic stabilization. Non-genetic perturbations can thus initiate evolutionary innovation. Genetic variation that is not usually phenotypically visible may play an important role in this process. Populations under stabilizing selection on a phenotype that is robust to mutations can accumulate such variation. After non-genetic perturbations, this variation can produce new phenotypes. We here study the relationship between a phenotype’s mutational robustness and a population’s potential to generate novel phenotypic variation. To this end, we use a well-studied model of transcriptional regulation circuits that are important in many evolutionary innovations. We find that phenotypic robustness promotes phenotypic variability in response to non-genetic perturbations, but not in response to mutation. Our work suggests that non-genetic perturbations may initiate innovation more frequently in mutationally robust gene expression traits.
Introduction

Two main perspectives exist about the origin of evolutionary innovations. The orthodox “genotype-first” perspective emphasizes the role of mutations in the production of new phenotypes. In this perspective, mutations produce individuals with novel phenotypes whose frequency in a population may increase through natural selection. The heterodox “phenotype-first” perspective (West-Eberhard, 1989, 2003; Hall, 2001; Price et al., 2003; Palmer, 2004; Newman et al., 2006; Pigliucci et al., 2006; Moczek, 2007; Gilbert & Epel, 2008) emphasizes the role of non-genetic perturbations, such as exposure to different temperatures, diets, or biotic interactions. Non-genetic perturbations also comprise fluctuations in an organism’s internal “microenvironment”, such as gene activity changes caused by noisy gene expression (McAdams & Arkin, 1997; Elowitz et al., 2002; Raj et al., 2010).

The phenotype-first perspective is based on the observation that organisms often have highly plastic phenotypes. That is, the same genotype has the potential to produce different phenotypes depending on non-genetic influences. A non-genetic perturbation can thus trigger a plastic phenotypic response in some individuals of a population. If the resulting novel phenotype provides a benefit to its carrier, it facilitates survival. Subsequently, selection may increase the frequency of those genotypes that produce the beneficial phenotype and of new or already existing genetic variants that exaggerate, refine, or “stabilize” this phenotype by making it independent of non-genetic factors. This process requires that the new phenotype appears recurrently, and hence, that the non-genetic perturbations that induced it either persist for many generations (Kim, 2007; Griswold & Masel, 2009) or that they produce a persistent epigenetic effect (Sollars et al., 2003; Gilbert & Epel, 2008). Waddington coined the term genetic assimilation for the stabilization of traits induced by non-genetic factors (Waddington, 1953).

Increasing amounts of evidence suggest that traits induced by non-genetic factors are important for innovation (for dissenting opinions, see Orr, 1999; de Jong & Crozier, 2003). First, theoretical work shows that assimilation can occur under broad conditions (G.P. Wagner et al., 1997; Rice, 1998; Masel, 2004; Ciliberti et al., 2007b; Lande, 2009; Espinosa-Soto et al., 2011). Second, laboratory evolution experiments show that assimilation does occur (Waddington, 1953, 1956; Rutherford & Lindquist, 1998; Suzuki & Nijhout, 2006; Eldar et al., 2009). Third, studies in natural populations suggest that genetic assimilation of traits induced by non-genetic factors is not rare (West-Eberhard, 2003; Pigliucci & Murren, 2003; Palmer, 2004; Aubret & Shine, 2009). For example, taxa with genetically determined dextral or sinistral morphologies are frequently
derived from taxa in which the direction of the asymmetry is not genetically fixed, but where it is a plastic response (Palmer, 1996, 2004). This occurs for many traits, such as the side on which the eye occurs in flat fishes (Pleuronectiformes), and the side of the larger first claw in decapods (Thalassinidea) (Palmer, 1996). Transitions like these indicate genetic assimilation of a direction of asymmetry originally induced by non-heritable factors. More generally, traits where fixed differences among closely related species are mirrored by plastic variation within populations are good candidates for genetic assimilation. For example, amphibian traits, such as gut morphology (Ledon-Rettig et al., 2008), limb length and snout length (Gomez-Mestre & Buchholz, 2006), follow this pattern.

A system is robust to genetic or non-genetic perturbations if its phenotype does not change when perturbed. Mutational robustness and robustness to non-genetic perturbations are correlated with one another in many cases (Rutherford & Lindquist, 1998; Ancel & Fontana, 2000; Meiklejohn & Hartl, 2002; de Visser et al., 2003; Ciliberti et al., 2007b; Proulx et al., 2007; Lehner, 2010), although exceptions exist (Cooper et al., 2006; Masel & Siegal, 2009; Fraser & Schadt, 2010). The ability to produce evolutionary innovation is linked to the robustness of a biological system (Ancel & Fontana, 2000; Wagner, 2005; Ciliberti et al., 2007a; Wagner, 2008b; Draghi & G.P. Wagner, 2009). For two reasons, robustness might seem to hamper innovation. First, a mutationally robust system produces less phenotypic variation in response to mutations. It may thus not facilitate the genotype-first scenario (Ciliberti et al., 2007a; Draghi & G.P. Wagner, 2009). Second, a system robust to non-genetic factors shows little phenotypic plasticity. Thus, it may not support innovation under the phenotype-first scenario. However, the role of robustness in innovation is subtler than it seems. This becomes evident when one considers how genotypes and their phenotypes are organized in a space of genotypes.

Genotypes exist in a vast space of possible genotypes. Two genotypes are neighbors in this space if one can be transformed into the other by a single mutation. The distribution of phenotypes in genotype space shows some qualitative similarities for different kinds of systems, from RNA and protein molecules to metabolic networks and transcriptional regulation circuits. First, large sets of genotypes produce the same phenotype. Each of these sets can be traversed through single mutation steps that leave the phenotype unchanged. Such a set is also referred to as a neutral network or genotype network (Schuster et al., 1994). Second, mutations of genotypes that lie in different regions of a genotype network can create very different novel phenotypes (Lipman & Wilbur, 1991; Schuster et al., 1994; Schultes & Bartel, 2000; Ciliberti et al., 2007a; Wagner, 2008a; Ferrada & Wagner, 2008; Rodrigues & Wagner, 2009).
To understand how mutational robustness relates to a system’s ability to produce evolutionary innovations, it is useful to distinguish between the mutational robustness of a genotype and that of a phenotype. A genotype $G_1$ is mutationally more robust than another genotype $G_2$, if $G_1$ is more likely to maintain the same phenotype than $G_2$ in response to mutation. By extension, a phenotype $P_1$ is mutationally more robust than $P_2$ if the genotypes that produce $P_1$ preserve $P_1$, on average, more often than the genotypes adopting $P_2$ preserve $P_2$ in response to mutations. Surprisingly, mutational phenotypic robustness can facilitate the production of novel RNA structure phenotypes (Wagner, 2008a). The reason is that genotypes with a more robust phenotype form larger genotype networks and have, on average, more neighbors with the same phenotype. A population of such genotypes encounters relatively few deleterious mutations that would slow its diversification and spreading through genotype space (while preserving its phenotype). The resulting higher genotypic diversity translates into greater phenotypic variability in response to mutations, even though every single genotype may have access to fewer novel phenotypes (Wagner, 2008b).

This mechanism, although corroborated for RNA and protein structural phenotypes (Wagner, 2008a; Ferrada & Wagner, 2008) may not lead to increased phenotypic variability in all systems. The reason is that it depends on how many different and unique phenotypes the neighborhood of different genotypes contains, and on how rapidly populations can spread through a genotype network. In other words, it depends on the organization of genotype networks in genotype space, which may differ among different system classes.

The above considerations pertain to phenotypic variability in response to mutations. Since robustness to mutations and to non-genetic factors are often positively correlated (Rutherford & Lindquist, 1998; Ancel & Fontana, 2000; Meiklejohn & Hartl, 2002; de Visser et al., 2003; Ciliberti et al., 2007b; Lehner, 2010), one might think that phenotypic variability in response to non-genetic perturbations may behave similarly. However, we show that this is not necessarily so for transcriptional regulation circuits. Such circuits direct the production of specific gene activity patterns at particular times and places in the developing organism. Changes in the expression of their genes are involved in many evolutionary innovations (Davidson & Erwin, 2006; Shubin et al., 2009). We study a generic computational model of transcriptional regulation in which the genotypes correspond to the cis-regulatory interactions in a transcriptional circuit. The phenotypes correspond to the gene activity pattern a circuit produces.

For this system, we have shown elsewhere that the organization of genotype space favors the evolution of new adaptive traits through a phenotype-first scenario. For example, we found that genotypes that can produce a new gene activity phenotype $P$ after non-genetic perturbations have easy mutational access to genotypes
where non-genetic perturbations are no longer necessary to produce \( P \). Thus, new phenotypes induced by non-genetic perturbations can easily undergo genetic assimilation (Espinosa-Soto et al., 2011). Because our previous results already show that the structure of gene circuit genotype space promotes assimilation, we here focus on the production of novel phenotypes. Specifically, we address how phenotypic robustness affects the potential of non-genetic perturbations and mutations to produce new phenotypes. We show that high phenotypic robustness to mutations increases the number of novel expression phenotypes that a population can produce in response to non-genetic perturbations. Thus, phenotypic robustness to mutation facilitates innovation under the phenotype-first scenario. It does so by allowing the accumulation of genetic variation that is not observed phenotypically under typical conditions, but that may be exposed after non-genetic perturbations (de Visser et al., 2003; Masel, 2006; Masel & Siegal, 2009).

**Methods**

**Model**

The model represents a regulatory circuit of \( N \) genes, where each gene’s activity is regulated by other genes in the circuit. The circuit’s genotype is defined by a real-valued matrix \( A = (a_{ij}) \), in which non-zero elements represent regulatory interactions between genes (Fig. 1a). An interaction \( (a_{ij} \neq 0) \) means that the activity of gene \( j \) can either have a positive \( (a_{ij} > 0) \) or a negative \( (a_{ij} < 0) \) effect on the activity of gene \( i \). We use \( m \) to refer to the number of interactions in a given circuit, and \( c \) to its interaction density, i.e. to the number of interactions \( m \) divided by the maximum possible number of interactions \( N^2 \). A vector \( s_t = (s_t^{(1)}, ..., s_t^{(N)}) \) describes the activity state of the circuit at time \( t \).

The activity of the genes in the circuit changes according to the difference equation

\[
s_{t+\tau}^{(i)} = \sigma \left[ \sum_{j=1}^{N} a_{ij} s_t^{(j)} \right]
\]

where \( \sigma(x) \) equals -1 when \( x < 0 \), it equals 1 when \( x > 0 \), and it equals 0 when \( x = 0 \).

Variants of this model have proven useful for studying the evolution of robustness in gene regulatory circuits (Wagner, 1996; Siegal & Bergman, 2002; Ciliberti et al., 2007b; Martin & Wagner, 2008), the effect of recombination on the production of negative epistasis (Azevedo et al., 2006; Martin & Wagner, 2009), the evolution of modularity in gene circuits (Espinosa-Soto & Wagner, 2010) and the evolution of new gene activity patterns (Kimbrell & Holt, 2007; Ciliberti et al., 2007a; Draghi & G.P. Wagner, 2009).
We consider asexual, haploid circuits that start their dynamics from a particular initial gene expression state $s_0$. One can view this initial state as being specified by factors external to the circuit, be they environmental factors, signals from adjacent cells, maternal regulators, or any genes “upstream” of the circuit. The phenotype is the stable (fixed-point) gene activity pattern $s_\infty$ that a circuit attains when starting from $s_0$. Throughout our work, we disregard circuits that do not produce fixed-point equilibrium states or that produced phenotypes in which the activity of a gene is equal to zero (neither active nor inactive), as in previous research (Ciliberti et al., 2007b). We consider circuits that attain the same $s_\infty$ as equal with respect to their gene expression phenotype. Under this assumption, a mutation that transforms two such circuits into one another would be neutral with respect to this phenotype (Fig. 1b).

**Determination of 1-mutant neighborhoods**

In several of our analyses, we explored properties of the circuits that differ from a reference circuit genotype $G$ by one single mutation. In our approach, a mutation affects a single regulatory interaction between two genes. That is, it changes a single entry $a_{ij}$ in the matrix $A$ of $G$. Our underlying assumption is that mutations occur in regulatory regions, where mutations in one enhancer often have no effect in other enhancers (Wray, 2007; Prud’homme et al., 2007). For simplicity, we also assume that every transcription factor binds to a different enhancer. We considered two kinds of single mutation for each entry $a_{ij}$ in the matrix $A$ of $G$: i) if $a_{ij} = 0$ we considered one mutant where $a_{ij} < 0$, and another in which $a_{ij} > 0$; ii) if $a_{ij} \neq 0$ we considered one mutant in which an interaction is lost ($a_{ij} = 0$), and another mutant in which we change the value of $a_{ij}$ while keeping its sign unchanged. Among all the possible variants in the one-mutation neighborhood of a circuit, we allowed exclusively those that maintained the number of interactions within an interval $[m_-, m_+]$, thus keeping interaction density at a value close to $c$. Throughout this manuscript, $m_+ - m_- = 5$. Whenever a new non-zero value was required for a given $a_{ij}$, we chose a normally distributed (N(0,1)) pseudorandom number, and forced its sign if needed. We defined the robustness to mutations of a genotype $G$ as the fraction of $G$’s 1-mutant neighbors that produce the same phenotype as $G$ when their dynamics start from the initial state $s_0$. To assess the phenotypes that $G$ can access through mutations, we registered and counted all the different phenotypes produced by the set of single mutant circuits that neighbor the reference circuit $G$. The approach is readily extended to entire populations. Whenever we applied it to entire populations, we counted phenotypes that occurred in the neighborhood of two or more circuits only once.
Evolving populations

For the model we use, a given pair of initial and final expression states \((s_0, s^\text{opt}_\infty)\) is representative of all pairs with the same fraction \(d\) of individual genes’ expression values that differ between \(s_0\) and \(s^\text{opt}_\infty\) (Ciliberti et al., 2007b). For a pre-specified \(d\), we thus chose an arbitrary such pair, and followed previously established procedures (Ciliberti et al., 2007b) to identify a circuit genotype \(G\) that is able to drive the system from \(s_0\) to \(s^\text{opt}_\infty\). The regulatory interactions in the initial genotype \(G\) are real numbers sampled from a normal distribution with mean 0 and standard deviation 1, i.e., an N(0,1) distribution. After having identified one such genotype \(G\), we created a population of 200 copies of it, and subjected this population to repeated cycles (“generations”) of mutations (with a probability of mutation of \(\mu = 0.5\) per circuit), and strong stabilizing selection on \(s^\text{opt}_\infty\). To mutate a circuit, we chose one of the circuit’s 1-mutation neighbors at random (see above).

Throughout, we interpret a circuit’s “fitness” as a survival probability. We followed the regulatory dynamics of each gene circuit with \(s_0\) as initial condition. We assigned circuits that attained an equilibrium state \(s_\infty\) that differed from \(s^\text{opt}_\infty\) in the activity state of \(k\) \((0 \leq k \leq N)\) genes a fitness equal to \((1 - k/N)^5\), which ensures a steep decrease in survival probability even for small deviations from \(s^\text{opt}_\infty\). Thus, \(s^\text{opt}_\infty\) represents a pre-determined optimal gene expression state, upon which stabilizing selection acts. Each generation, we constructed a new population by sampling individuals with replacement from the previous generation, and subjecting copies of them to mutation with a probability \(\mu\). We kept each of these new individuals with a probability equal to its fitness, and continued sampling until the newly generated population had 200 members. For all the populations we study, we let the initial population of identical genotypes evolve for \(10^4\) generations under selection for \(s^\text{opt}_\infty\), before collecting any simulation data. This allows the population to erase any traces of the initial genotype, and to reach a plateau where phenotypic variability in response to either mutations or non-genetic perturbations varies little across generations.

In a distinct set of simulations, we explored how exposure to non-genetic perturbations in this preliminary period of stabilizing selection could affect a population’s phenotypic variability. In these simulations, each circuit in a population was subject to non-genetic perturbations with probability \(\beta\) every generation. For each circuit undergoing non-genetic perturbations we set the initial state of one of the circuit’s genes picked at random to a random activity state (either \(-1\) or \(1\)).

We define the genotypic distance between two circuits as the minimum number of mutations needed to
transform one circuit into the other, normalized by the maximally possible number of such mutations. The minimum number of mutations that set two genotypes apart is the number of differences between their matrices $A$. Whenever a pair of corresponding regulatory interactions $a_{ij}$ are both different from zero and have opposite signs, we count the difference twice. The reason is that in our mutation procedure, changing the sign of a regulatory interaction $a_{ij}$ requires at least two mutations. The maximal number of mutations between circuits with the same number of regulatory interactions is given by the sum of the number of interactions of both circuits.

**Implementation of noise**

We emulated the perturbations produced by noise in two complementary ways. Firstly, we changed the activity state of single genes in the initial state $s_0$ for each gene in a circuit, and we determined the new phenotypes $s_{new}^{\infty}$ that resulted from such change.

Secondly, we perturbed the developmental dynamics (‘noisy dynamics’) as follows: For each circuit in a population, we generated $5N$ dynamic trajectories, each of which started from $s_0$. For each of these trajectories, and for each step of the regulatory dynamics, we perturbed the activity of a randomly picked gene with a probability of 0.5. We then followed each trajectory until an activity pattern $s$ had consecutively repeated itself, and labeled this pattern as $s_{\infty}$. We then counted the number of different fixed-point equilibrium states that each circuit could attain in these $5N$ trajectories.

**Random sampling of genotypes in genotype networks**

In order to sample properties of a given genotype network uniformly, we performed a random mutational walk restricted to this genotype network, that is, to circuits that attain a given $s_{\infty}^{opt}$ from the initial state $s_0$. We then examined properties of genotypes every $n$ steps of this random walk, where $n$ equaled 5 times the upper limit $m_+$ of the number of interactions in the circuit. This sporadic sampling serves to erase correlations in genotypes along this random walk.

**Results**

**Genotype networks of gene expression phenotypes have different sizes**

For our model, most or all genotypes that produce the same phenotype form large connected genotype networks (Ciliberti et al., 2007a,b). The size of any one phenotype’s genotype network depends only on the fraction $d$ of genes whose expression state differs between the initial state $s_0$, and the steady state activity
phenotype $s_{\infty}^{opt}$ (Ciliberti et al., 2007b). Specifically, phenotypes where these two states (regardless of their actual expression values) are more similar have larger genotype networks (Fig. S1 in the Supporting Information). One can view regulatory circuits as devices that compute an expression state $s_{\infty}^{opt}$ from the initial state $s_0$. From this perspective, a larger number of gene expression differences between these states means that the computation becomes increasingly difficult, in the sense that fewer genotypes can perform it.

We examined in our model the relationship between the size of a phenotype $P$’s genotype network and the robustness of circuits with this phenotype $P$ to mutations. To this end, we pursued the following procedure for genotype networks of different sizes (different $d$). We uniformly sampled $10^6$ genotypes from a genotype network and determined their mean robustness to mutations, that is, the mean fraction of their neighbors with the same phenotype. For all examined cases, the average mutational robustness (i.e. phenotypic robustness) is higher for genotypes on larger genotype networks when we control for the number $N$ of genes in a circuit and the interaction density $c$ (Fig. S2). Thus, phenotypic robustness to mutations increases with genotype network size, just as for RNA (Wagner, 2008a). Therefore, we can simply use $1 - d$ as a proxy for genotype network size and phenotypic robustness to mutations.

**Phenotypic robustness to mutations facilitates phenotypic variability in response to noise**

In this paper, we are concerned with the production of new steady-state gene expression patterns $s_{\infty}^{new}$ that are different from $s_{\infty}^{opt}$. We refer to such activity patterns as new phenotypes. They could result from mutations that change regulatory interactions in a circuit. They could also result from non-genetic perturbations (Fig. 1c). We here consider two kinds of non-genetic perturbations, noise in a cell’s internal environment, and change in the organism’s (external) environment. Both kinds can induce dramatic gene expression changes in organisms ranging from bacteria to metazoans (Elowitz et al., 2002; Raj et al., 2010; Snell-Rood et al., 2010). We first focus on noise, which includes stochastic changes in protein or mRNA copy numbers in a cell, and which can cause phenotypic heterogeneity in clonal populations (McAdams & Arkin, 1997; Elowitz et al., 2002; Raj et al., 2010). Such noise may affect the activity or expression of circuit genes at a given time, which may alter a circuit’s gene expression dynamics, and lead to a new steady-state activity pattern $s_{\infty}^{new}$.

We emulated the perturbations produced by noise in two complementary ways. First, we perturbed the activity state of single genes in the initial state $s_0$. Secondly, we randomly perturbed the dynamic trajectory from $s_0$ to $s_{\infty}$ (‘noisy dynamics’; see details of both implementations in Methods).
We asked how the mutational robustness of a gene expression phenotype affects the number of new phenotypes that these two kinds of noise can produce in populations of evolving circuits. This number reflects the potential of a population to produce phenotypic variation through noise. We evolved populations of 200 circuits under stabilizing selection on a given gene expression state $s_{\infty}^{opt}$, as described in Methods. We found that noise can produce more new and different phenotypes in populations evolving on large genotype networks. Fig. 2 shows pertinent data for circuits with $N = 20$ genes and an interaction density $c \approx 0.2$. These observations also hold if we vary the numbers of genes and regulatory interactions in a circuit (Figs. S3,4), with a single exception for perturbations in $s_0$ when the number of regulatory interactions is very low (Fig. S3d).

**Populations with more robust phenotypes harbor more diverse genotypes**

Increased genotypic diversity in populations evolving in large genotype networks might aid in producing increased phenotypic variability, as discussed in the Introduction. We next asked whether this mechanism may apply to our system.

As a measure of a population’s genotypic diversity, we estimated the mean pairwise circuit genetic distance, as well as its maximum, in each of 500 populations evolved under stabilizing selection on a phenotype $s_{\infty}^{opt}$ (see Methods). We did so for two classes of populations that differ in the robustness of their phenotypes, and found that the mean genotypic distance is significantly higher for populations with a robust phenotype. The same holds also for the maximum genotypic distance. These observations are not sensitive to the number of genes and interactions in a circuit (Table S1). Thus, populations with a robust phenotype are genetically more diverse than populations with a less robust phenotype. These observations hint that the higher genetic diversity of populations with robust phenotypes may be exposed as phenotypic variability in response to noise.

**Phenotypic robustness does not facilitate phenotypic variability caused by mutations**

We next asked whether phenotypic robustness also facilitates phenotypic variability in response to mutations for the regulatory circuits we study. We again studied populations of circuits evolved under stabilizing selection on a phenotype $s_{\infty}^{opt}$. In such populations, we determined the number of unique new gene activity phenotypes in the population’s 1-mutation neighborhood (see Methods). This number of unique phenotypes is a measure of the population’s phenotypic variability in response to mutations. It thus reflects a population’s potential to produce phenotypic variants through mutation.
We found that populations with a highly robust phenotype show lower phenotypic variability in response to mutations. This holds despite their somewhat higher genotypic diversity (Table S1, discussed above). Fig. 3 shows pertinent data for circuits with 20 genes and interaction density $c \approx 0.2$. The same behavior holds for populations of circuits with different number of genes and different interaction densities (Fig. S5). In sum, robustness of a phenotype to mutations impairs phenotypic variability to mutation, as opposed to what we saw for variability in response to noise.

Our results suggest that a phenotype’s mutational robustness promotes phenotypic variability in response to noise, but hinders such variability in response to mutations. This may seem surprising, because robustness to mutations increases with robustness to noise for individual circuits (Ciliberti et al., 2007b). One might thus think that phenotypic variability also behaves similarly in response to these perturbations. However, robustness to mutations explains less than 25 percent of the variance in robustness to noise, as a new statistical analyses of our previously published data (Ciliberti et al., 2007b) demonstrates (results not shown). Thus phenotypic variability in response to noise and to mutation are only weakly coupled.

With these observations in mind, we analyzed the phenotypic variability in response to noise and mutations of individual circuits in populations evolving on different genotype networks (Table S2). After having obtained this data, we compared the mean number of new phenotypes that mutations or noise could produce from circuits in populations with different levels of phenotypic robustness (Table S3). We found that phenotypic variability in response to gene expression noise decreases less with phenotypic robustness to mutations than phenotypic variability to mutations (Table S3). It may even increase with phenotypic robustness. These observations suggest that the increased genotypic diversity attained on larger genotype networks is insufficient to compensate for the reduction in variability in response to mutations. It is, however, sufficient to compensate for the smaller (or null) reduction in phenotypic variability in response to noise in gene expression.

**Phenotypic robustness increases phenotypic variability after environmental change**

Thus far, we focused mostly on phenotypic variability in response to small, random non-genetic perturbations, such as single gene expression perturbations along a gene expression trajectory. For such perturbations, we found that phenotypic robustness favors phenotypic variability. We now turn to the question of what happens when a whole population is subject to the same non-genetic perturbation. In nature, this may occur because of environmental change outside the organism or colonization of a new habitat.
The environment can have two different roles in this context. The first is an inducing role, where the environment acts as an “agent of development” (West-Eberhard, 1989). In this role, it affects the phenotype produced from a genotype. In many cases, environmentally induced phenotypic change is linked to major changes in gene expression (Snell-Rood et al., 2010). The second role is an evaluating role, where the environment acts as an “agent of selection” (West-Eberhard, 1989). In anthropomorphic terms, the environment in this role distinguishes well-adapted from poorly adapted phenotypes.

Conveniently, our model allows us to study these roles independently. We model a change in the environment’s evaluation role as a change in the identity of the optimal phenotype \( s_{\infty}^{\text{opt}} \), for all circuits in the population. We model a change in the environment’s inducing role as a change in the initial state \( s_0 \) in the whole population. Such a change could occur, for example, through a signaling pathway that detects an environmental change, and that affects genes upstream of the circuit. Put differently, changes in \( s_0 \) reflect the environment’s effect on phenotype production, while changes in \( s_{\infty}^{\text{opt}} \) affect the survival probability of individuals, without inducing novel phenotypes. We note that other factors, such as mutations in upstream genes, might also lead to changes in \( s_0 \). Any one such change, however, would initially affect only one individual in a population, and not the whole population at the same time.

We first asked how an environmentally induced change in the initial gene activity pattern \( s_0 \) affects the number of different actual phenotypes that a population displays. We note that our populations may contain a few individuals with phenotypes different from the optimal phenotype \( s_{\infty}^{\text{opt}} \). The reason is that, in contrast to previous formulations (Ciliberti et al., 2007b), we here represent fitness as a continuous variable that depends on the similarity of a circuit’s phenotype \( s_{\infty} \) to \( s_{\infty}^{\text{opt}} \) (see Methods). We started out with a population evolved under stabilizing selection on an optimal expression phenotype \( s_{\infty}^{\text{opt}} \) and a given gene activity pattern \( s_0^{a} \) as initial condition. We then counted the number of phenotypes in the population, and compared it with the number of different phenotypes that the same population displays when \( s_0^{a} \) is replaced by a random gene activity pattern \( s_0^{b} \) as an initial condition. We found that phenotypic diversity increases after substitution of \( s_0^{a} \) with \( s_0^{b} \) (Figs. 4 and S6). In addition, the magnitude of this increment increases with phenotypic robustness (Fig. 4 and Table S4). This last observation is generally not sensitive to the number of genes and regulatory interactions in a circuit (Fig. S6). The single exception to these observations were circuits of very low interaction density \((N = 20; c \approx 0.1 ; \text{Fig. S6d})\), that also show other non-typical behaviors (Ciliberti et al., 2007b). Our results suggest that, after environmental change, observable phenotypic diversity increases to a larger extent in populations with a robust phenotype. We note that because the identity of \( s_{\infty} \) does not affect
the production of phenotypes, but only their viability, it is not appropriate to carry out an analogous analysis for changes in $s_{\infty}^{\text{opt}}$.

In earlier sections, we have shown that phenotypic robustness impedes phenotypic variability after mutations in populations evolving in a constant environment (Fig. 3). We next asked whether this also holds after a change in the inducing role of the environment. We started out, as in our last analysis, with populations of circuits evolved under stabilizing selection on an optimal expression phenotype $s_{\infty}^{\text{opt}}$, and with a given gene activity pattern $s_{0}^{a}$ as initial condition. Then, we changed the initial condition $s_{0}^{a}$ for all the circuits to a new random initial condition $s_{0}^{b}$, and allowed evolution to proceed. Before and after this change, we recorded the number of different phenotypes accessible from the population through mutations. Under the new condition the population effectively searches genotype space for optimal phenotypes. During this search, many variant circuits may not survive and be passed on to subsequent generations. Here, however, we do not focus on this search but on the effect on a population’s potential to produce phenotypic variation through mutation immediately after environmental change.

Before environmental change, populations with a robust phenotype have access to fewer phenotypic variants, just as in our previous observations (Fig. 3). Immediately after environmental change (at $t = 1$), the number of new phenotypes accessible through mutations increases, in a burst, in all populations. Importantly, this increase is higher in populations with a robust phenotype (Figs. 5a and S7). This means that phenotypic robustness facilitates the phenotypic variability caused by mutations, but only after environmental change. As in our analysis above, the only exception occurs when interaction density is very low (Fig. S7d).

We next asked whether the evaluation role of the environment has similar effects on mutational access to new phenotypes. To this end, we repeated the above analysis, but replaced, at $t = 1$, the optimal phenotype $s_{\infty}^{\text{opt},a}$ by a randomly chosen optimal $s_{\infty}^{\text{opt},b}$ (without changing $s_{0}$). We also observed a transient, albeit delayed and more gradual, increase in the number of phenotypes that are mutationally accessible. In this case, phenotypic variability after environmental change is lower for populations with a robust phenotype (Figs. 5b and S8). Thus, the inductive role of environment, but not its evaluative role, causes higher phenotypic variability in populations with robust phenotypes.

An open question is how mutation-accessible phenotypic variability changes when both the inductive and the evaluation roles of the environment change. This question is important because a change in the inductive role favors phenotypic variability to a larger extent in populations with a robust phenotype, whereas a change in the evaluative role particularly favors variability in populations with less robust phenotypes. Thus, a
combination of both effects could result in a negligible effect of phenotypic robustness on variability after environmental change. To answer this question, we repeated our analysis from the previous paragraph, but replaced the original pair of states \((s_0^a, s_{\infty}^{opt,a})\) with a new pair \((s_0^b, s_{\infty}^{opt,b})\), such that the distance \(d\) between \(s_0\) and \(s_{\infty}^{opt}\) was the same for both pairs.

In this new analysis, populations evolving on a large genotype network show greater phenotypic variability in response to mutations immediately after this change (Fig. 5c). These differences are statistically highly significant (Table S5). The same observations hold for circuits of different sizes and different interaction densities (Fig. S9 and Table S5). As in our analysis above, the only exception occurs when the interaction density is very low (Fig. S9d). These observations imply that the inductive role dominates in its immediate effect on phenotypic variability when both roles of the environment change. In sum, populations with a robust phenotype have mutational access to more phenotypic variants after environmental change. This increased access is caused by the inductive role of the environment, that is, by the new phenotypes that a new environment can bring forth.

**The effect of phenotypic robustness on variability also occurs after long-term stabilizing selection in the presence of non-genetic perturbations**

In all the simulation results that we report above, we evolved populations under stabilizing selection in the presence of mutations only, before collecting any data. One may ask what happens in a different scenario, when these populations are also subject to recurrent non-genetic perturbations. Long periods of stabilizing selection in the presence of non-genetic perturbations may promote the accumulation of circuit genotypes that only rarely produce (maladaptive) new phenotypes after non-genetic perturbations. The resulting differences in the distribution of circuits in genotype space may alter the positive effect of phenotypic robustness on variability. We next determined whether this is the case.

To this end, we allowed populations of circuits to evolve under stabilizing selection on a given phenotype for \(10^4\) generations. Throughout this period, small random non-genetic perturbations altered the dynamics of some fraction of the circuits in the populations (see Methods). We found that in these populations, phenotypic robustness still has a positive effect on variability caused by noise (Figs. S10). The effect of phenotypic robustness on variability is clearly visible as long as the probability \(\beta\) of a circuit undergoing non-genetic perturbations throughout the period of stabilizing selection is not too high, that is, when populations are evolved under recurrent but not too *intense* noise (Figs. S10). Moreover, populations with a robust phenotype
still form more novel phenotypic variants after a change in the environment’s inducing role (not shown).

The distribution of a population’s circuits in genotype space also depends on whether or not genotypes with a high mutational robustness are favored in evolution. This difference may also alter the relationship between phenotypic robustness and variability that we observe. Stabilizing selection favors genotypes with high mutational robustness if the product of population size $M$ and mutation rate $\mu$ is much greater than one (van Nimwegen et al., 1999). All of our analyses above pertained to this case. However, we also studied the opposite extreme, in which selection cannot lead to the accumulation of genotypes with high mutational robustness: a single individual (a population of size one) exploring a genotype network through random mutations. In this analysis, we focused on the cumulative number of new phenotypes that this “population” can explore as a result of noise and mutation. We found that with increasing phenotypic robustness the cumulative number of phenotypes accessible through noise increases (Fig. S11a,b), but cumulative phenotypic variability after mutation decreases (Fig. S11c). Thus, whether selection can or cannot increase genotypic mutational robustness does not affect qualitatively the effect of phenotypic robustness on phenotypic variability after non-genetic perturbations and mutations.

**Discussion**

If non-genetic change is to be causally involved in evolutionary innovation, it needs to generate novel, potentially beneficial, phenotypes. Genetic assimilation can then stabilize one such beneficial phenotype, if the non-genetic perturbations that induced it either appear recurrently (Kim, 2007; Griswold & Masel, 2009), or if they have effects that persist for several generations (Sollars et al., 2003; Gilbert & Epel, 2008). Previously, we studied the structure of the genotype space associated with a model of gene regulatory circuits. We showed that this structure facilitates the genetic assimilation of adaptive phenotypes that initially appear only after non-genetic perturbations (Espinosa-Soto et al., 2011). Therefore, we here left genetic assimilation aside, and concentrated on earlier evolutionary events, namely the origins of novel traits. Specifically, we used the same model of gene regulation to ask whether the robustness of an existing phenotype and non-genetic change can facilitate the origin of new phenotypes.

We focused on gene regulatory circuits for two reasons. First, many important adaptations appear at the level of gene regulation (Davidson & Erwin, 2006; Prud’homme et al., 2007; Shubin et al., 2009); and second, empirical evidence is especially supportive of the phenotype-first scenario for morphological and developmental traits [e.g. directional asymmetry in diverse taxa (Palmer, 2004), head size in Australian tiger...
snakes (Aubret & Shine, 2009), or amphibian limb or gut morphology (Gomez-Mestre & Buchholz, 2006; Ledon-Rettig et al., 2008). The production of these traits depends to a great extent on the dynamics of gene regulation. In the generic model of transcriptional regulation circuitry that we examined, the relationship between genotypes (patterns of regulatory interactions) and phenotypes (gene activity or expression patterns) is well-studied (Wagner, 1996; Ciliberti et al., 2007a,b; Martin & Wagner, 2008, 2009). In this model, we can use the size of a phenotype’s genotype network as a proxy for a phenotype’s robustness to mutations.

We analyzed the potential of different kinds of perturbations to generate phenotypic variation in populations that were subject to stabilizing selection for many generations. Such populations accumulate genetic variation that is not phenotypically visible (Gibson & G.P. Wagner, 2000). New genetic perturbations, such as gene knock-out mutations, have the potential to expose this hidden genetic variation (Bergman & Siegal, 2003; Tirosh et al., 2010) and facilitate adaptation to a new optimum (Bergman & Siegal, 2003). In addition, non-genetic perturbations may convert the accumulated genetic variation into evolutionarily meaningful phenotypic variation (Schmalhausen, 1949; West-Eberhard, 2003; Hermisson & G.P. Wagner, 2004).

We broadly distinguished two kinds of non-genetic perturbations. The first corresponds to fluctuations in a gene circuit’s microenvironment that have important effects on gene expression phenotypes (McAdams & Arkin, 1997; Elowitz et al., 2002; Raj et al., 2010). The second kind comprises changes in the (macro)environment external to an organism. For brevity, we refer to these kinds of change as noise and environmental change.

We first found that phenotypic mutational robustness increases phenotypic variability of populations in response to noise but not in response to mutations. This last finding differs from observations for RNA secondary structure, where phenotypic robustness facilitates the mutational access to phenotypic variants (Wagner, 2008a). The reason stems from differences in the organization of genotype space for these two system classes, i.e. in the arrangement of different genotypes and genotype networks in genotype space (Ancel & Fontana, 2000; Ciliberti et al., 2007a; Wagner, 2008a; Espinosa-Soto et al., 2011). For example, non-genetic perturbations do not favor increased access to new phenotypes for RNA structures (Ancel & Fontana, 2000), but they do so for gene activity phenotypes (Espinosa-Soto et al., 2011). A recent mathematical model (Draghi et al., 2010) shows that mutational access to new phenotypes can depend on the organization of genotype space and on details of a population’s evolutionary dynamics. It shows that phenotypic variability may vary non-monotonically with mutational robustness, reaching a peak at intermediate values of robustness. Because multiple factors can affect phenotypic variability, it is not surprising that in system classes as different as molecules and regulatory circuits robustness affects variability in different ways.
Next, we showed that environmental change, besides increasing observable phenotypic variation, transiently increases phenotypic variability caused by mutations. Because mutational access to most novel variants is only possible in the new environment, these variants can be considered environmentally-induced phenotypes, supporting the phenotype-first scenario. Importantly, this increase in phenotypic variability is higher in populations that had a more robust phenotype before environmental change.

In sum, we found a positive effect of phenotypic robustness on phenotypic variability after non-genetic perturbations. This positive effect is not sensitive to the magnitude of non-genetic perturbations. Phenotypic robustness favors phenotypic variability after single-gene perturbations in the initial gene activity state, which is the smallest possible non-genetic perturbation in our model (Fig. 2a). It also favors phenotypic variability after replacing the initial state by a completely new random gene activity state (Figs. 4 and 5a). In contrast, populations with robust gene expression phenotypes are phenotypically less variable in response to mutations. Thus, a mechanism that relies exclusively on mutation to produce novel phenotypes becomes less important for innovation as a phenotype’s robustness increases. Our results suggest that plasticity-mediated innovation may be especially important for gene expression traits with high mutational robustness. Our work thus hints under what circumstances the phenotype-first scenario is more likely to underlie the origin of new traits. In this regard, we note that non-genetic induction of novel traits is not expected exclusively for gene circuits with (mutationally) robust phenotypes. We observe a general increase in phenotypic variability after environmental change (Figs. 4 and 5). This increase is just especially marked for robust phenotypes.

We note that the effects of phenotypic robustness on variability in response to non-genetic perturbations or mutations that we observe are qualitatively the same when populations evolve in the presence or absence of indirect selection for mutational robustness (Fig. S11). Rather, the manner in which phenotypic robustness affects variability in response to perturbation may be specific to the kind of perturbation.

Our observations also hold when populations evolve under stabilizing selection and in the presence of recurrent but mild non-genetic perturbations (Fig. S10). However, the positive effect of phenotypic robustness on phenotypic variability may not occur in populations that have evolved under intense and recurrent non-genetic perturbations. Whether this observation undermines in a significant manner the generality of our conclusions is an open question. The answer will not only depend on how often real gene circuits are subject to non-genetic perturbations along their evolution, but also on how frequently such perturbations change the activity of genes in a given gene regulatory circuit. Additional factors, such as the sensitivity to perturbation of gene regulatory circuits ‘upstream’ of the circuit under study will also affect whether the effect that we
describe will occur for a specific circuit.

We emphasize that our conclusions are not in conflict with previous theoretical (e.g. Bergman & Siegal, 2003; Hermisson & G.P. Wagner, 2004) and experimental (Tirosh et al., 2010) research which shows that major genetic alterations can expose hidden genetic variation. Our results merely suggest that phenotypic robustness does not favor phenotypic variability caused by mutations, not that mutations do not increase variability. Also, our results are consistent with the observation that mutations can cause increased sensitivity to non-genetic perturbations (Levy & Siegal, 2008). In fact, we show that a non-genetic perturbation such as an environmental change, can enhance the potential of mutation to generate variation (Figs. 5a and 5c). This last observation is also consistent with previous work on niche evolution, which shows that non-genetic perturbations can accelerate adaptation to a new environment (Kimbrell & Holt, 2007).

The general increase in phenotypic variability we observe is consistent with many empirical observations on phenotypic variation that is conditional on the environment. For example, severe environments enhance phenotypic differences among fruit fly strains (Kondrashov & Houle, 1994), and a temperature rise caused by a lack of shade increases the frequency of abnormal morphologies in fruit flies (Roberts & Feder, 1999). Moreover, population genetic theory predicts that the release of hidden genetic variation after environmental change should be very common (Hermisson & G.P. Wagner, 2004).

In conclusion, our observations suggest that phenotypic robustness to mutations can play a positive role in phenotypic variability after non-genetic perturbations. To see this, one needs to study the role of population level processes, as we did. We caution that we made our observation in the context of a specific model of transcriptional regulation circuits. The gene expression phenotypes of such circuits play central roles in many evolutionary innovations (Davidson & Erwin, 2006; Shubin et al., 2009). However, phenotypes may be distributed differently in genotype space in other classes of biological systems. Whether our observations hold in these systems remains to be seen.

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References


**Figure legends**

**Fig. 1** Gene regulatory circuit model. (a) A gene regulatory circuit. Black bars indicate genes that encode proteins which regulate the activity of other genes in a hypothetical circuit. The regulatory interactions are described by a matrix $A = (a_{ij})$. An interaction means that the activity of gene $j$ can either have a positive ($a_{ij} > 0$, red rectangles) or a negative ($a_{ij} < 0$, blue rectangles) effect on the activity of gene $i$. (b) Gene circuits that differ in a single interaction are neighbors in genotype space. Each large circle surrounds a distinct gene regulatory circuit. Red arrows represent activating interactions, and blue lines represent repressing interactions between different genes (black rectangles). Dashed lines represent the interactions that are necessary to convert the indicated circuits into the middle circuit. a) and b) are modified with permission from (Ciliberti et al., 2007b). (c) Example of novel phenotypes caused by three kinds of perturbations. A reference gene circuit produces phenotype $s_\infty$, in which four genes are active (genes 5-8; yellow) and four genes are inactive (genes 1-4; red). In a one-mutation neighborhood (all circuits that differ from the reference one by a single interaction) we find four phenotypes $s_{new}^\infty$ different from the original $s_\infty$ (left). If we perturb the system state of the reference circuit without altering its genotype, other novel phenotypes are encountered (center and right panels). The perturbations we used are either all single-gene perturbations in the initial condition $s_0$ (center), or perturbations of the dynamical trajectory of the circuit (‘noisy dynamics’; right).

**Fig. 2** High phenotypic robustness facilitates phenotypic variability in response to noise in gene expression. The distance $d$ between the initial state $s_0$ and the optimal phenotype $s_{\infty}^{opt}$ is strongly associated with genotype network size and with phenotypic mutational robustness (“phenotypic robustness”) hereafter. ‘High’, ‘medium’ and ‘low’ correspond to expression phenotypes with high ($d = 0.1$), intermediate ($d = 0.25$), and low ($d = 0.5$) robustness. The figure shows results for $N = 20$ genes and a fraction $c \approx 0.2$ of non-zero regulatory interactions. Both panels show mean numbers of novel phenotypes averaged over 500 independent populations, for each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars indicates one standard deviation. The number of different new phenotypes that a population can access after perturbations of a) single genes in the initial state $s_0$, or b) a circuit’s gene expression trajectory, increases with phenotypic robustness.

**Fig. 3** High phenotypic robustness does not facilitate phenotypic variability in response to mutations without
preceding environmental change. ‘High’, ‘medium’ and ‘low’ correspond to expression phenotypes with high \(d = 0.1\), intermediate \(d = 0.25\), and low \(d = 0.5\) robustness. The figure shows results for \(N = 20\) genes and a fraction \(c \approx 0.2\) of non-zero regulatory interactions. The panel shows the mean number of novel phenotypes averaged over 500 independent populations, at each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars indicates one standard deviation.

**Fig. 4** High phenotypic robustness increases phenotypic diversity in populations of gene circuits after environmental change. The number of different phenotypes that populations display increases after changing the initial expression state \(s_0\). Such an increase is greater for populations with mutationally more robust phenotypes. The figure shows results for \(N = 20\) genes and a fraction \(c \approx 0.2\) of non-zero regulatory interactions. The panel shows the mean number of observed phenotypes averaged over 500 independent populations, for each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars indicates one standard deviation.

**Fig. 5** High phenotypic robustness allows mutational access to more phenotypes after an environmental change produces novel phenotypes. The figure shows the number of different phenotypes in the 1-mutant neighborhood of a population, for three different scenarios of environmental change at generation \(t = 1\). The insets show the number of phenotypes accessible through mutation immediately before \((t = 0)\) and immediately after \((t = 1)\) environmental change. The figure shows results for \(N = 20\) genes and a fraction \(c \approx 0.2\) of non-zero regulatory interactions. ‘High’, ‘medium’ and ‘low’ correspond to expression phenotypes with high \(d = 0.1\), intermediate \(d = 0.25\), and low \(d = 0.5\) phenotypic robustness. Data are mean values averaged across 500 independent simulations for each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars (in the insets) indicates one standard deviation. (a) The initial state \(s_0\) changes. (b) The identity of the optimal phenotype \(s_{\text{opt}}^\infty\) changes. (c) The original pair \((s_0^a, s_{\text{opt},a}^\infty)\) changes to a new pair \((s_0^b, s_{\text{opt},b}^\infty)\).