EVOLUTIONARY CONSEQUENCES OF EPIGENETICALLY INDUCED PHENOTYPIC SWITCHING

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In this book chapter, we discuss the importance of phenotypic switching with respect to adaptation. Hereby, we focus on epigenetic mechanisms of phenotypic switching that are involved in gene expression regulation. We start by placing the discovery of phenotypic switching in the context of the history of evolutionary biology. We highlight how the controversy about whether phenotypic switching can contribute to adaptation arose. We then present a non-exhaustive list of examples of epigenetic phenotypic switching in nature. Finally, we discuss several evolutionary hypotheses about the role of phenotypic switching in adaptation to new and fluctuating environments, and give examples of how these hypotheses have been addressed experimentally.

Biological systems are characterized by stunning diversity. This diversity is reflected in variation of observable characters (i.e. phenotypes) both between individuals within the same and between different species (Darwin, 1859). Studying how this variation among individuals in the same population is generated and maintained, and how it translates into the differences that we can observe within and between species is the essence of evolutionary biology. The main sources of observable phenotypic diversity are genetic changes that are stably transmitted to subsequent generations (Fig. 1). Natural selection acts on these inherited phenotypes by preserving the beneficial and erasing the deleterious ones, causing a corresponding change in frequency of the underlying genetic determinants in the population. However, phenotypes can also be determined and maintained by molecular mechanisms that are independent from the underlying functional DNA sequence. These epigenetic mechanisms act through the regulation of gene expression causing different developmental outcomes.

Epigenetically induced phenotypic states are not as stable as genetically determined ones, and are characterized by high reversion rates to the original phenotype. Due to the unstable nature of epigenetic inheritance, its contribution to the process of evolution is questioned. In this chapter, we cover the current knowledge of molecular mechanisms of epigenetic inheritance and its evolutionary consequences, specifically in the context of discrete phenotypic switching. To better understand why the role of epigenetic mechanisms in the process of evolution is contentious, it is important to first introduce the historical development of evolutionary thought and the importance of inheritance in this process.

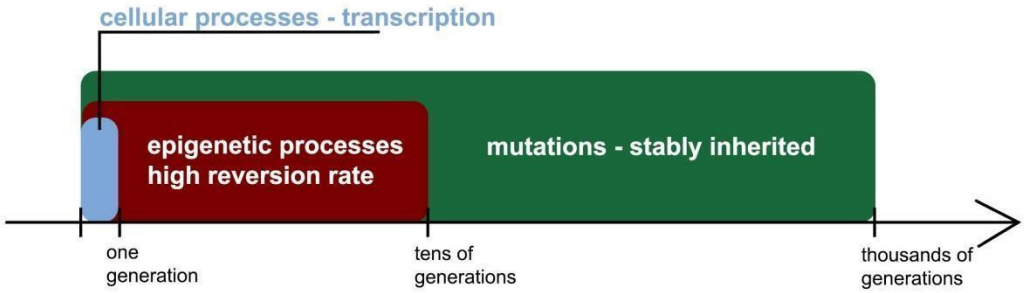

Figure 1. The timescale of inheritance. The boxes represent the time period for which particular forms of inheritance (differently colored boxes) persist. Mutations are stably inherited and are a substrate for natural selection. The timescale over which the inherited mutation is maintained in the population depends on its consequences on the phenotype. Certain cellular processes are maintained for only one generation and are of no direct importance for evolution. Epigenetic states of gene expression can be maintained for tens of generations. The figure was designed and made by Inês Amaro.

Evolution and the principle of inheritance

During the 17th century, the vast diversity of the natural world became apparent to the scientific community, and the first attempts to catalogue and document phenotypic variation began. One of the scientific pioneers in this field was John Ray (1627-1705). He classified groups depending on their similarities, and termed as species any group that shares characteristics, which distinguish them from another group. For species to exist, he realized that these characteristics must be stably transmitted from parents to their offspring, making him one of the first to recognize the connection between inheritance and interspecies variation.

A similar stance was adopted by Carl Linnaeus (1707-1778), who embarked on one of the most comprehensive efforts to catalogue and classify all living groups. He devised a system of classification in which species were grouped into higher order associations depending on their morphological similarities. For Carl Linnaeus, and most of his contemporaries, species were unchangeable and were created to perfectly fit the environment they inhabit (Mayr, 1982).

The view of immutability of species was challenged by, among others, Jean-Baptiste Lamarck (1744-1829), who provided the first theoretical framework for the evolution of species. In his work "Zoological Philosophy-an exposition with regard to the natural history of animals", Lamarck postulated that through constant use or disuse of organs an individual would further develop the organ or degenerate it. Moreover, this change in the morphology of the organ would then be passed on to the next generation (Lamarck, 1809). Whether an organ would be used or not depends on the environmental conditions. In Lamarck's view, environmental cues act as inducers of a morphological change, creating in such a way variation within a population that is beneficial in the

given environment. Inheritance in Lamarck's theory plays a significant role, as it enables the transfer of environmental information between generations.

The crucial importance of inheritance was also acknowledged in the fundamental formulation of evolutionary theory by Charles Darwin (1809-1882), and independently, by Alfred Wallace (1823-1913). Darwin conducted research on understanding the variation between individuals, as well as the geographical distribution of species. The results from his studies led him to the formulation of the theory of evolution through natural selection that he presented in his work "On the origin of species". For Darwin, variation between individuals is the basis of the evolutionary process. On the other hand, the mere existence of variation is not enough for evolution to proceed. Inheritance of variation is of paramount importance, or how Darwin formulated it: " Any variation that is not inherited is unimportant for us" (Darwin, 1859). In Darwin's view, evolution stems from the Malthusian principle by which populations with infinite resources will geometrically increase in number. However, since resources are always limited, competition arises between individuals. Those phenotypic characteristics that enable better survival in a given environment will be preserved. On the other hand, those that are not beneficial will be purged, over time, from the population. In contrast with Lamarck's theory of inheritance of acquired characteristics, where the environment acts as both an inductive and selective force, in the theory of natural selection the environment has the sole role to select for beneficial variation. Variation is created in the parents' germline and transmitted to the offspring. Darwin's theory holds as long as there is heritable variation within a population, no matter the underlying mechanism of inheritance and the generation of the variation. In Darwin's words "owing to this struggle of life, any variation, however slight and from whatever cause proceeding, if it be in any degree profitable to an individual of any species…, if useful, is preserved".

As we have seen, inheritance is an inseparable part of the evolutionary process. At the time Darwin published "On the Origin of species" the nature of inheritance was a complete mystery. Discovery of its underlying mechanism only happened during the end of the 19^{th} and the beginning of the 20^{th} century. Today, we know that phenotypic information is transferred through the genetic code. The discovery of genes as the carriers of the inheritance of phenotypic characters proved to be paramount for explaining evolutionary processes. However, observations of phenotypes that can be transmitted by mechanisms that are on first sight independent of the genetic code, i.e. epigenetic inheritance, in the 20^{th} century brought the question of inheritance back into evolutionary theory. In the following paragraphs we will cover the relevant discoveries of the genetic basis of inheritance and its importance for the evolutionary theory.

Unknown to Darwin, and in parallel to his work, Gregor Mendel (1822-1884) was conducting his research on the nature of inheritance. In his studies on the phenotypic variation in plants he crossed pea plants with different phenotypic variants (seed shape, flower color, plant length) and examined the phenotype of their offspring. He concluded that the subsequent generation (F_1 generation) will usually inherit the characteristic form of one parent, which he termed as dominant, and the other character as recessive. However, after another round of crossing (F_2 generation) the recessive character would reappear always in a 1:3 ratio within the offspring, compared to the dominant character. As Mendel pointed out, this particular pattern of inheritance is possible only if these

characters are determined by independent particles that are segregated in the parents' reproductive organs and assorted independently in the offspring (Mendel, 1865). This was the first evidence of the particulate basis of the inheritance and a first hint of its molecular mechanism. At first thought incompatible, Darwin's theory and Mendel's discovery complemented each other and proved crucial for the further development of evolutionary theory.

The nature of Mendel's factors remained a mystery until the end of the 19th century. It was Wiliam Johannsen (1857-1927) who coined the term „genes" for these factors (Churchill, 1974), as well as the terms „phenotype" for the observable characters of an individual and „genotype" for the underlying genetic determinants. Around the same time in his work Hugo de Vries (1848-1935) refered to „mutations" as any changes in the observable characters that deviate from those found in other individuals of the same species (Lenay, 2000). With the work of Thomas Morgan (1866-1945) and co-workers it became evident that genes are located on chromosomes, cellular structures that were suggested previously to be carriers of phenotypic information (Gilbert, 1998). Moreover, Morgan was also the first to suggest that mutations are alterations in these cellular structures.

However, since chromosomes were shown to be complex structures that are composed of proteins and deoxyribonucleic acid (DNA), the molecular basis of inheritance was elusive. Two schools of thoughts competed, one claiming that the proteins are the carriers of information and the other pointing out the importance of DNA in the inheritance process. The debate was put to rest by the experiments conducted by Oswald Avery (1877-1955) and co-workers. In his studies, he examined the transformation of a non-virulent bacteria (rough colony, R strain) into a virulent phenotype (smooth colony, S strain) when exposed to heat inactivated, virulent S strain. He noted that if the solution with mortalized virulent bacteria was treated with proteases, enzymes that degrade proteins, before the transformation procedure, the R strain would acquire the virulence capacity. However, if the solution was treated with enzymes that specifically degrade DNA, the R strain would not become virulent. These results clearly showed that the phenotypic information, in this case virulent phenotype, is encoded within the DNA (Avery et al., 1944). The discovery of its chemical composition and of the secondary structure showed that the information is contained in the sequence of the nucleotides within the DNA chains and the mutations are the result of the alteration of this sequence. These discoveries shed light on evolutionary processes as well, and completed the theory of natural selection.

The final unification of the particulate theory of inheritance and evolutionary theory happened in the works of Ronald Fisher (1890-1962), Sewall Wright (1889-1988), Sergei Chetverikov (1880-1959) and John Haldane (1892-1964). Fisher in his work "Genetical theory of natural selection" showed that fitness increase depends on the additive genetic variation in the population (Fisher, 1930). Here, fitness is considered as the long-term reproductive success of an individual. Mutations appear randomly in genes and are the source of phenotypic variation. Some mutations are neutral and have no observable effect, whereas others decrease (deleterious mutations) or increase (beneficial mutations) fitness.

All of the four mentioned scientists showed independently how the frequency of alleles, i.e. different genetic variants, in the population would change when we take into account natural selection (Provine , 1971). In an infinitely large, randomly mating, population that is not under selection, and in which different alleles exist, an equilibrium frequency of these alleles will be established (Hardy, 1908; Weinberg, 1908). If selection acts on such a population, the equilibrium will be changed. Here, the change in frequency of an allele depends on its fitness effect. Moreover, the new equilibrium frequency of alleles in the population under selection depends on the relative difference between their corresponding fitness effects (Provine, 1971).

Independently, Fisher, Haldane, and Wright also devised ways of representing the evolutionary process by means of a fitness landscape (Fig. 2; Fisher, 1930; Wright, 1932; Haldane, 1932). The concept of the fitness landscape aims at constructing a map of the connection between phenotype/genotype and fitness. In a two-dimensional fitness landscape, two axes represent phenotypic values or genotypes and a third one fitness. This results in a three dimensional landscape in which valleys represent genotypes with low fitness effects and peaks represent genotypes with great reproductive success. During evolution, mutations change the genotype which causes populations to move on the landscape until they reach a fitness peak. According to this simplistic theoretical concept, reaching a peak means that no single step mutations are available that would further increase fitness.

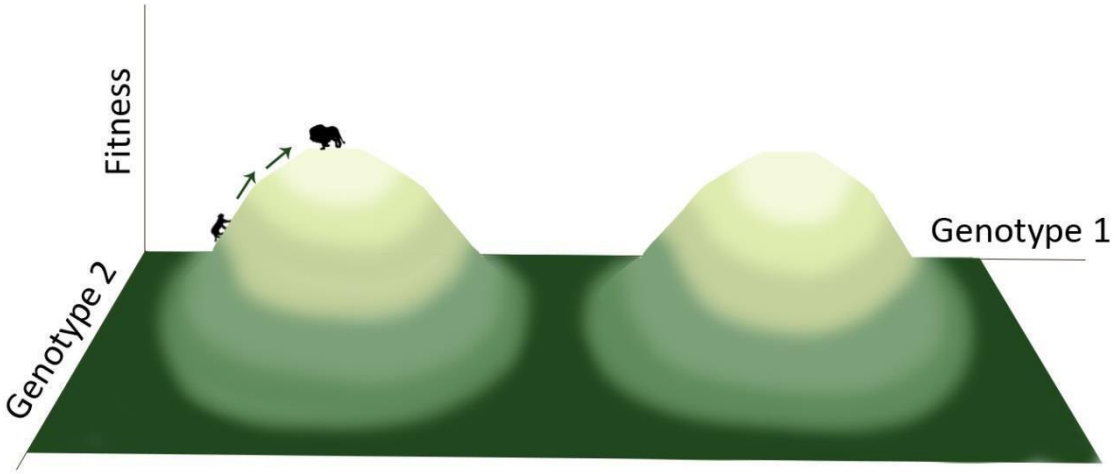

Figure 2. Representation of a fitness landscape. The scheme represents a two-dimensional fitness landscape with genotypes as two axes and fitness as the third. The landscape consists of two fitness peaks separated by a fitness valley. A population (here represented as a figure of a cat) evolves by acquiring mutations (represented by arrows) that move it towards the fitness optimum (represented by a figure of a lion) at one of the fitness peaks. The figure was designed and made by Inês Amaro.

Although the concept of the fitness landscape can be misleading because of the gigantic complexity of the true genotype space, various evolutionary hypotheses have been derived from fitness landscapes models. For example, if the population is well-adapted and thus at a fitness peak, most new mutations are deleterious or neutral. On the other hand, if the population is further away from the peak, for example due to a change in the

environment, there is a greater proportion of mutations that are beneficial (Fisher, 1930; Fragata et al., 2019; Orr, 2006).

Following these works, the Modern Synthesis, the final unification of Mendelian laws of inheritance and Darwin's theory of natural selection was born (Huxley, 1942). In summary, heritable variation is created by random changes in the DNA sequence. These mutations can have a significant impact on reproductive success. Depending on their fitness effect and the population size and structure, evolution happens when the frequency of genotypes changes over time.

However, the phenotype is not only the result of a nucleotide sequence. It is an outcome of the complex interactions that occur between the organism and the environment. Since the beginning of the 20th century it is known that the same genotype can produce different phenotypes depending on the environment that the organism is experiencing during the development, a phenomenon known as phenotypic plasticity (Bradshaw, 1965). Moreover, in some cases the phenotype of an organism can switch between generations at a certain rate, a phenomenon known as phenotypic switching. This can happen due to the stochastic nature of the underlying developmental mechanisms or it can be induced by an environmental cue.

A beautiful example of an environmentally induced phenotype is seen in the butterfly *Byciclus anynana*. This butterfly exists in two distinct morphological forms depending on the season. During the rainy season, the butterfly develops colorful wings with ring patterns, whereas in the dry season it produces wings that are more conspicuous and resemble the color of its surroundings (Shapiro, 1984). It was shown that this phenomenon is adaptive, since the color patterns help the butterfly to avoid predators (Lyytinen et al., 2003). On first sight, this case resembles the principles of Lamarck's view of evolutionary mechanisms. However, the phenomenon is quite distinct. In Lamarck's view, an environmental cue acts as an inducer of phenotypic change but at the same time as the selective force. In this example of environmentally induced phenotypic switching, the inductive cue (availability of water) is quite different from the selective force (predators).

The first attempt to explain phenotypic plasticity and its possible contribution to evolution was made by Conrad Waddington (1905-1975). To this end, he introduced the concept of the epigenetic landscape. According to this concept, genes that determine a phenotype are functionally interacting with each other resulting in a network that interacts with complex environmental cues. Out of this interaction, an epigenetic landscape arises that dictates how an organism will develop. The environmental cues can alter the landscape through epigenetic changes, resulting in the development of different phenotypes(Waddington, 1957). Today, the concept of epigenetic inheritance goes beyond Waddington's model. In modern terms, epigenetic inheritance is comprised of many different mechanisms (some of which can be inherited seemingly independently of the underlying DNA sequence) that modulate gene expression and cause production of different phenotypes. In the next part, we will introduce the molecular mechanisms that can create epigenetically determined phenotypic switching and highlight examples of epigenetic mechanisms of gene expression and its effect on phenotypes and their stability.

Molecular basis and examples of epigenetically determined phenotypic switching

Epigenetic inheritance represents the transfer of information from one generation to the other independently of the underlying DNA sequence, both in the case of mitotic and meiotic transmissions. Epigenetic inheritance is enabled by many interconnected molecular mechanisms that mainly involve the regulation of gene expression: DNA methylation, histone modification, positive protein feedback loops, and transfer of regulatory RNAs (Jablonka and Raz, 2009). As explained below, through these mechanisms different phenotypic states can be achieved in the same population. As a result of environmental input or due to an intrinsic stochastic nature, phenotypes induced by epigenetic means can switch between generations (i.e. phenotypic switching). Here, we mainly focus on the molecular basis and phenotypic effects of stochastic epigenetically determined phenotypic switching. Before we explain the molecular mechanisms in detail, it is important to note that even though an epigenetic state is inherited independently of the underlying DNA sequence, the ability to produce epigenetic changes is encoded genetically. Particular epigenetic states are determined by the underlying sequence and are strongly dependent on this sequence. What makes the described mechanisms epigenetic is that for the same genotype different gene expression states, e.g. active or silent, can be observed and sometimes propagated through cell divisions during the development of an individual, or from one generation to the other (Moazed, 2011).

Firstly, we will briefly describe each of the four known epigenetic mechanisms that are involved in gene expression regulation and their mode of inheritance. DNA methylation is a process that consists of the addition of methyl group residues to the cytosine ring of the DNA chain. Methylation does not happen randomly along the genome, but on sequence specific stretches of DNA that are recognized by specific enzymes, called methyl-transferases. The sequences that are methylated are usually palindromic and are methylated on both strands. After cell division, the next generation inherits DNA in hemi-methylated state, where only one strand contains a methyl group. In the subsequent step, this methyl group acts as an anchor for the molecular machinery that adds a methyl group to the complementary strand, which restores the methylation pattern (Heard and Martienssen, 2014).

Transfer of information through modifications of histones is similar, in principle, to DNA methylation. Here, the modification happens on the protein level. Histones are proteins that form multi-protein complexes around which DNA is wrapped. Some of these histones (H3 and H4) have extensive N-terminal arms protruding from the complex that can be modified by the addition of different chemical groups such as phosphoryl, acetyl, methyl or ubiquitin. The effect of modifications on gene expression depends on the chemical group that is added, the genomic context, and also on the position and amino acid to which the chemical group is attached. For example, the addition of a methyl group on the lysine residue of histone H3 at positions 9 or 27 leads to gene repression. However, the same modification on lysine at position 4 leads to gene activation (Berger, 2007). The principle of inheritance of histone modifications is similar to DNA methylation. The modified histones are distributed randomly between cells during the division and act as anchors for histone modifying protein complexes

that, in turn, modify neighboring histones, which reestablishes the local chromatin state (Fig. 3).

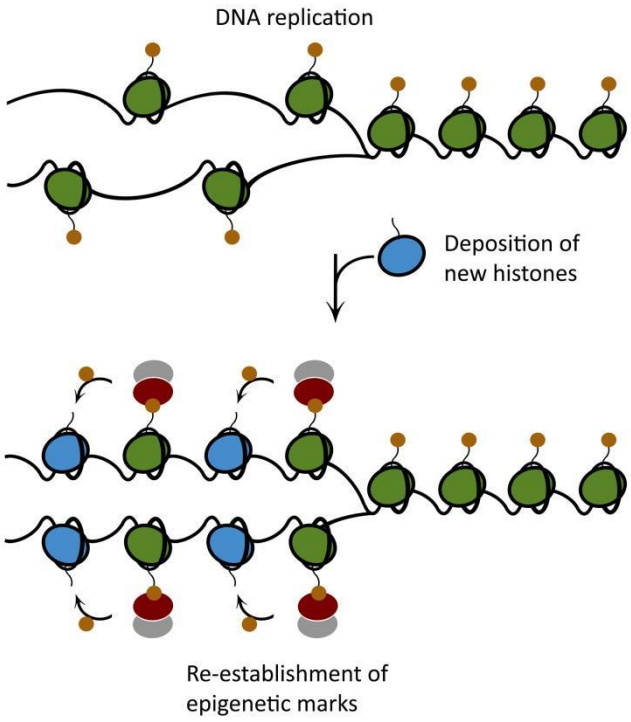

Figure 3. Inheritance of histone modifications. Histones are proteins that form multi-protein complexes around which DNA is wrapped. During DNA replication parental histones (green) with particular epigenetic modifications (orange) are randomly distributed between the two newly synthetized DNA strands. Subsequently, newly produced histones (blue) are incorporated. The chromatin remodelling complex, consisting of a subunit that recognizes the parental histone modification (red) and an enzyme that modifies in the same pattern the neighboring histone (grey), re-establishes the pattern of paternal epigenetic marks in the daughter cells. The figure was designed and made by Inês Amaro.

Regulatory RNAs (such as siRNAs and miRNAs) are short 20-25 nucleotide-long chains that bind to the growing mRNA during transcription through complementary binding, which forms a short double strand stretch that represents a recruiting platform for histone modifiers that modify the local chromatin, usually causing its compaction and gene inactivation. During meiosis, regulatory RNAs are maternally inherited via the cytoplasm of the oocyte where they are usually stored during oogenesis.

Transcription regulating protein feedback loops represent a system of gene regulation in which a protein is acting as an indirect (e.g., via cross-feedback) or direct transcriptional activator of the gene that encodes its own synthesis. The protein itself is usually inherited through the cytoplasm as is the case with regulatory RNAs. The transcriptional state of the gene in this case is determined by the protein concentration thresholds in the cytoplasm.

All these mechanisms can have a profound effect on the phenotype (as we will discuss via examples below) and create phenotypic variation within a population, which, as we saw, is the first necessary prerequisite for evolution. However, they are all characterized by low stability and low fidelity of inheritance compared to genetic changes. Since inheritance is paramount for evolution, the effect of such a system of generation of phenotypic variance on adaptation is questionable. However, there is increasing evidence that they provide short-term adaptive value, which we will cover in the rest of this chapter.

Phenotypic switching and epigenetic inheritance are present in all life forms, from bacteria to mammals and plants. One of the oldest examples of phenotypic switching

was described by Carl Linnaeus. As a strong advocate of the immutability of species, he thought that each group of beings was created to fit perfectly to its natural surroundings. However, this view was challenged in 1742 by the finding of student Magnus Zioberg, who discovered, on an island near Stockholm, a particular variety of a common toad-flax (*Linaria vulgaris*, an outcrossing plant). This variety exhibited a completely different flower shape and morphology from the more common plant form. Regular flowers of *Linaria* have five petals that are united to form a corolla tube, which is characterized by clear dorso-ventral asymmetry (Fig. 4A). The variety that was found by Zioberg contained flowers with petals that formed five spurs with a distinct radial symmetry (Fig. 4B) (Gustafsson, 1979).

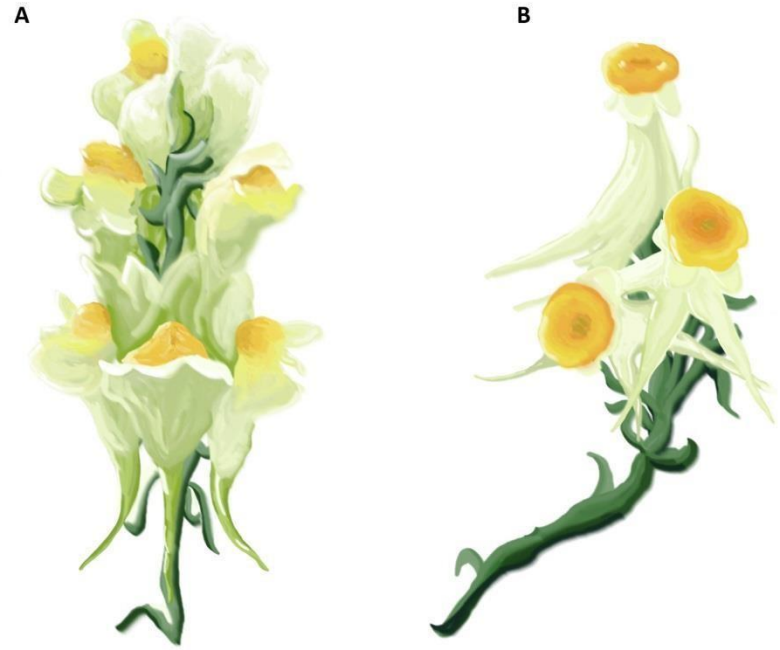

Figure 4. Pelorism in *Linaria vulgaris.* (A) Common form of flower shape in *Linaria.* The petals form a corolla tube with a distinct dorso-ventral asymmetry. **(B)** Peloric flower shape in *Linaria.* Petals form five spurs with a distinct radial symmetry. The figure was designed and made by Inês Amaro.

However, the peculiarity of the specimen was not only in the flower morphology, but was reflected also in the fact that from one generation to the other the "aberrant" plants would produce offspring with a regular flower shape, more typical to the common toad-flax. Carl Linnaeus noted what is probably one of the oldest observations of phenotypic switching: "Nothing can, however, be more fantastic than that which has occurred, namely that a malformed offspring of a plant which has previously always produced irregular flowers now has produced regular ones. As a result of this, it does not only deviate from its mother genus but also completely from the entire class and thus is example of something that is unparalleled in botany so owing to the difference in the flowers no one can recognize the plant anymore". This plant eventually led Linnaeus to revise his position on immutability of species and adopt a view in which species could change through the process of hybridization, the view that he would also use to explain the appearance of the variety of toad-flax with malformed flowers. However, as a

consequence of this, the "reputation" of the plant would suffer as Linnaeus infamously named it a "monster" (Peloria in Greek) (Gustafsson, 1979).

The phenomenon of peloric flower morphology was, soon after, observed in other plant species as well, and was taken up by the first pioneers of genetic theory of inheritance as an example of the effect of random mutational changes in the genetic code (de Vries, 1901). In his studies of the mutations, De Vries estimated the mutation rate for the peloric variant of toad-flax to be around 1%, which is, as we now know, an unusually high mutation rate for a genetic change. Further observations of the nature of pelorism, made not in toadflax, but in a related species, *Antirrhinum*, showed that the peloric phenotype is inherited in Mendelian fashion and results in a 1:3 ratio of phenotypes in the F_2 generation, indicating that a genetic change is responsible for the phenomenon. This observation was first made by Charles Darwin in experiments in which he crossed the mutant specimens with the regular plant variety (Darwin, 1868). Even though he essentially obtained the same result as Mendel did in his studies on the nature of inheritance, Darwin did not understand the importance of the findings. It would take almost a hundred years to discover the *cyc* gene that is responsible for the regulation of flower shape. The mutation in this gene was associated with the peloric phenotype (Stubbe, 1967). Although this was discovered in another species, the principle of genetic change was used to explain the pelorism phenomenon in *Linaria* as well, and for most of the scientific community the story of "monster" was put to rest. Probably this is the reason why it took until the end of 20^{th} century to discover the molecular mechanisms of pelorism in toad-flax.

The symmetry of the flowers in *Linaria* is controlled by a homolog of the *cyc* gene of *Antirrhinum*, *Lcyc*. In the study of the nature of pelorism in toad-flax, it was shown that the expression pattern of this gene in the peloric form of *Linaria* corresponds to the pattern observed in mutant *Antirrhinum*. However, no genetic change was associated with the peloric form of toad-flax. The phenotypic information and its inheritance in this case was determined to be dependent on one of the molecular mechanisms of epigenetic inheritance. The mechanism in question, in the case of *Linaria*, is DNA methylation (Cubas et al., 1999). This modification leads to a change in gene expression. In the peloric form of toad-flax, the *Lcyc* gene was shown to be highly methylated in contrast to the form with regular flowers. Two hundred years after the discovery of pelorism, this study clearly showed that the phenomenon was independent of genetic change. In one of the finest examples of scientific irony, what was considered as the first example of the effect of genetic mutations on the phenotype became the oldest example of the effect of the epigenetic inheritance on selectable traits, and the first measurement of mutation rate turned out to be the oldest analysis of the rate of phenotypic switching.

Examples of phenotypic switching are not exclusive to plants. However, due to the non-existence of a clear distinction between somatic and germ cell lines, the evidence of epigenetic inheritance is more abundant in plants than in animals. In animals, where the germline-soma distinction is established very early on during the post-fertilization development, epigenetic reprogramming erases most epigenetic marks (Monk et al., 1987; Platz et al., 1975). However, some of these marks can still be transmitted and affect the phenotype of subsequent generations (Brykczynska et al., 2010; Hammoud et al., 2009; Messerschmidt, 2012). One such case was observed in *agouti* mice. These

mice carry an insertion of a transposable element just upstream of the *agouti* gene that determines, among other phenotypic traits, the color of the fur. The transposon itself can be differentially methylated and, consequently, change the transcription level of the neighboring gene (Morgan et al., 1999). This results in mice with variegating yellow color of the fur and altered susceptibility to obesity. The methylation state of the locus is maternally transmitted and can also be influenced by the environment. Food with high levels of methyl donors can increase the methylation state and increase the frequency of yellow fur color in the offspring (Daxinger and Whitelaw, 2012). The phenotypic trait in this case can persist for up to two generations before switching to the initial methylation state (Morgan et al., 1999).

Phenotypic switching is also present in unicellular organisms. The bacterium *Photorhabdus luminescens*, a mutualistic partner of the nematode *Heterorhabditis bacteriophora*, can exist in two distinct phenotypic forms, long-shaped rods and shorter rods. Additionally, the form with long rods produces enzymes and metabolites such as proteases and cell-clumping factors that the shorter form cannot produce (Akhurst, 2009). This affects the capability of the bacteria to establish a mutualistic relationship with the nematode. Shorter rods are no longer capable of supporting nematode growth and development. The switch from the long form to the shorter one occurs upon infection of an insect larva by the nematode that contains the bacteria. Once the nematode is inside the insect, the bacteria are released into the hemolymph of the larva, causing an infection and subsequent death of the infected victim that then serves as food for the nematode (Eckstein and Heermann, 2019). The phenotypic switch is induced by stress that bacteria experience upon their release into the hemolymph of the insect (Joyce et al., 2006). The molecular basis of this phenotypic change is a positive protein feedback loop. The responsible protein, HexA, acts as an auto-activator of its production and was shown to be responsible for the increased pathogenicity of the shorter form (Joyce and Clarke, 2003; Langer et al., 2017). It is produced as part of cellular stress response mechanism and its levels are maintained by self-perpetuating mechanisms that consequently preserve a certain proportion of the shorter form of bacteria in the population.

These examples clearly show that epigenetic phenotypic switching can have a profound effect on phenotype and on the generation of phenotypic variation. In order to determine its effect on adaptation, it is of paramount importance to understand the stability of inheritance and the rate of switching. This rate is dependent on the organism, the mechanism of switching, and the environment. However, in all identified examples, it is consistently higher than mutation rate.

In plants, particular epigenetic marks can be maintained for several generations. Studies that used *Arabidopsis* epigenetic recombinant inbred lines (epiRILs), that were created through several rounds of inbreeding of epigenetically diverse population with varying DNA methylation patterns (Johannes et al., 2009; Reinders et al., 2009), showed that certain unmethylated loci can be inherited for up to 7 generations (Johannes et al., 2009). Moreover, particular epigenetic marks explained up to 90% of the broad-sense heritability of flowering time, and >50% of the observed phenotypic variance (Cortijo et al., 2014).

Epigenetic inheritance can be maintained in animals to a similar extent. In the nematode *Caenorabditis elegans*, high temperature was used as an inducing cue that caused a derepression of the heat-shock responsive HSP90 gene array. This change in expression was maintained for up to 14 generations and was clearly linked to the changes in the methylation levels of lysine at position 9 of histone H3 (Klosin et al., 2017).

The direct measurement of the rate of switching is an experimental challenge. Firstly, this is due to the difficulty in choosing the phenotypic readout to measure in the process, and secondly, because the switching rate tends to be much higher than the genetic mutation rate. Therefore, the traditional protocols for the measurement of mutation rate, such as the fluctuation test, cannot be used. Nevertheless, in a recent study using budding yeast, a more direct experimental estimation of the rate was made (Dodson and Rine, 2015). The yeast strain was labeled using a GFP marker (conferring green fluorescent color to the cells) flanked by 3´and 5´parts of RFP gene (conferring red fluorescent color to the cells). In a constitutively epigenetically silenced region, a gene encoding Cre recombinase was introduced. Upon the activation of the gene due to epigenetic switching, the produced Cre recombinase cut out the GFP gene and reconstituted the RFP gene, causing a switch in the color of the cells from green to red. The frequency of the switch in color allowed for the measurement of switching rate from one epigenetic state to the other. It was determined that the epigenetic switching rate was on the order of 10^{-3} to 10^{-4} (Dodson and Rine, 2015), which is much higher than the mutation rate in yeast, which is estimated to be on the order of 10^{-7} to 10^{-8} (Lang and Murray, 2008).

In summary, phenotypic variation can be maintained through mechanisms independently of the DNA sequence and result in epigenetically determined phenotypic switching. This epigenetically generated variation can be maintained for several generations, though not as stably as mutations (Fig. 1). In the rest of the chapter, we will present possible mechanisms by which phenotypic switching might shape evolutionary outcomes.

Evolutionary consequences of phenotypic switching

As new examples of epigenetic phenotypic switching were discovered, the question about its role in evolution became more pertinent. Conrad Waddington, who was the first to introduce and define the epigenetic landscape, also made the first attempt to define the role of phenotypic switching in evolution. As we described previously, a single genotype can produce several phenotypes randomly or through the interaction with its environment. These phenotypes could serve as a transition step for new or pre-existing genetic variation, which, when subsequently fixed in the population, make the respective phenotype permanent. According to this view, the phenotype appears first in the population (Waddington, 1959; Behera and Nanjundiah, 2004). This process is known as genetic assimilation and represents the general theoretical framework that underlies modern concepts of evolutionary consequences of phenotypic switching.

From a theoretical point of view, the presence of stochastic phenotypic switching can have converse effects. In the simplest scenario of a stable, homogeneous environment,

phenotypic variation produced in the same genotype should result in a fitness disadvantage, because not all individuals express the optimal phenotype. On the other hand, in a fluctuating or heterogeneous environment or upon environmental change, phenotypic switching can theoretically provide a fitness advantage for two reasons. Firstly, an existing phenotypic switch can create the crucial phenotypic variation to deal with a new challenge more rapidly and at higher proportions than (even pre-existing) genetic mutation. Here, the epigenetic switching provides a bet-hedging strategy (Cohen, 1966), where the fitness disadvantage in the current environment is compensated later by the potential advantage upon the environmental change (Carja et al., 2014; Furrow and Feldman, 2014). Secondly, this absolute fitness advantage that phenotypic switching confers in a new environment enables populations to persist at higher numbers, which results in a higher probability of subsequent genetic mutations (Bonduriansky and Day, 2009; Klironomos et al., 2013). Thus, phenotypic switching might greatly promote the survival of populations in new environments, especially in scenarios of evolutionary rescue, in which a population is exposed to a deleterious environment where it would face extinction if it remained at its current phenotype.

Furthermore, theoretical studies have proposed that epigenetic switching can facilitate the transition of a population across a fitness valley (Fig. 5). For example, if both a genetic mutation and an epigenetic switch confer the same fitness effect, i.e. a knock-out mutation versus the transcriptional silencing of a gene, then the epigenetic switch could alter the fitness landscape and the distribution of fitness effects of genetic mutations (Klironomos et al., 2013). That is because the silencing of the gene would render all genetic mutations in the respective gene neutral. Genetic mutations that are deleterious in the active transcription form of the gene would have no effect on the phenotype once the gene is silenced. This could allow a population to maintain cryptic genetic diversity and to explore a greater part of the fitness landscape. Ultimately, this can change the evolutionary fate of the population (Klironomos et al., 2013). Here, the buffering mechanism of epigenetic switching attenuates deleterious fitness effects of mutations, which makes the valley appear shallower (Tadrowski et al., 2018).

Epigenetic switching cannot only affect evolution by changing the fitness effect of genetic mutations, but also by modifying the local mutation rate. It is known, for example, that methylated cytosine tends to convert to thymidine at a higher rate than the non-methylated form, usually through the process of spontaneous deamination (Ehrlich et al., 1986). As a result, methylated DNA has a higher propensity of acquiring mutations. On the other hand, methylation of the DNA can inactivate transposable elements and prevent their jumping across the genome. Indeed, it was shown in plants that lack of the methylation machinery causes an increase in the rate of transposable element insertions (Kakutani et al., 1995; Mlura et al., 2001). Histone modifications can also affect the mutation rate, because highly compacted regions of the genome are thought to protect the DNA from possible mutagenic factors. For example, it was shown that in human cancer cell lines, variation in a particular histone mark, the methylation of lysine on histone H3, can account for up to 40% of the variation in mutation rate (Schuster-Böckler and Lehner, 2012). Finally, in addition to altering mutation rates, chromatin structure can also alter the frequency of recombination, as it was shown in yeast where the absence of epigenetic silencing increases recombination events (Batté et

al., 2017). On the other hand, a genetic mutation can cause a change in the epigenetic switching rate, or its presence altogether. For example, mutations of cytosine in CpG islands (a type of DNA methylation) remove the epigenetic marks from that locus and thus change the epigenetic state. This is also true for histone marks that are dependent on the underlying genetic sequence.

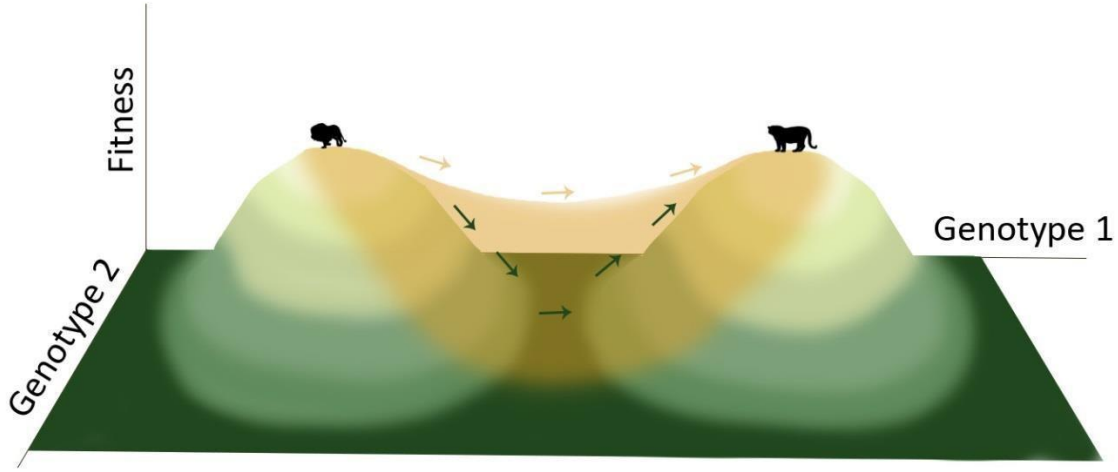

Figure 5. Representation of the possible effect of an epigenetically induced phenotypic switching on fitness landscape. A two-dimensional fitness landscape with two fitness peaks with a valley between them (see Fig. 2) is presented. A population in one fitness peak (here represented with a figure of a lion) could move to another fitness peak (represented with a figure of a panther) by acquiring mutations that would alter its fitness (darker arrows). These genetic mutations require the population to cross a fitness valley which might result in the possible extinction of the population and would ultimately inhibit the transition. Here, epigenetic switching could buffer the deleterious effects of the mutations, resulting in the alteration of the fitness landscape (yellow transparent cloud connecting the peaks) by making the fitness valley shallower. This would, consequently, facilitate the transition between the peaks. The figure was designed and made by Inês Amaro.

With respect to genetic adaptation, theoretical models predict that the effect of an epigenetic switch depends on the rate of switching itself and the fitness effect of epigenetic states (Kronholm and Collins, 2016; Tadrowski et al., 2018). When the switching rate is high, adaptation proceeds slowly and is mostly driven by genetic mutations, regardless of the fitness effect of the epigenetic change, due to the cost of switching to the suboptimal phenotype. Lower switching rates tend to speed up adaptation, but the final fitness value reached is usually smaller than in the case of high reversion rates.

A second determinant of the contribution of an epigenetic switch to genetic adaptation is the relative fitness effect of the switched phenotype compared to the effect of the respective genetic mutation. If those fitness effects have the same value, genetic adaptation will be slow and genetic mutations might never fix in the populations. On the other hand, if the effect of an epigenetic switch is too small there would be no benefit of switching at all. Populations reap the largest benefit if the epigenetic switch has a fitness effect that is close to the effect of mutations, but not the same. Thus, theory proposes that there exist intermediate optimal switching rates and fitness effects of epigenetic

changes that are most beneficial for adaptation (Kronholm and Collins, 2016; Tadrowski et al., 2018).

In spite of extensive theoretical work, experimental evidence for the contribution of epigenetically induced phenotypic switching to evolution is still scarce (Charlesworth et al., 2017). The reason for this, as exemplified above, is due to the intertwined connection between the epigenetic and genetic systems of inheritance and the difficulty of disentangling the phenotypic effect of one system from the other. However, certain attempts were made in this direction recently.

One experimental way of understanding the contribution of epigenetic phenotypic switching to evolution is to observe adaptation in an organism in which the known epigenetic machinery was shut off. In such a study (Kronholm et al., 2017), conducted in *Chlamydomonas*, histone acetylation and DNA methylation were manipulated both by knocking-out the responsible gene and by using an inhibitor of the enzymes likely to be involved in epigenetic regulation. The resulting strains that had an impairment in the epigenetic machinery were exposed to different stressful environments (salt stress, nitrogen starvation or high concentration of carbon dioxide). The results showed that the impaired strains did not adapt to these stressors as easily as the epigenetically capable wild type. The genomic and epigenomic data showed differences in methylation pattern in evolved clones, indicating that new epigenetic patterns arose or became active during the experiment.

One route to quantifying the adaptive value of epigenetic phenotypic switching is the use of controllable model systems where epigenetic and genetic effects can be more easily distinguished. Yeast offers an experimental system with great possibilities for genetic manipulations. This advantage was used in a study that examined the impact of protein feedback loops (i.e., dynamic regulatory interactions between genes) on adaptation (Bódi et al., 2017; see also Braun, 2015 and David et al., 2013). In the study of Bódi and co-workers, two yeast strains were constructed, one in which a positive feedback loop controlling the expression of a multidrug transporter gene was engineered (i.e., gene expression was variable and self-reinforcing, resulting in potential phenotypic switching), and another strain in which the expression of the transporter gene was enforced and constant. The expression of the transporter gene in the strains that contained the positive feedback loop showed bimodal expression patterns (implying phenotypic switching) and a higher variance in expression compared to the strain with constant expression. During a subsequent adaptation experiment, the strain with the ability to switch phenotypes evolved to higher levels of drug resistance. Moreover, the effect of the beneficial mutations that accumulated during the adaptation experiment depended on the presence of the feedback loop. This was shown by experimentally shutting off the feedback loop in the evolved strains, which resulted in lower fitness.

Another recent study conducted in budding yeast provided direct evidence that epigenetic phenotypic switching can aid genetic adaptation (Stajic et al., 2019), thereby confirming theoretical predictions (Behera and Nanjundiah, 2004; Lande, 2009). *Saccharomyces cerevisiae* has the particular characteristic that heterochromatin-like epigenetic silencing of genes occurs at three distinct locations in the genome. In one of these locations, the subtelomeric region, a *URA3* reporter gene was inserted at different

distances from the telomere (the end of the chromosome). Because distance to the telomere correlates with epigenetic silencing, this resulted in several yeast strains with different epigenetic silencing/switching rates. The reporter gene *URA3* is an essential gene that is responsible for the production of uracil, an essential nucleotide component of RNAs. On the other hand, in the presence of a particular drug (5-FOA), the activity of Ura3 protein is deleterious, since it converts the drug into a toxin that eventually kills the cell (Boeke et al., 1987). In an evolution experiment, the drug was used to select against the activity of the gene. Moreover, by analyzing the proportion of cells that were resistant to the drug but at the same time able to produce uracil, the experimental system allowed to easily distinguish phenotypically switching clones from carriers of genetic mutations that accumulated during the evolution. The authors observed that as the rate of gene silencing increases (up to 10^{-2}) the populations survived better in the drug environment. Consequently, the proportion of populations that escaped extinction was lowest in cases when the rate of silencing was low (10^{-6}). However, too high rates of switching delayed the spread of beneficial genetic mutations, probably because the efficiency of the switching system reduced the effective selective advantage of adaptive genetic changes. This supported the above-mentioned hypothesis that there exists an optimal switching rate that enables the fast appearance and spread of beneficial genetic mutations. Moreover, Stajic et al. showed that the spectrum of genetic mutations depended on the rate of epigenetic phenotypic switching. At low rates of silencing, most beneficial mutations were found in the uracil biosynthesis pathway. At higher rates of switching, mutations were increasingly observed in genes known to control epigenetic silencing. Interestingly, these mutations were shown to directly change the epigenetic switching rate by making an epigenetic state more stable. Thus, not only the presence but also the rate of phenotypic switching affected the speed and mechanism of evolution.

The empirical and theoretical examples highlighted in this chapter show that phenotypic switching might indeed be a significant factor for evolution. An important step is to quantify how much of the observed phenotypic variation in nature is due to epigenetic variation. This is a challenge due to the intricate connection between the epigenetic and genetic systems, as highlighted above. Moreover, our experimental examples show the potential power of epigenetic switching for adaptation. However, the above-mentioned studies were conducted in organisms that reproduce asexually. Sexual reproduction adds another level of complexity, because epigenetic marks tend to be erased during the process of gametogenesis. To demonstrate the importance of epigenetic switching for adaptation across the tree of life, it will be important to perform similar studies in more complex organisms in the future.

For a better assessment of the contribution of epigenetic phenotypic switching to evolution it is crucial to understand the fitness effects of epigenetically determined phenotypes across environments. It is likely that the effect size of functional epigenetic changes tends to be smaller than that of functional genetic mutations. That is because genetic mutations can completely alter the role and activity of a protein, whereas epigenetic changes only alter the gene expression patterns. Nevertheless, this can have profound effects on the interacting genes in biological pathways and the resulting phenotype. Considering the difficulties of detecting genetic mutations of small effect, it

will be a challenge for the field of evolutionary biology to develop approaches to detecting epigenetic mechanisms of small effect in natural data.

In this chapter we have argued that any variation in a phenotype that is inherited can be the basis for evolutionary processes. Specifically, epigenetically induced phenotypic switching can cause considerable changes to the phenotype and influence how organisms adapt to their environment. Considering epigenetic switching in evolutionary studies can be important, especially with respect to the response of populations to rapid environmental change or environmental fluctuations.

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