Regulating Evolvability and Robustness under Environmental Variability

Anke Posma
S5412935

Abstract

Life has evolved the ability to be both robust, as well as adaptable to environmental changes. Moreover, environmental variability can open up evolutionary paths as it shapes the genotype-phenotype-fitness mapping. In this essay, the effects of a variable environment on evolvability and robustness are discussed on the basis of the architecture of gene regulatory networks. Additionally, environmental effects are studied at different levels of complexity within a gene regulatory network. I expect to observe increased evolvability and robustness at higher levels of complexity leading to a reduced effect of environmental variability. At the lowest level of complexity - where transcription factors directly interact with their cognate binding sites - mutational trajectories can become accessible under environmental fluctuations, overcoming evolutionary constraints. Additionally, initiation complexes - in which a hierarchy exists between the relative contributions of transcription factors - can promote evolvability and robustness. At the intermediate level of complexity, particularly within regulatory pathways, evolvability and robustness is mainly due to promiscuous binding of transcription factors. The chance of promiscuous binding as well as crossing a certain activation threshold can be achieved by increased transcription factor levels induced by an environmental change. These newly established connections allow for control over novel downstream effectors. Lastly, the entanglement of networks exhibits high evolvability and robustness at the highest level of regulatory complexity. This entanglement is essentially a combination of hierarchical clustering and promiscuous binding as observed at the lower levels of organisation. Since a variable environment can overcome evolutionary constraints, by affecting the expressed phenotype, as well as oppose constraints, by defining the selective forces, analysing its effects is a complex task. However, the general trend suggests that an increasing level of complexity coincides with smaller environmental effects, as robustness and evolvability grow with complexity.

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Introduction

Selection pressures that lead to the adaptation of a population are shaped by the environment. This environment can be variable, both in time and in space and thus constantly change the imposed selection pressures. For a biological system, two traits are of importance under changing environmental conditions: 1) exhibiting robustness, while at the same time 2) being evolvable. Robustness can be defined as the maintenance of a structure or function in the presence of genetic variation (Wagner, 2008; Payne & Wagner, 2014). Evolvability is the ability to generate heritable phenotypic variation. A highly evolvable population therefore has an easy access, i.e. within a few mutational steps, to novel adaptations (Wagner, 2008; Payne & Wagner, 2014). To function properly, organisms must preserve their functioning under environmental or genetic perturbations, while at the same time welcoming adaptations to improve their functioning within the environment. Being too preservative or too welcoming, however, can decrease the long-term survival chances at the population level. Despite these opposed limitations in the degree of evolvability and robustness, evolution has given rise to adequate levels of both.

Evolvability and robustness both function at the genetic level. Changes at this level can have an additive effect, by which the total effect is the effects of all individual genetic changes combined (Kogenaru, de Vos & Tans, 2009). Often, however, the effect of a change is dependent on the presence of other genetic changes, a phenomenon defined as epistasis. These gene-to-gene interactions (G x G) can oppose evolutionary constraints, as an evolutionary path may only be accessible in a certain genetic context. The ultimate epistatic constraint is reciprocal sign epistasis. In this case, two mutations have a negative fitness effect when they occur on their own, but a positive effect when they happen simultaneously (Poelwijk *et al.,* 2011b). The sign of the fitness effect is therefore dependent on the genetic background. A fitness landscape presents reciprocal sign epistasis as the presence of multiple peaks (Wright, 1932; Poelwijk et al., 2011b; Bank, 2022). In such a landscape, populations can evolve via positive selection at mutational steps towards the fittest genotype, i.e. the fitness peak. It is based on the mapping from genotype to phenotype (often gene expression) to some measure of fitness (GPF mapping). In the case of a rugged fitness landscape harbouring multiple peaks, a population can get stuck at a suboptimal, local peak (Poelwijk *et al*., 2011b). Epistasis therefore drastically affects the accessibility of evolutionary paths and opposes constraints to the evolvability of populations.

Neutral walks within a fitness landscape can promote evolvability and robustness (Maynard Smith, 1970), thereby overcoming epistatic constraints. Smith depicted a protein space that harbours a continuous network of functional proteins in which the connections represent a single mutational step, similar to a fitness landscape. He argued that all functional variants can be reached from a random starting protein without going through non-functional variants. Neutral mutations contribute to the robustness of a network by allowing the protein to change its genetic sequence without changing its phenotype, and consequently maintaining its fitness. By ‘walking’ over these horizontal paths in the protein space or fitness landscape, a greater number of functional phenotypes can be accessed, thereby promoting evolvability. Indeed, mapping of genotypes to phenotypes have shown a high occurrence of neutral correlations at various levels of organisation (Wagner, 2008; Payne & Wagner, 2014; Greenbury *et al*., 2016).

Another possibility to overcome evolutionary constraints is with help of the environment. Environmental conditions can change the sign of a mutational effect as well as its epistatic interactions (de Vos, Schoustra & Visser, 2018). Environment-dependent epistasis (G x G x E) in combination with variations in the environment can therefore open up novel evolutionary paths (Steinberg & Ostermeier, 2016). A mutation that is deleterious in one environment, can be favoured in another. Fluctuating environments can thus represent the solution for a population to escape a suboptimal fitness peak (Flynn *et al*., 2013). It should be noted that a GPF map is shaped by two environmental effects: it affects the mapping from genotype to phenotype, as well as phenotype to fitness. Most studies up to date, however,

have focused mainly on evolvability within a constant environment thereby ignoring the effects of a variable environment (Bank, 2022).

The architecture of gene regulatory networks (GRNs) provides an optimal system to study the mechanisms underlying the environmental effects on GPF mapping (Nge *et al*., 2020). These networks regulate gene expression, thereby determining cellular functioning, while responding to environmental conditions. The mediators in gene expression are transcription factors (TFs), which are often trans-regulated, i.e. originating from a different gene than their target gene (Signor & Nuzhdin, 2018). TFs bind to specific DNA sequences within cis-regulatory regions, often promoters or enhancers, to govern gene expression. The architecture of GRNs allows the networks to be divided into different levels of organisation or complexity: regulatory interactions, regulatory pathways, and regulatory networks (Nge *et al*., 2020). GRNs involved in developmental processes are not discussed in this essay, as these are often regulated by morphogen gradients which makes environmental effects harder to define.

Due to their crucial role in cell functioning, GRNs must exhibit robustness (Baier *et al.,* 2023), while they offer at the same time a logical target for adaptation (Hsu *et al.*, 2021). Indeed, GRNs have shown to maintain expression patterns in the presence of mutations in the DNA binding sites (Payne & Wagner, 2015; Dalal & Johnson, 2017). This robustness allows genetic rewiring of the network under conservation of the phenotype, a process called phenotypic or system drift, promoting evolvability (Crombach *et al*., 2016; Dalal & Johnson, 2017). The environment in which a GRN functions can be both external as well as internal, provided that it is communicated to the cell via an extracellular signal. The environmental variability a GRN is exposed to should occur over time, be fast enough to prevent populations adapting completely to one environment, as well as slow enough to restrain individuals from experiencing only the average between the two environments (Suiter, Bänziger & Dean, 2003).

In this essay I will discuss the state-of-the-art research on the effects of environmental variability on the evolvability and robustness of GRNs and the underlying mechanisms that are evolved to do so. More specifically, I will consider the effects of environmental variability at different levels of complexity within a GRN to investigate how it influences the evolvability and robustness. I expect that environmental variability will generally increase the robustness and evolvability of regulatory networks, in line with G x G x E interactions. Furthermore, I hypothesise that the effect of environmental variability is more substantial on a smaller scale, or the lowest level of complexity. Previous research has hinted that higher complexity structures can enhance both evolvability and robustness of a system (Catalán *et al*., 2018; Houle & Rossoni, 2022). Higher evolvability and robustness are likely to dampen the environmental effects, therefore I expect to see less pronounced effects of environmental fluctuations at higher complexity levels.

Regulatory interactions

At the most basic level of GRNs, TFs directly interact with their DNA binding sites to coordinate gene expression. Often, this binding represents a so-called lock-key system, whereby the key must accurately fit the lock (Poelwijk *et al*., 2007). A modification in only one of the interactors will consequently lead to a mismatch (Nge *et al*., 2020), thereby exhibiting a form of reciprocal sign epistasis. In this first chapter, I will discuss some mechanisms induced by environmental variability that are able to overcome the evolutionary constraints caused by reciprocal sign epistasis at the direct interaction level.

The *lac* regulatory system in *Escherichia coli* (Figure 1A) provides an optimal model system to study the effect of environmental variability on this level of complexity. In the presence of the inducer ligand IPTG, the artificial mimic of allolactose, binding of LacI with the *lac* operon is inhibited. Consequently, the *lac* genes in this environment are expressed which allow *E. coli* cells to import and metabolise lactose. In the absence of lactose, the alternative environment, LacI represses the expression of the *lac* genes, thereby reducing unnecessary costs (Dekel & Alon, 2005). The two environments thus

represent opposing selection pressures. LacI functions in this reaction as a TF, regulating the expression of the *lac* genes by binding to the *lac* operator. This lock-key system between LacI and the operator is dependent on two amino acid residues at the repressor side, and four base pair positions in the operator (Lehming *et al*., 1990).

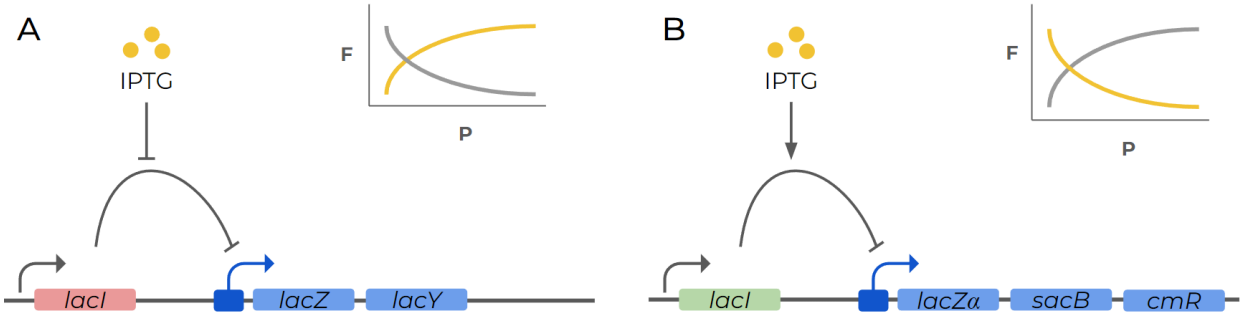

Figure 1. Simplified visualisation of the lac regulatory system in E. coli. Dark blue shows the operon of the *lac* genes, which are coregulated by the repressor LacI. The graph on the top right presents a schematic correlation between phenotype and fitness for an environment with IPTG (yellow) and without (grey). A) shows the wildtype system in which IPTG induces expression of LacZ and LacY (de Vos *et al*., 2015), B) shows the inverse variant of LacI in which IPTG represses expression of SacB, CmR and LacZα (Poelwijk, de Vos & Tans, 2011).

De Vos and colleagues (2015) compared two *lac* repressor-operator pairs that show equal expression levels but differ in their ability to repress expression of the operon. In either the absence or presence of IPTG, none of the mutational trajectories between the two variants were accessible under positive selection. Alternation between the two environments, however, allowed for gradual adaptive evolution under positive selection by single mutational steps. The ability to repress expression of the *lac* genes can be increased under positive selection by a mutation in the environment without IPTG. Switching to an environment with ligand selects for mutations that increase the ability for expression. After reaching the local optimum in this environment, another environmental switch allows for an additional increase in the repressor ability. An essential factor in allowing adaptation by positive selection within these alternating environments is the decrease in global fitness that follows from an environmental switch. This causes adaptive pathways to open up as a higher number of mutations provide a relative fitness gain. Thus, positive selection can drive adaptation of a molecular interaction under reciprocal sign epistasis when there is temporal environmental variation and cross-environmental trade-offs.

Environmental trade-offs in combination with alternation between these environments can even access evolutionary paths that lead to the inversion of a response (Poelwijk, de Vos & Tans, 2011; de Vos *et al*., 2013). In one of these studies, the *lac* operon is synthetically modified and contains the *sacB* and *cmR* genes which affect the growth rate of *E. coli*, as well as the *lacZ*α gene which is used as an indicator for the expression level (Poelwijk, de Vos & Tans, 2011). The authors designed two environments that both oppose negative selective pressures on the expression of the operon. One medium contains sucrose and IPTG. Whereas the presence of sucrose indicates the lack of need to express, the addition of IPTG forces expression. In the other environment the antibiotic chloramphenicol (Cm) is present which opposes a selection pressure for increasing expression, as the *cmR* gene product inactivates the antibiotic. However, in this environment no IPTG is present and therefore the gene expression is repressed. The environments therefore oppose fluctuating demands on the system. By letting synthetically produced *E. coli* mutants evolve under fluctuating environments, the population was able to reach the global optimum phenotype. This phenotype is inverted to the wild-type system as LacI adapted an altered regulation towards binding of IPTG, which now acts as a co-repressor instead of an inducer (Figure 1B). This shows that different regulatory functions, even those with reversed effects, are connected in genotype space by just a few mutational steps. To be more precise, only three mutations are needed to evolve an inverse

variant from the wild-type LacI repressor (de Vos *et al*., 2013). The effects of these three mutations are highly dependent on the environment as well as previous mutations (G x G x E). Environmental fluctuations and cross-environmental trade-offs combined with negative selection pressures in both environments can lead to reverse functioning of a TF, showing the high evolvability and robustness of these factors.

However, not all TF bindings exhibit the lock-key binding. Expression of one gene is often regulated by multiple cooperating TFs and/or multiple binding sites at the DNA sequence (Taatjes, Marr & Tjian, 2004; Spivakov, 2014). An example is the transcription of the tetracycline resistance gene in *E. coli* that is able to export the antibiotic tetracycline from the cell (Shultzaberger *et al*., 2010). The promoter region of the gene has three TF binding sites. MarA binds to one of the sites (Martin *et al*., 1999), whereas σ70 binds to the other two (Hawley & McClure,1983). The stability of the entire initiation complex is dependent on the cooperative binding of the two TFs and can be measured as the expression rate of the *tet* gene. The environment, the concentration of the tetracycline drug, determines the optimal expression rate as the tradeoff between exporting enough tetracycline to avoid toxicity and limiting the cellular cost of overexpression (Lenski *et al*., 1994). To evolve to this optimal expression rate, not all TFs appear to contribute equally (Shultzaberger *et al*., 2010). A single mutation in one of the σ70 sites greatly reduced the stability of the initiation complex, whereas a similar effect for MarA binding site was only reached by complete removal of the site. So, the most contributing TF can access a broad range of fitness values, even within one mutational step. A particular combination of TFs under certain environmental conditions can enhance the accessibility of evolutionary paths. Therefore, a strict GPF mapping does not exist, but rather a hierarchy. This hierarchy within an initiation complex leads to higher order interactions (G x G x G) which can promote evolvability and robustness.

To conclude, environmental variability and cross-environmental tradeoffs can lead to positive selection, thereby overcoming evolutionary constraints opposed by epistasis at the direct interaction level of TF and DNA binding site, or TFs and ligands. This could even lead to the inverse response of a TF to an inducer when both environments oppose negative selection pressures. Quite often, transcriptional regulation is mediated by an initiation complex of multiple TFs which have varying contributions to the expression level, depending on the environment. These hierarchical effects on the phenotype, and thereby fitness, are another mechanism by which evolvability and robustness is established at the level of direct interactions within a GRN.

Regulatory Pathways

Regulatory pathways compose an intermediate level of organisation within a GRN. In this essay, I define a regulatory pathway as a set of proteins that perform one regulatory function together, but not necessarily directly bind to one another (Nge *et al*., 2020). Additionally, I will consider the cases in which one regulator controls multiple downstream effectors and discuss how novel links can be gained.

In contrast to strict lock-key bindings as discussed for the LacI repressor and its cognate binding sites, promiscuous binding of TFs is very common. This promiscuity leads to the expression of non-target genes, a mechanism defined as ‘regulatory cross-talk’ (Friedland *et al*., 2016). Although this can have negative effects on the fitness of the individual, it can also offer opportunities for new interactions that facilitate adaptability (Wagner, 2021). If such a newly established connection appears to be beneficial, mutations that strengthen the binding affinity between TF and promoter sequence are selected for (Lamrabet *et al*., 2019).

A two-component regulatory system (TCS) can illustrate the adaptive benefits of regulatory crosstalk upon environmental change (Taylor *et al*., 2022). A TCS is a common component of a cell's regulation system that links environmental signals to gene expression. The system is composed of a sensor histidine kinase (HK) and its cognate response regulatory (RR). Under normal conditions, the HK

picks up extra-cellular signals upon which it phosphorylates RR. RR in its active state will bind to its cognate DNA promoter sequences and initiate gene expression. Additionally, HK can also dephosphorylate RR, thereby regulating the concentration of RR in the cell. Environmental change can induce mutations that suppress the ability of HK to dephosphorylate RR (Lozada-Chavez, Janga & Collado-Vides, 2006; Taylor *et a*., 2015). Upon increased concentration of RR, the chance of binding between RR and a non-cognate binding site also increases and consequently provides opportunities for cross-talk. Changes in the extracellular environment can therefore strengthen pre-existing links between TFs and non-cognate binding sites, causing rewiring of the regulatory pathway. In the case of TCSs, which are in close contact with the external environment, RR can replace the function of original TFs in activating gene expression. Crosstalk can thus be used as a mechanism of evolvability by gaining novel connections, induced by increased expression of TFs upon environmental fluctuations.

Similarly, regulatory crosstalk allows TFs to take over the function of a master regulator. Loss of the master regulator FleQ in the bacterium *Pseudomonas fluorescens* leads to the loss of motility (Shepherd *et al*., 2023). The researchers identified a group of 13 TFs that share structural similarity with FleQ. Half of them (seven) were able to take over the lost function upon induced high expression levels. This increased expression was modulated by mutational changes to the TF or by feedback mechanisms. Additionally, increased expression can also be induced by the environment that activates the TF for its original function, which eventually leads to the occurrence of crosstalk. Thus, provided an initial high similarity, hyperactivation is sufficient to gain a novel function. It is worth noting that the studied group of TFs (RpoN-EBPs) are known to be evolutionary versatile. Their relatively low binding affinity makes them prone for regulatory innovation, especially in variable environments. Hence, upon environmental alterations, increased expression of a TF can lead to crosstalk thereby promoting the robustness of a pathway, even without the interference of mutations.

Some TFs are known to bind thousands of DNA sites in both active and inactive regions (Li *et al*., 2008; Biggin, 2011). Many of these bindings are effectively non-functional, as a certain ‘threshold of activation’ must be exceeded to acquire a considerable level of gene expression (Spivakov, 2014). Increased activation of the yeast master regulator Sef1 by fusion with a VP16 activation domain showed upregulation of 92 genes whereas the wild-type Sef1 upregulates ‘only’ 85 genes (Hsu *et al*., 2021). The seven different-acting genes harbour a non-functional binding under normal activity levels which becomes functional under increased activity levels. Increased activity of Sef1 can therefore lead to additional control over target genes that were not affected before. This master regulator controls the expression of iron-uptake genes. Whereas iron is needed for cellular processes, it can also be toxic when concentrations are too high. Additionally, the effect of a variable environment was studied. The wild-type Sef1 and the Sef1-VP16 cells were grown in an iron-rich and an iron-low environment. Iron-rich conditions revealed no difference in fitness between the two types. In an iron-low environment, however, the wildtype was outcompeted by the Sef1-VP16 variant, as the latter one exhibited a higher tolerance to drought-like conditions. Since a different environment opposes new selection pressures, non-functional TF binding provides adaptational opportunities that can relatively easily be accessed by increased TF activity, a mutant TF, or a perturbed regulatory network. Fluctuating environments therefore allow mutational trajectories towards gaining control over novel downstream effectors performing one regulatory function together.

At the level of regulatory pathways, promiscuous binding allows for a high evolvability and robustness. Upon environmental fluctuations, adaptive paths may induce high expression levels, increasing the number of both target and non-target binding sites to which a TF binds. The TF can thereby gain control over novel downstream effectors. This promotes both the evolvability and robustness of the pathway, as regulation of downstream effectors can be interchanged. Increase in gene expression has been shown to be more evolutionary accessible compared to a decrease in gene expression (Poelwijk *et al*., 2011a), supporting induced expression levels as an adaptive mechanism within GRNs.

So, at the level of a regulatory pathway, evolvability and robustness is mostly determined by the flexibility of TFs to gain control over novel effectors.

Between Gene Regulatory Networks

So far, I have discussed mechanisms that enable evolvability and robustness at the level of direct interactions as well as within regulatory pathways. These mechanisms include accessible mutational trajectories between different variants of a TF, hierarchical binding within an initiation complex and crosstalk or non-functional binding, which are all mediated by environmental fluctuations. At the network level, high entanglement between different pathways and networks constitutes the most important feature in providing evolvability and robustness to environmental perturbations (Inoue & Kaneko, 2021). Interestingly, one can view this entanglement as a combination of mechanisms at lower levels, namely hierarchical clustering and promiscuous binding.

In their model, Inoue and Kaneko (2021) considered three different types of regulatory network structures that can react to an environmental input signal: the direct network type, the feed-forward network, and the entangled network (Figure 2). They tested how well the different types of networks are able to cope with noise, both intrinsic and external, mutations and changes in parameter values. As expected, the entangled network showed the highest robustness to all types of perturbations. This can be attributed to the high number of detour paths accessible in this network that induce a noise-cancelling effect. Furthermore, entangled networks are the most evolvable due to their structure as they can adapt faster to unforeseen challenges compared to the other network types. Entangled types of networks in the model evolved when binding affinity was low and gene expression responses were therefore ‘sloppy’. High binding affinity, on the other hand, led to the evolution of direct networks. Therefore, the authors propose that the entangled networks evolved to deal with the sloppiness of genes. I, however, argue that the sloppiness evolved to generate such entangled networks enabling high evolvability and robustness. Evolvability and robustness are, after all, essential traits of a biological system and therefore under selective pressures. Assuming these traits to be a byproduct of sloppy gene binding underestimates, in my opinion, the dependence of any species’ survival on evolvability and robustness.

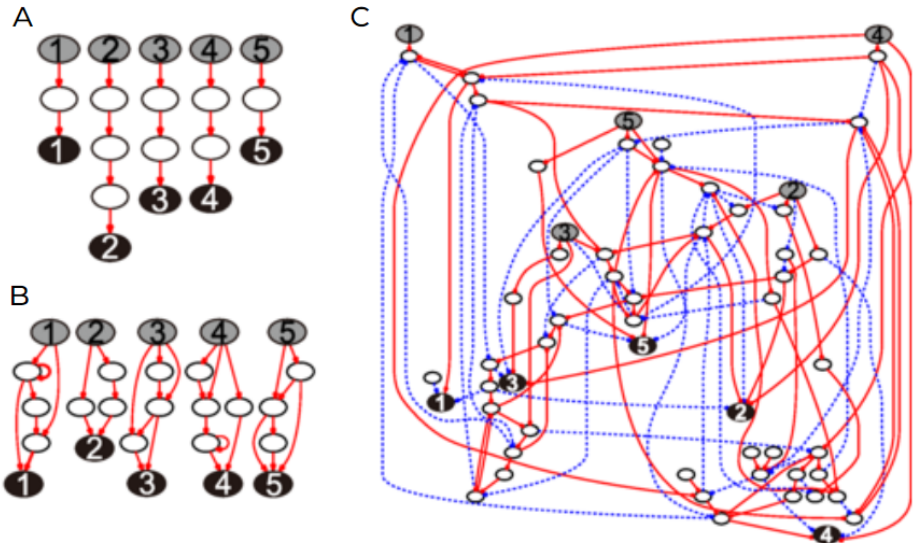

Figure 2. Three possible types of a gene regulatory network structure. A) represents the direct type, B) the feed-forward network, and C) the cooperative type. Reprinted from Inoue & Kaneko (2021).

Since regulatory networks are this highly entangled, you could also speak of one general GRN. Most TFs regulate only a minor part of the GRN, while there are several master regulators. In the case of *E. coli* nine master regulators affect half of the genes, by both direct and indirect regulation (Martinez-Antonio & Collado-Vides, 2003), while the genome encodes for roughly 300 TFs (Pérez-Rueda & Collado-Vides, 2000). One experimental study tested the robustness of *E. coli*'s GRN by adding new connections to different hierarchy levels (Isalan *et al*., 2008). An event was reconstructed by which a gene or an open reading frame was duplicated and linked to a regulatory input. For instance, an original linear interaction from TF 1 to TF 2 can become a positive feedback loop by establishing a new connection between the promoter of TF 2 and the reading frame of TF 1. From the total of 598 rewired plasmid clones, 95% were able to survive within *E. coli*, with 84% showing a similar growth response as the wildtype. Furthermore, different hierarchical levels appeared to be similarly robust to additional connections. Next, the authors considered the evolvability of the rewired networks upon different environmental stressors, such as a heat shock and environmental fluctuations. Whereas most of the rewired networks gave no evidence of new phenotypes, a few provided a selective advantage upon the stressors. One rewired GRN showed a fitness advantage in the fluctuating environment, as it was able to repress certain flagellar genes. Since the ability to move was not crucial, these variants could save energy on the flagellar genes and instead increase cell division. Rewiring at a high organisational scale therefore shows great robustness, while it can also provide adaptation to new and fluctuating environments, thus promoting evolvability.

As a follow-up on the previous experiment, researchers investigated whether the GRN of *E. coli* could be rewired in a more effective way than the wildtype (Carrera, Elena & Jaramillo, 2012). An ODE-based theoretical model was built using a combination of transcriptomic sequence data and a fitness function based on the link between gene expression and cell growth. Various GRNs were generated which had the same expression profile as the wildtype under varying environments, but a distinct rewiring, by which the number of interacting components was allowed to vary as well. The synthetic GRNs could evolve and were exposed to environmental perturbations. Several of these rewired GRNs showed higher growth values compared to the wildtype and a more robust responsiveness to environmental fluctuations. This difference was mainly due to the number of interacting components, as the rewired GRNs had up to 69% reduction in regulatory interactions and 73% reduction in number of operons. The fewer number of regulatory interactions allowed for a higher evolvability as the regulators had a larger overall effect. The authors therefore suggest that evolvability and robustness are not evolved to optimality in wild-type *E. coli* cells. Their finding is really interesting, as it suggests that in this case a reduction in complexity allows for a higher evolvability and robustness.

Viewing transcriptional regulation as one general GRN can provide valuable insights, considering that one unit is highly affected by the larger network (Isalan *et al*., 2008). The entanglement of the GRN allows for a high evolvability and robustness due to widespread effects of the components and the high number of possible evolutionary paths. Entanglement as a mechanism of evolvability and robustness proves to be highly efficient, as rewiring the network has a minimal effect on an organism's survival. At the same time, a reduction in complexity, realised by a smaller number of interacting components, can promote evolvability and robustness at this level of organisation.

Discussion

In this essay I studied the effects of environmental variability on the evolvability and robustness at different levels of complexity on the basis of the architecture of gene regulatory networks. Mechanisms that improve evolvability and robustness can be viewed as tools on a GPF map. These mechanisms vary according to the complexity level. At the lowest level of organisation, environmental variability can overcome evolutionary constraints under positive selection. The main mechanism of evolvability and

robustness are mutational changes that can alter the binding affinity under different environments, but also hierarchical contributions of different TFs play a role. At an intermediate level of complexity, a TF can gain control over a novel downstream effector upon increased expression induced by an environmental change. Evolvability and robustness at this level can be mainly attributed to the promiscuous nature of TF binding. At the highest complexity level, the entanglement of networks allows for a high evolvability and robustness. This entanglement is essentially a combination of hierarchical clustering and promiscuous binding. However, a slight decrease in complexity at this level can actually enhance the evolvability and robustness of the system. Generally, it appears that the higher the level of complexity, the higher the level of entanglement, accompanied by an increase in evolvability and robustness.

The effect of environmental variability seems to decrease with higher complexity. Whereas environmental variability at the lowest level of organisation can help to overcome evolutionary constraints, its effect on higher levels of organisation merely shifts towards a selective pressure. The effect of environmental variability therefore appears to be dual in the sense that the environment opposes evolutionary constraints as well as opportunities. This can be explained by the fact that two environmental effects shape the GPF map (Figure 3A). First, the environmental effect that works on the mapping from genotype to phenotype can function as an inducer, helping to overcome evolutionary constraints. This environmental effect can be clearly observed in the *lac* regulatory system, as the environment determines whether the genes are expressed. Since one environmental condition modulates gene expression in a binary way, the environmental effect of genotype to phenotype mapping on this lowest level of complexity is rather large. Gene expression at higher levels of complexity is often more gradual and controlled by multiple environmental and internal variables. Second, the environment may affect the mapping from phenotype to fitness, as it shapes the selective forces that define the fitness of an organism. This effect opposes evolutionary constraints, as the fitness of a certain phenotype in one environment can greatly differ from the fitness of the same phenotype in another environment (Figure 3B). This environmental effect determines the direction and magnitude of selection, whereas the environmental effect on genotype to phenotype mapping merely impacts the phenotype which can be selected on. Again, the environmental effect from phenotype to fitness mapping is most prominent at lower levels of complexity. Due to the high robustness of higher complexity systems, fitness changes between different environments are minimal. At lower levels of complexity, an environmental switch can open up adaptive trajectories by lowering the overall fitness levels, whereby the environmental effect on phenotype to fitness mapping can also help to overcome constraints. Therefore - besides presenting evolutionary constraints - both environmental effects can also overcome evolutionary constraints, supporting the hypothesis that environmental variability can promote evolvability and robustness. Additionally, both environmental effects seem to decrease with higher levels of complexity. This is in correspondence with the hypothesis that robustness and evolvability are higher at higher complexity levels causing a smaller environmental effect.

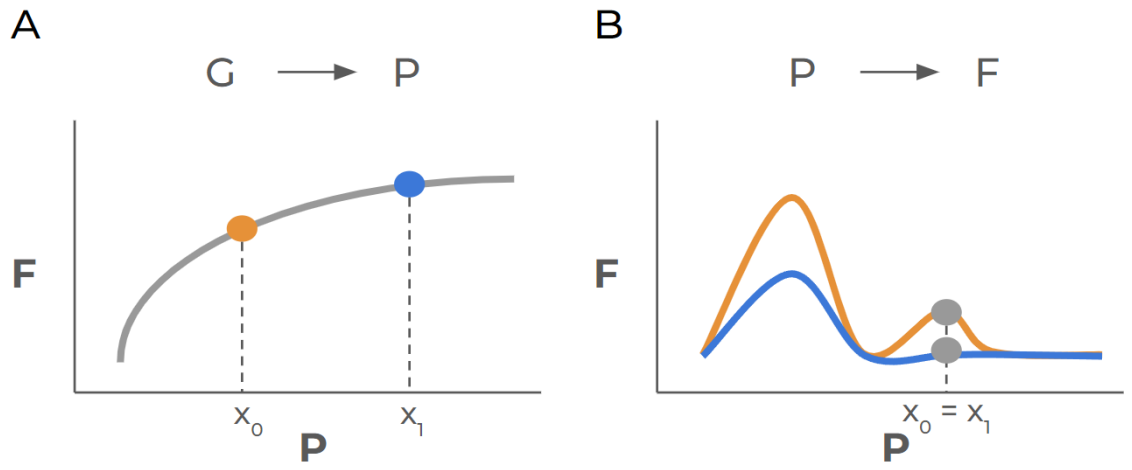

Figure 3. Environmental variability can overcome evolutionary constraints at two levels. A) shows a possible effect of an environmental change on the genotype (G) to phenotype (P) mapping. In this case, phenotype x_0 is converted into x_1, thereby increasing the fitness, B) shows a possible positive effect of environmental change on the phenotype to fitness (F) mapping. Here, an environmental switch reduces the fitness levels for all phenotypes, opening up the adaptive path towards the global fitness peak. In this case, the environment does not affect the phenotype ($x_0 = x_1$). In both plots, orange represents the state before the environmental switch, and blue the state after.

Ultimately, environmental variability is the driver behind evolvability. Under a constant environment, evolvability would be redundant, while robustness would only be required for internal changes. Rather, evolvability and robustness evolved as an adaptation to fluctuating environments, closely linked to the occurrence of neutral mutations (Kirschner & Gerhart, 1998). Therefore, a variable environment opposes selective pressures on the ability to evolve. Consequently, the current architecture of a biological system can largely be explained by past environmental fluctuations (Poelwijk, de Vos & Tans, 2011). Despite the essential role of environmental variability in evolvability and robustness of populations, this factor is still too often neglected in studies concerning this topic (Bank, 2022). While I acknowledge that the interaction between the environment and biological systems is highly complex, especially at higher levels of organisation, the only way to increase our knowledge on the topic is by investigating it. Until then, we can only be fascinated by the ability of living organisms to be simultaneously evolvable and robust, and the many underlying mechanisms shaped by evolution to do so.

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