MINI-REVIEW

The Boggarts of Biology: How non-genetic changes influence the genotype

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Abstract

The notion that there is a one to one mapping from genotype to phenotype was overturned a long time ago. It is now well established that besides the genetic background, environmental inputs guide the development of phenotype. In addition, altered RNA and protein molecules also influence phenotype in conjunction with the external environment, leading to 'non-genetic' changes. The phenotypic variation we see across all living organisms therefore results from a combination of genetic and non-genetic changes. In spite of the prevalent idea that variability arising from non-genetic changes is transient and does not influence evolution, recent work has shown that it can impact both short and long-term adaptation. In this review, I propose that one way in which non-genetic inputs can affect evolution is by indirectly influencing genetic change. I classify and review the many ways in which non-genetic changes influence genotype and impact cellular fitness across generations, with an emphasis on the enticing idea that non-genetic changes act as stepping stones for genetic adaptation. Overall, I review how non-genetic changes impact phenotype via their influence on the genotype, and thus can play a role in evolutionary change.

Keywords

Non-genetic change, translation errors, phenotypic variability, adaptation, evolution

Introduction

The saying 'The more things change, the more they stay the same' aptly describes the constant activity required to maintain the status quo within biological systems. However, living systems are complex, and function with the help of multiple error prone chemical processes. They also innovate, adapt and respond to environmental stimuli, creating a foundation for phenotypic variation to emerge. In fact, heritable phenotypic variability, i.e, phenotypic changes that are faithfully passed on from generation to generation, lies at the heart of Darwinian evolution. Typically, heritable variation stems from differences in the genetic material (usually DNA) of the organism. In turn, these differences manifest as phenotypic variation via RNA and protein transactions within the cell. However, unlike DNA, for the most part, RNA and protein molecules cannot be used as templates to generate more copies of themselves (For exceptions, see True and Lindquist 2000; de Farias et al. 2017). Changes in these molecules are therefore typically short lived, often dictated by changes in the external environment; and thought to have a limited impact across generations (reviewed in Bonduriansky and Day 2009; Jablonka and Raz 2009; Charlesworth et al. 2017). Thus, any phenotypic variation that alters cellular fitness in the long term might be expected to stem only from changes in the genetic material.

In contrast to this thought, a large body of work has shown that non-DNA based phenotypic alterations can both impact fitness, and be inherited (reviewed in Bonduriansky and Day 2009; Ribas de Pouplana et al. 2014; Ackermann 2015). Many terms have been coined to refer to non-DNA based changes, and 'epigenetic changes' such as DNA and histone modifications and RNA editing in particular have been extensively reviewed (Gott and Emeson 2000; Jablonka and Raz 2009; Charlesworth et al. 2017). For the purpose of this article and to avoid confusion, I define 'non-genetic changes' as changes that impact phenotype without altering the primary DNA sequence.

While it is clear that non-genetic changes can increase phenotypic variability and affect adaptation in the short term, their role in long term evolutionary processes has remained controversial. Even when such a role is discussed, it is usually limited to the sub-set of non-genetic changes that can be inherited (reviewed in Bonduriansky and Day 2009; Jablonka and Raz 2009). (Box 1 and Fig. 1). However, non-genetic changes can influence evolution in another major way: by indirectly affecting the genotype. Non-genetic changes co-exist with genetic change in all cells; they interact and have mutual influence. Yet, their effects are

often considered separately, and their impact on phenotypic variation is also viewed through this lens. In this review, I discuss the various ways in which non-genetic changes can influence the genotype and therefore evolution, with a focus on molecular mechanisms. I begin with a brief background, go on to classify examples, and finally highlight some open questions.

Background

Non-genetic changes can impact phenotype in multiple ways

Boggarts are mythical creatures popularized by J.K Rowling. They are shape-shifters with a temporary existence. Like them, non-genetic changes too come in various flavours, and are characterized by rapid response to a new stimulus (reviewed in Fox et al. 2019) (Tyedmers et al. 2008; Tadrowski et al. 2018). They can also help organisms navigate changing environments via 'phenotypic switching', i.e, by switching to one of two or more possible stable phenotypes within the same genotype (reviewed in Fox et al. 2019; Stajic and Bank 2020). From a molecular point of view, quantitative or qualitative differences in RNA and protein molecules (leading to altered transcription and translation) underlie nearly all non-genetic changes that alter phenotype. In general, such changes could either be triggered by variation in the external environment or be independent of the external environment (reviewed in Vogt 2020).

For example, an increase in temperature (environmental change) leads to the loss of a histone modification (non-genetic change) in the germline of *C. elegans* embryos (Klosin et al. 2017). In turn, this results in the activation of several normally silenced genes for over 10 generations, after which the original pattern of modification is re-set. Non-genetic changes that are independent of the environment are usually associated with stochastic differences in the level and composition of intracellular molecules (reviewed in Krishna and Laxman 2020; Vogt 2020). For example, Novick and Weiner showed that after prior exposure to different amounts of lactose, clonal *E. coli* cells in the same environment could be transcriptionally 'induced' or 'uninduced' for lactose utilization depending on stochastic variation in the levels of the lactose transporter protein (galactoside permease) they carried (Novick and Weiner 1957). Cells previously exposed to high lactose remained induced, while those exposed to low lactose were not induced; the states of gene expression persisted stably for several generations. However, environment independent non-genetic changes need not arise only from stochastic events. For instance, alternate decoding of the genetic code via frameshifting

and stop codon readthrough can affect expression of the genotype (Farabaugh 1996; Ivanova et al. 2014; Fan et al. 2017). Recently, a rare +1 frameshift mutation in the essential *rpoB* gene (encoding RNA polymerase) in *E. coli* was identified in a screen for resistance to the antibiotic rifampicin (Huseby et al. 2020). Surprisingly, although they showed no additional mutations, mutant cells continued to retain viability (for which functional RNA polymerase is essential) as well as rifampicin resistance. Further investigation revealed that the frameshift mutation was suppressed by a second phenotypic frameshift downstream ~5% of the time. This non-genetic change restored the original reading frame with just one amino acid change. In addition, translation was upregulated in response to RpoB depletion, resulting in ~70% functional protein being formed (Huseby et al. 2020), and minimising the cost of antibiotic resistance.

Therefore, non-genetic changes can be either induced by the environment or independent of the environment; they can arise from stochastic processes or be part of a stable gene regulatory network. Overall, non-genetic changes can influence phenotype via multiple routes (reviewed in Ackermann 2015; Ling et al. 2015; Stajic and Bank 2020).

Limits to the impact of non-genetic changes: Heritability and Penetrance

Since RNA and protein regulatory changes underlie most non-genetic changes, a substantial proportion of the population can respond immediately and simultaneously to an external stimulus. In contrast, genetic change is slow. Even when a beneficial mutation or gene combination arises, it takes a while (several generations) for such a change to spread across a substantial proportion of the population. Just like genetic change, non-genetic changes can be deleterious, beneficial, or neutral with no apparent fitness related effect on the phenotype in a given environment (Goldsmith and Tawfik 2009; Bullwinkle et al. 2014; Bodi et al. 2017; Carey et al. 2018). The precise distribution of such effects is not well worked out, and is likely to differ depending on the mechanism by which the non-genetic change operates, as well as the linked genotype and environment (Klironomos et al. 2013). Whatever their distributions, the rate at which non-genetic changes occur as well as the number of distinct processes feeding into such changes (transcription, translation, secondary modifications and protein folding, to name a few) likely outnumber those associated with DNA mutations (Drummond and Wilke 2009; Gout et al. 2013; Mordret et al. 2018). Although high in number, two factors have been seen as a limit to their impact:

(i) Lack of heritability: As discussed already, many non-genetic changes occur in conjunction with an environmental change, and may not last once the environment changes again. This is often seen as a limit to their long-term impact. However, experiments over the years have uncovered heritable non-genetic changes across model systems, reducing the strength of this argument (See Box 1).

(ii) Low penetrance of the phenotype: Non-genetic changes may mediate phenotypic change in a sub-population of cells, or alter a sub-population of intracellular RNA or protein in all cells. For instance, stable transcriptional regulation (guided by environmental cues) ensures 100% penetrance of one of two phenotypes in clonal cells. A *Bacillus subtilis* cell is either a dormant endospore or an actively growing cell (Tan and Ramamurthi 2014), *Caulobacter crescentus* cells are either stalked or swarmers (Tsokos and Laub 2012). On the other hand, phenotypic changes that arise from errors in transcription, translation and protein folding are unpredictable, and the altered molecules form only a small percentage of the total population (Drummond and Wilke 2009). In turn, any phenotypic change associated with such an altered molecule will have much less visibility than a mutation that leads to the same change. Although this remains a factor in considering the impact of non-genetic changes, experiments have shown that even seemingly small contributions from altered tRNAs and proteins can impact phenotype and short-term adaptation. For example, 5% of altered RNA polymerase molecules are sufficient to confer resistance to low amounts of rifampicin (Javid et al. 2014); a ~1% misacylation of tRNAs is sufficient for *E. coli* cells to show a growth advantage under oxidative stress as compared with unmodified cells (Schwartz et al. 2016). Therefore, experimental evidence suggests that low penetrance need not be a barrier for non-genetic changes to impact phenotype and fitness. In addition, the many examples discussed later in this article strengthen this view.

Ways in which non-genetic changes can lead to genetic change

As mentioned previously, the role of non-genetic changes in evolution has remained controversial; partly due to the paucity of experimental evidence in support of such a contribution. Recent experiments have shown that non-genetic changes can influence long term adaptation as well as the future path of genetic change (discussed in the sections below). By doing so, they assume relevance for evolution. We could visualize the overall phenotype as a body whose skeleton is determined by the underlying genotype, with the flesh and blood supplied by a combination of genetic and non-genetic changes in a given environment (Fig.

2). In the section below, I categorise three ways in which non-genetic changes can impact the genotype (Fig. 3), and discuss evidence for each with a focus on the molecular mechanisms. I review specific examples within each category where non-genetic change either precedes or succeeds genetic change in shaping phenotype. Finally, I discuss the limited experimental evidence available for phenotypic changes that act as stepping stones for genetic change. I review this in light of our recent finding that mistranslation increases early survival under DNA damage in *E. coli* cells (non-genetic change) followed by beneficial mutations in the gene *gyrA* (genetic change) (Samhita et al. 2020a). Overall, I present evidence suggesting that non-genetic changes can have a significant impact on evolution via their impact on the genotype.

Box 1

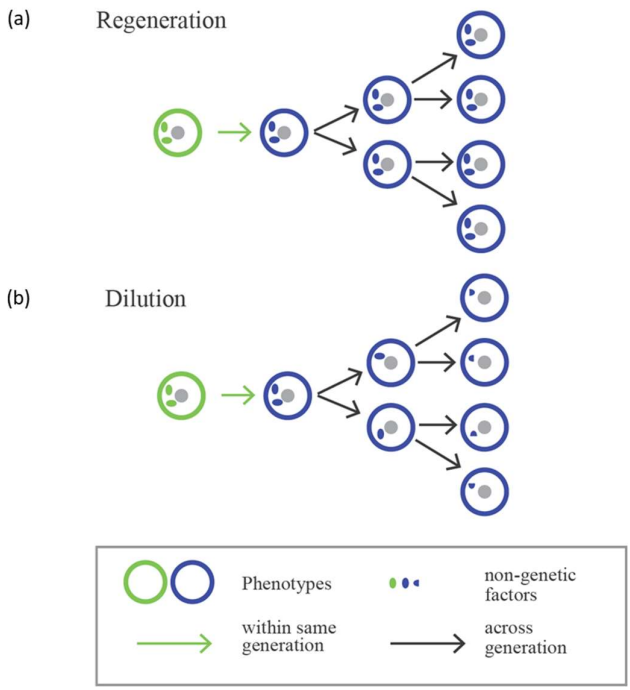
Direct inheritance: The simplest way for non-genetic change to be perpetuated is to directly pass on from one generation to the next, much like DNA does (Fig. 1). There are two ways in which this can happen. One, where the non-genetic change is generated or established afresh every generation from a template (Fig. 1a). Second, where the same factor is propagated directly and diluted over time (Fig. 1b).

DNA and histone modifications fall under the first category, and have been extensively reviewed elsewhere (reviewed in Moazed 2011). Typically, progeny inherit 'directions' to regenerate a given pattern. For example, when a cell carrying DNA methylations on both strands divides, one methylated DNA strand inherited from the parent cell orients the molecular machinery in the daughter cells and enables methylation on the complementary strand. Of all examples that fall under direct inheritance, prions are perhaps the most unique. A prion (proteinaceous infectious particle- (Prusiner 1982)) arises spontaneously as an alternately folded version of a normal cellular protein (with a probability ranging from 10^{-2} to 10^{-7}, depending on the prion- (Tuite 2020)) with the added ability of converting the normal form into the prion form by assisted folding. Prion based inheritance is best studied in laboratory strains of *Saccharomyces cerevisiae* where they can be stably maintained and propagated across several generations (Halfmann et al. 2012). However, there is at least one example of a natural prion that is propagated in a fungus and even serves a critical cellular function: the [Het-s] prion in *Podospora anserina* (Debets et al. 2012). The prion version (but not the normal form) mediates heterokaryon incompatibility (a form of self versus non-self

recognition between fungal hyphae (Glass and Kaneko 2003), where genetically different nuclei are prevented from co-existing in one cytoplasm). The prion ensures that vegetative mixing cannot happen, a result that is also thought to keep the spread of cytoplasmic infections at bay (Debets et al. 2012). There is no genetic basis for this change, it is passed on as a dominant trait through the cytoplasm.

A second way to carry over a non-genetic change into the next generation is through direct transfer without regeneration (Fig. 1b). This occurs when the factor causing the non-genetic change (often altered protein) remains stable across generations. Such a factor could either be diluted at every cell division and be inherited by both daughter cells, or be asymmetrically inherited by only one of two daughter cells. By tracking fluorescently tagged LacY proteins, Lambert and Kussell found that *E. coli* cells retain a 'phenotypic memory' of past exposure to lactose by retaining stable LacY from cytoplasmically inherited protein for ~10 generations (Lambert and Kussell 2014). In turn, this reduces the time taken to re-start exponential growth (lag phase) when cells are moved from glucose to lactose as the carbon source. In contrast to even distribution among daughter cells, protein aggregates that arise from heat shock are asymmetrically inherited by one daughter cell for several generations in *E. coli* (Govers et al. 2018). These cells go on to show higher heat shock tolerance although they have no history of exposure to high temperature. In most of these cases (with the exception of prions which show dominant cytoplasmic inheritance (Halfmann et al. 2012; reviewed in Tuite 2020)), the inheritance is unstable and often does not last beyond a few generations. Therefore, although they are fascinating and clearly impact short term adaptation, it is not clear to what extent such processes can impact long term evolution.

Figure 1

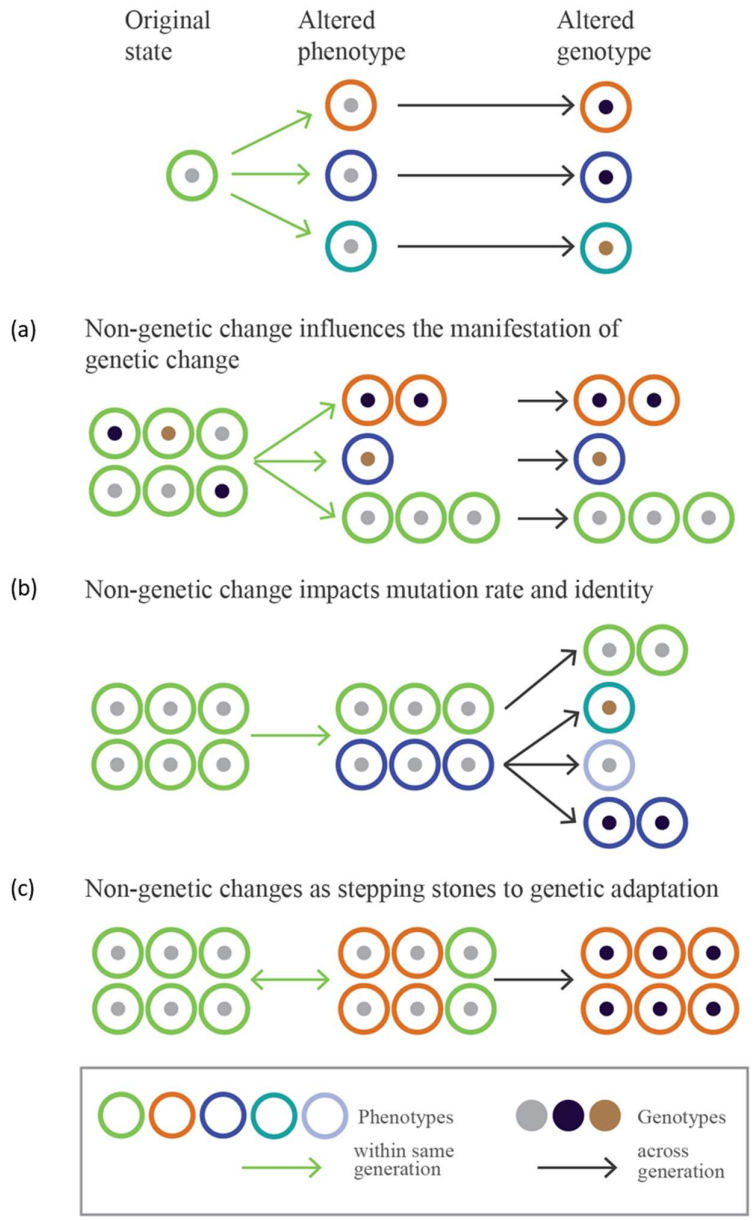


Direct inheritance: Non-genetic changes can be passed on by direct inheritance of altered factors (altered DNA, RNA or protein). In this representation, an altered phenotype (green to blue) occurs within a generation without any change in the genotype. It is then transmitted across generations either by (a) regeneration of the factor or by (b) dilution of the factor every generation, with the genotype still remaining intact.

Figure 2

Phenotype is a composite of genetic and non-genetic changes: The figure shows a Persian watercolour sketch of a composite camel (~1500 A.D). Several animals (analogous to many non-genetic changes) form the body of the camel and co-exist within it, each with their own thoughts and impulses. The camel's body provides a broad shape and structure within which these exist (analogous to the genotype). External factors such as the man (environment) guide and can change the direction in which the overall body moves. *Image source:* Online repository of open access images from the Metropolitan Museum of Art

Figure 3



Non-genetic changes influence genetic change: (Summary) Altered phenotypes can lead to altered genotypes in various ways (a-d) Four ways in which non-genetic changes can impact the genotype are shown here. A non-genetic change is represented by a change in phenotype while the genotype remains the same (a) Non-genetic changes can expose pre-existing genetic variation, uncovering diverse phenotypes (b) Non-genetic changes can alter the nature and rate of mutations, leading to genotypic change (c) Adaptive non-genetic changes can help cells tide over a challenging environment, while paving the way for genetic change

Non-genetic change influences the manifestation of genetic change

Non-genetic changes can expose, suppress or enhance already existing genetic variation in a population (Fig. 3a). Variation in the untranslated 3' region of genes can be exposed by stop codon read-through during translation and also impact the evolution of protein coding genes (Kosinski and Masel 2020). The yeast prion [*PSI* $^{+}$] provides a good example of an altered protein that uncovers already existing genetic variation. [*PSI* $^{+}$] is an alternately folded version of the translation termination protein eRF3. eRF3 normally promotes the disassembly of translation termination complexes after a protein chain is terminated at a stop codon. In prion plus cells, eRF3 is sequestered into prion aggregates, thereby leading to a loss of function phenotype and increased stop codon read-through (Chernoff et al. 1993; True and Lindquist 2000) . Extended proteins are made across cellular mRNAs, exposing cryptic genetic variation in normally untranslated regions and leading to diverse phenotypes across different carbon sources and antibiotics (True et al. 2004) .

Classic experiments by Waddington demonstrated the power of non-genetic change in controlling the penetrance of genetic variation (Waddington 1942). Waddington exposed *Drosophila melanogaster* to heat shock or ether (neither is mutagenic), and showed that exposure to these stimuli led to two effects: (a) They generated new phenotypes (crossveinless and bithorax-like, respectively) and (b) These phenotypes became independent of the stimulus after ~20 generations of repeated exposure to heat or ether and selection for crossveinless and bithorax-like flies, respectively (reviewed in Crispo 2007). Many years later, experiments with heterozygous *D. melanogaster* mutants of the gene HSP90 encoding a chaperone recapitulated the same findings under heat shock, leading to the hypothesis that differences in HSP90 may underlie Waddington's observations (reviewed in Zabinsky et al. 2019).

Given that protein structure and folding is sensitive to a range of environmental stresses, it is perhaps not surprising that a chaperone that aids in the correct folding of other proteins has emerged as a central molecule in uncovering diverse phenotypes (corresponding to diverse genotypes) that are otherwise 'canalized' or invisible to selection. Inhibition of Hsp90 function leads to a plethora of new morphologies and fitness related diversity across several model systems (reviewed in Zabinsky et al. 2019). Although Hsp90 is upregulated at high temperature, it is also functionally compromised and unable to aid in the correct folding of all its client proteins (Queitsch et al. 2002). A loss of function phenotype of Hsp90 can lead to

client proteins (signal transducers, transcription factors and members of multi protein complexes) that are degraded, misfolded or alternately folded (Rutherford and Lindquist 1998; Queitsch et al. 2002), and expose phenotypes associated with previously buffered mutations. All of these outcomes can lead to the manifestation of otherwise hidden (cryptic) genetic variation.

For example, the influence of Hsp90 on the transcription factor Ste12 was studied using *S. cerevisiae* as a model system. Ste12 regulates the choice between mating and invasion of host tissue in many fungi (Dorrity et al. 2018); it is not an Hsp90 client protein. However, several mutant variants of this factor are chaperoned by Hsp90 under normal conditions in *S. cerevisiae*, masking the mutant phenotype. When exposed to high temperature, Hsp90 is unable to maintain the buffering. Cells carrying the Ste12 variants lose the ability to mate and demonstrate a dominant hyperinvasive phenotype, unmasking the cryptic variation (Dorrity et al. 2018). Conversely, Hsp90 can also allow new mutations to have immediate phenotypic consequences. The tyrosine kinase c-Src interacts poorly with Hsp90. However, a mutated oncogenic variant of this protein called v-Src is stabilized by Hsp90, activating its promiscuous kinase activity and capacity for oncogenesis (Boczek et al. 2015).

Other chaperones such as GroEL in bacteria have also shown similar though not quite such far ranging effects (Sabater-Munoz et al. 2015), suggesting that chaperones in general are good candidates to act as a conduit between genotype, environment and phenotype.

Non-genetic change impacts mutation rate and identity

Altered transcription and translation can indirectly impact mutation rates via the generation of novel transcripts and proteins, and interestingly, even by altering the degree and timing of gene expression. In particular, several examples of translation errors affecting the nature of mutations have surfaced in the past decade. In one example, genes evolved in a translation error prone background showed different sets of mutations as compared with those evolved in a wild type background. Bratulic and co-workers propagated a plasmid borne antibiotic resistance gene (TEM-1 beta lactamase) through multiple rounds of mutation and selection in the bacterium *E. coli*, selecting for resistance against a different class of antibiotic (cefotaxime) (Bratulic et al. 2017). The antibiotic resistance gene was propagated in either wild type or translation error prone genetic backgrounds. After four rounds of experimental evolution, resistance genes from the two backgrounds showed distinct mutations. Deleterious mutations were more effectively purged in the error prone background, likely due to the

increased cost of such mutations in an error prone background, and selection on destabilizing protein sequences (Bratulic et al. 2015).

Translation errors can also impact global mutation rate, both via an overall increase in mistranslation (Krisko and Radman 2013) and by specific amino acid changes (Humayun 1998; Bacher and Schimmel 2007). In general, mistranslated proteins tend to misfold and aggregate (Drummond and Wilke 2008), and so are typically unable to carry out their normal cellular functions efficiently. When such dysfunctional proteins are involved in DNA replication and repair, they can introduce mutations. Studies carried out over 20 years ago isolated an *E. coli* mutant carrying a mutation in the anticodon of a glycine tRNA gene, resulting in increased substitutions of glycine in place of aspartate in cellular proteins. Such cells also showed an increase in mutagenesis (Slupska et al. 1996). Recently, it was found that DNA polymerase III (Pol III) isolated from these cells showed significantly higher error prone replication *in vitro* as compared with those isolated from WT cells (Al Mamun et al. 2006). Given that Pol III is the chief replicating polymerase in bacteria and also responsible for correction of mismatches post replication (reviewed in Sutton and Walker 2001), it is reasonable to hypothesize that heterogeneity in DNA Pol III sequence contributed to the increased mutagenesis.

In addition to acting via altered proteins, mistranslation can trigger stress responses and in turn briefly elevate the basal mutation rate. For example, global mistranslation (Samhita et al. 2020a) as well as mistranslation induced by a defective alanyl tRNA synthetase (Bacher and Schimmel 2007) trigger a DNA damage response (SOS response) which can be mutagenic (Baharoglu and Mazel 2014). Overall, both mutation rate and identity can be impacted in multiple ways by non-genetic changes (Fig. 3b) such as translation errors.

Changes in transcription can also affect mutagenesis. An elegant experimental system set up in clonal yeast cells recently showed that changing the amount of transcription from a gene can impact not just the degree of adaptation, but also the kinds of mutations sampled by the organism (Stajic et al. 2019). Stajic and Banks inserted a reporter gene URA3 (required for uracil biosynthesis) at different chromosomal positions associated with different degrees of transcriptional silencing. URA3 expression is toxic in the presence of 5-Fluoro-orotic acid (5-FOA); the authors grew three versions of the silenced yeasts corresponding to how much the gene was silenced (high-H, low-L and intermediate-M) in the presence of 5-FOA and selected for resistant strains. Interestingly, the M lines (intermediate silencing) adapted

fastest, with the rapid appearance and spread of several mutants where URA3 expression was abrogated. In addition, both M and L lines showed mutations in genes other than URA3, whereas the H lines only showed resistant mutations in URA3. While the mutation rates remained unchanged across the three lines, the kinds of mutations sampled and the rate of adaptation were clearly influenced by the degree of gene expression. This work also helped to disentangle the genetic from non-genetic contributions in a given phenotype, something that has been a challenge for experimentalists.

Therefore, both mutation rates and specific mutational paths can be influenced by non-genetic changes (Fig. 3b).

Non-genetic changes as stepping stones to genetic adaptation

Perhaps the most exciting aspect of non-genetic changes has been the speculation that when beneficial, they may 'buy time' for genetic change, thus linking short term adaptation to long term evolutionary change (Fig. 3c and Fig. 4). The hypothesis has been laid out in several forms over the years, but experimental evidence remains extremely limited. A specific case of this situation where the beneficial non-genetic change is induced by the environment, was proposed by Baldwin (Baldwin 1896). He postulated that an organism acquired adaptive phenotypic changes as a consequence of its interaction with the environment. With time, 'heritable characters' that produced the same changes would be favoured by natural selection and spread in the population (reviewed in Crispo 2007). Multiple theoretical models have shown that this can happen, both with environmentally induced change and with environment independent changes such as alterations in RNA and protein sequence or protein folding (Hinton and Nowlan 1987; Whitehead et al. 2008; Bonduriansky and Day 2009; Klironomos et al. 2013).

For example, the 'look- ahead' effect postulates that when a beneficial trait requires two mutations, cells that acquire one mutation can use protein synthesis errors to phenocopy the effect of both mutations together, and thus reach the fitness peak fast (Whitehead et al. 2008). This model explores the specific case where the first mutation is neutral or mildly deleterious, and so cannot spread rapidly in the population on its own. In this scenario, the phenotypic impact of translation errors in addition to the first mutation can give the cell an adaptive edge, allowing the corresponding genotype to spread in the population. Experimental evidence for the first step exists; i.e, protein synthesis errors can lead to a fitness advantage in a specific genetic background (Kramer and Farabaugh 2007; Javid et al. 2014; Fan et al. 2015). Clear

evidence for the second step (second beneficial mutation) has been elusive for some time. However, recent work with antibiotic tolerant cells offers one instance where a beneficial phenotype is achieved through two independently beneficial mutations; where the first can potentially be substituted for, or enhanced by, a phenotypic change. Liu and co-workers examined clinical samples of *Staphylococcus aureus* from patients under treatment in a multi drug regime (Liu et al. 2020). They found that, as per previous predictions and observations (Levin-Reisman et al. 2017) , mutations leading to antibiotic tolerance (slower killing of the population) preceded the appearance of resistant mutants. In addition, the presence of mutations that conferred tolerance (first beneficial mutation) enhanced resistance in the multi-drug regime, when compared with cells that only carried resistance mutations (second beneficial mutation). Tolerance can also be achieved by non-genetic means (Levin and Rozen 2006; Cohen et al. 2013); however, the current study mapped all tolerant phenotypes to mutations. The pattern of antibiotic resistance evolution was also duplicated in laboratory experiments (Liu et al. 2020), suggesting that the evolution of antibiotic resistance in *S. aureus* and other bacteria might occur through this two-step process.

Klironomos et al modelled a population where both genetic and non-genetic changes occur independently (Klironomos et al. 2013). Non-genetic changes were assigned a higher rate of appearance than genetic change (mutations). In addition, non-genetic changes could revert to the original state at a given probability. The authors found that populations with options for both kinds of change adapted faster than those that relied only on mutations, a finding that is broadly supported by both earlier (Hinton and Nowlan 1987; Behera and Nanjundiah 2004) and later work (Kronholm et al. 2017). In addition, because non-genetic changes are fast, they rapidly lead to a fitness peak. In such populations, non-genetic changes do ‘buy time’ for adaptive genetic change, however, mutations can accumulate neutrally in the meantime. This leads to increased standing genetic variation; another way in which non-genetic changes can indirectly impact the supply of genetic variants. The model also highlights the fact that current mutations observed in populations could well have been preceded at one time by non-genetic change, creating a stepping stone to the current phenotype.

Excitingly, we have evidence that at least in one case, something like this may have happened. Versions of the essential metabolic protein isocitrate dehydrogenase (IDP) are found in the mitochondria (IDP1), cytoplasm (IDP2), and peroxisomes (IDP3) of *S. cerevisiae*; the peroxisomal version is evolutionarily the most recent. Yanagida and co-workers (Yanagida et al. 2015) found that an ancestral IDP2 gene taken from distantly related

yeast strains such as *Ashbya gossypii* carries possible signals for peroxisomal targeting beyond the stop codon, but they are in a +1 translational reading frame, and therefore cryptic. When the *A. gossypii* IDP2 was expressed in *S. cerevisiae*, ~30% of the total protein product could be detected in the peroxisome. Further, a protein with size corresponding to the frameshifted product was detected by mass spectrometry, showing that frameshifting must have taken place and made the peroxisomal targeting signal effective. The authors also mutagenized another ancestral IDP2 (from *Kluyveromyces waltii*) in the region around the stop codon, followed by selection on petroselinate containing medium (peroxisomal IDP is essential for growth on petroselinate). This led to the rapid selection of mutant proteins carrying genetic single base deletions (one per mutant) that brought the peroxisomal targeting signal in frame. Therefore, the ability to generate an alternate protein product may have served as a stepping stone for mutations that fixed novel cellular localization for this protein (c). However, although this is a novel finding that highlights the role of translational errors in adaptation, it still involves some speculation about past evolution. That is, it falls short of a demonstration that adaptive non-genetic change can be a precursor to genetic change.

Recently, we showed that generalized (non-directed) mistranslation can increase survival when *E. coli* cells are treated with the DNA damaging antibiotic ciprofloxacin (Samhita et al. 2020a). Irrespective of the non-genetic route by which basal mistranslation in *E. coli* is elevated, there is a common consequence: the level of the protease Lon, a key player in degrading misfolded proteins, also goes up. Increased Lon leads to an increase in the levels of RecA, a protein that is essential for DNA repair and recombination. As a consequence, when faced with DNA damage from ciprofloxacin (cip), mistranslating cells are already closer to the threshold for activation of the DNA repair response (the SOS response) than wild-type (WT) cells that retain a basal (high) translational fidelity and constitute the control population. Therefore, they begin DNA repair earlier, and display ~4-fold higher survival than the WT. At this point (~2 hours post exposure to ciprofloxacin), mistranslating cells are genetically identical to the WT (excluding the genetically altered mistranslating strain, Samhita et al. 2020a). However, 24 hours later, both WT and mistranslating cells carry point mutations known to confer resistance to cip, in the gene *gyrA*. Thus, the increased early survival of mistranslating cells (Fig. 4c) relative to the WT (Fig. 4a) occurs via a relatively higher population size for subsequent mutations to occur in. This finding provides a direct experimental example for the stepping stone effect: A non-genetic change (elevated translation errors) provides a temporary survival advantage via an altered phenotype

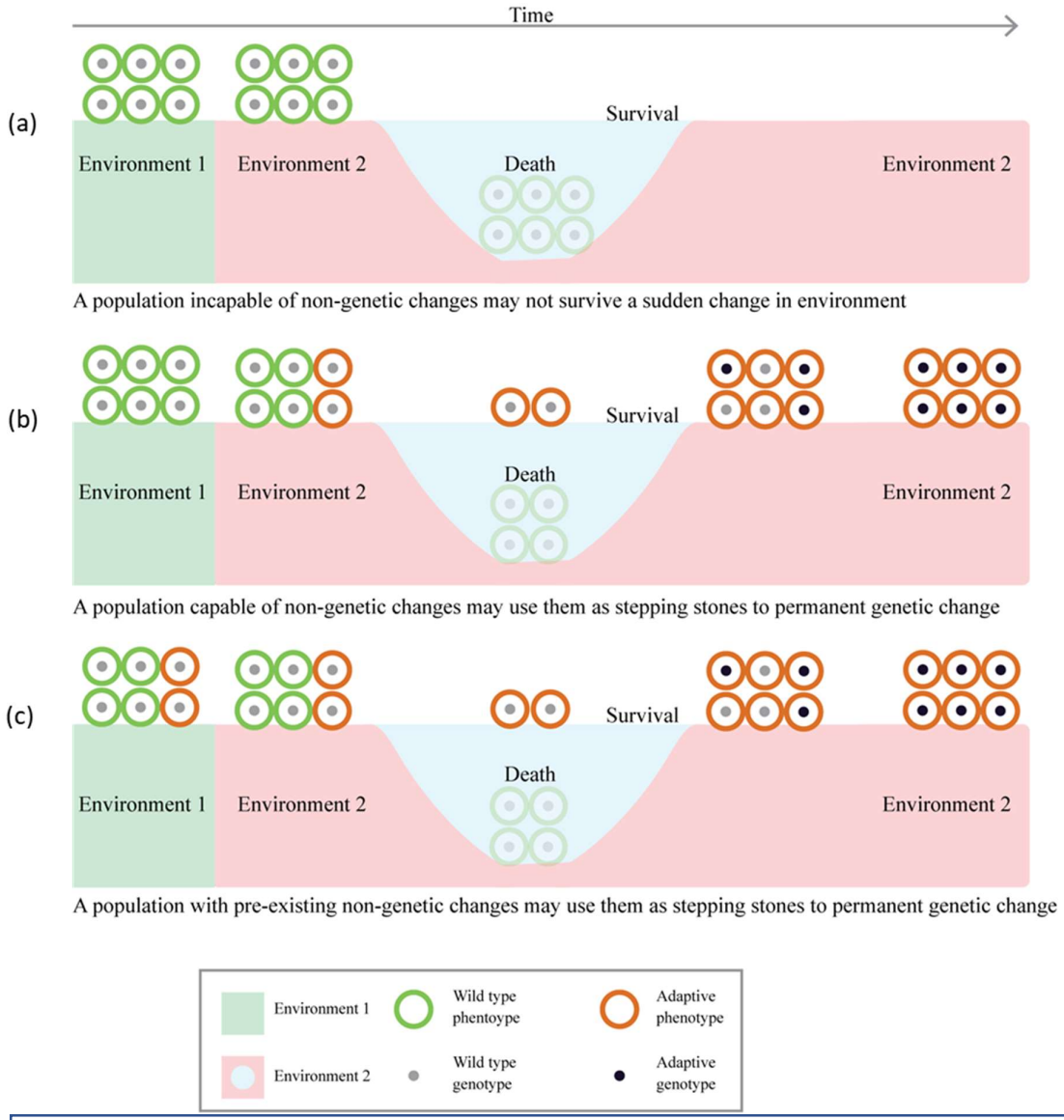
(resistance to ciprofloxacin), and the same phenotype is subsequently fixed by genetic change (Fig. 4c). Interestingly, *reducing* the basal level of mistranslation in the wild-type concomitantly lowered its resistance to ciprofloxacin, suggesting that translation error and DNA damage repair remain relevant even in the wild-type under normal conditions, and perhaps giving a broader context to the stepping stone effect.

Among the ways in which a non-genetic change induced by stress can act as a stepping stone, two broad mechanisms deserve attention. They are (a) an elevated population size, which increases the number of cells within which a favourable mutation can take place, and (b) an elevated mutation rate, which increases the probability per cell of a favourable mutation taking place. Experiments and modelling work with persister cells (metabolically inactive non-dividing cells that survive antibiotic treatment) in *E. coli* suggests that both methods may be at play in moving from persistence to antibiotic resistance (Windels et al. 2019). Persisters could be a pre-existing part of the population (Fig. 4c) (Cohen et al. 2013), or be generated by exposure to the antibiotic itself (Fig. 4b) (Dorr et al. 2010). Persister numbers are strongly correlated with the number of resistant cells that arise later; and genetic mutants that show a constitutively high level of persistence also show a higher mutation rate (Windels et al. 2019), which may help in the generation and establishment of resistant mutations. However, more work is needed to establish a direct link here from an adaptive phenotype, to genotypic change.

The idea that short lived phenotypic changes can pave the way for specific genetic change is conceptually different from the other ways in which non-genetic changes can impact the genotype (discussed above: uncovering cryptic change and altering mutation supply or identity). In some ways, it is the simplest category, merely requiring a phenotypic change that can provide a short-term survival benefit. Mutations will then occur over time irrespective of the nature of the change as long as the population has a reservoir of viable cells; and some of these mutations will be beneficial. Why then do we not see more examples of such phenomena? One possibility is that experimental designs have failed to capture situations where this might occur. Alternately, perhaps evolution does not often take this path. If the former is true, more experiments particularly with microbial systems (where it is relatively easy to track both kinds of changes) should produce further examples. If the latter is true, it opens up the question of what sorts of constraints may be operating against such a strategy. Overall, although experimental examples of these phenomena remain tantalisingly few, a

case can be made for non-genetic changes to act as stepping stones for genetic adaptation (Fig. 4).

Figure 4



Non-genetic changes act as stepping stones to genetic adaptation: Non-genetic changes can lead to increased survival in a new stressful environment, relative to the wild type. (a) Populations incapable of mounting a short term advantageous non-genetic change (green cells) may perish under the stress (b) Populations capable of mounting a beneficial non-genetic change (green to orange transition) in response to environmental change survive (c) Populations that carry a pre-existing beneficial change also survive (b) and (c) lead to survival and allow time for spontaneous beneficial mutations to appear, thereby 'fixing' the beneficial phenotype.

Perspective

From the examples reviewed above, it is clear that non-genetic changes can alter mutational trajectories, uncover cryptic genetic diversity, and influence the direction of future genetic change. By doing so, they gain the power to affect long term adaptation and evolution. The categories discussed here are by no means comprehensive. For instance, some microorganisms show deviations from the universal genetic code (Ling et al. 2015), and decode the same codon differently depending on environmental cues (Prat et al. 2012). Altered decoding changes the codon to amino acid mapping, but can also go on to influence genotypic change. Jing Ma and Isaacs (Ma and Isaacs 2016) found that a 'recoded' *E. coli* strain in which the standard stop codon UAG was re-assigned to UAA (Lajoie et al. 2013), was resistant to several viruses. A reversal of this genome wide recoding from UAA back to UAG restored viral infectivity. Interestingly, after just five days of propagation in recoded *E. coli*, bacteriophage MS2 regained the ability to infect the strain via mutations in two genes; one mutation altered its own UAG stop codon to UAA, while the other created a new premature stop codon in lieu of UAG (Ma and Isaacs 2016). In addition, the recoded *E. coli* strain was unable to take up conjugative plasmids, potentially altering future genotypic change through horizontal gene transfer. Therefore, re-assignment of even one stop codon to another stop codon (UAG to UAA) can have consequences on host genotype as well as the genotype of co-evolving organisms.

Other non-genetic changes have been demonstrated to impact long term adaptation even though they are neither directly inherited nor impact DNA sequence. For example, Bodi et al constructed two gene expression systems for a gene encoding a multidrug transporter pump in *S. cerevisiae*. One involved a positive feedback loop, the other did not (Bodi et al. 2017). During selection for drug resistance, the one with the positive feedback loop — which also showed greater variance in gene expression —adapted faster. There was no difference in mutation rate between the two evolving sets. While phenotypic heterogeneity arising from increased variance in gene expression levels can seemingly contribute to adaptation under stress (Carey et al. 2018), it also extracts a cost during normal conditions (Bodi et al. 2017), creating a trade-off. Recently, we found that mistranslating *E. coli* cells show greater variability than the WT in cell size and division time (Samhita et al. 2020b). Again, this is correlated with higher survival under some stresses. We do not know if specific protein variants consistently dictate the survival advantage under specific environments, nor in what manner these may go on to influence genetic change. As is clear from the examples discussed

in this article, in recent years, translation errors have emerged as important contributors to altered phenotype and adaptation. DNA remains the primary code of life. Like with all codes though, the key that decodes it can conceal or reveal it to different degrees. How much of the genetic change that we measure today was preceded by non-genetic changes? Do cells employ errors in translation and transcription as strategies to generate variation? Have cells evolved to rely more on genetic change in some environments and more on non-genetic changes in others? These and several other open questions remain, as experiments broaden our knowledge of non-genetic changes.

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