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Abstract

The role of natural selection in the evolution of adaptive phenotypes has undergone constant probing by evolutionary biologists, employing both theoretical and empirical approaches. As Darwin noted, natural selection can act together with other processes, including random changes in the frequencies of phenotypic differences that are not under strong selection, and changes in the environment, which may reflect evolutionary changes in the organisms themselves. As understanding of genetics developed after 1900, the new genetic discoveries were incorporated into evolutionary biology. The resulting general principles were summarised by Julian Huxley in his 1942 book *Evolution, The Modern Synthesis*. Here, we examine how recent advances in genetics, developmental biology and molecular biology, including epigenetics, relate to today's understanding of the evolution of adaptations. We illustrate how careful genetic studies have repeatedly shown that apparently puzzling results in a wide diversity of organisms involve processes that are consistent with neo-Darwinism. They do not support important roles in adaptation for processes such as directed mutation or the inheritance of acquired characters, and therefore no radical revision of our understanding of the mechanism of adaptive evolution is needed.

"Darwinism has been under constant scrutiny ever since On the Origin of Species *was published. The theory of evolution by natural selection, based on variation and selection, provided a hitherto unparalleled explanation of life's diversity and change, invoking no forces other than simple biological ones, such as heredity and mutation. One of the main ideas that derive from Darwinism – and, in my view, one of the most powerful ideas in the history of science – is that adaptation and design can arise without any ... guiding hand"* [1].

1. Introduction

During the 1930s and 1940s, the findings of classical and quantitative genetics were integrated into general evolutionary biology, in response to the population genetic models of evolutionary processes pioneered by Fisher, Haldane and Wright. The Modern Synthesis of evolution (MS) was named by Julian Huxley [2] to emphasise the wide acceptance of its principles as a framework for understanding the mechanisms of evolution, and for interpreting data on a wide range of biological phenomena. Its basic ideas remain central to contemporary biology, despite enormous advances over the past 80 years, especially those connected with the rise of molecular biology.

The core tenet of the MS is that adaptive evolution is due to natural selection acting on heritable variability that originates through accidental changes in the genetic material. Such mutations are random in the sense that they arise without reference to their advantages or disadvantages (i.e. their fitness effects), although their phenotypic effects are necessarily constrained by organisms' developmental systems [3, 4], as was recognised by the founders of the MS, e.g. [5]. Because this viewpoint asserts that natural selection acts to increase the frequencies of advantageous variants within populations, it is often referred to as neo-Darwinism.

Processes other than natural selection and mutation were, however, also included in the MS – most notably genetic drift (random fluctuations in the frequencies of variants in finite populations), which is the basis of the neutral theory of molecular evolution [6] that is widely used as a null model for interpreting data on DNA sequence variation and evolution. But a random process such as drift cannot explain adaptation, except when it acts in conjunction with selection, as in Wright's shifting balance theory [7]. A powerful theoretical argument for the predominant role of selection in adaptive evolution was provided by Fisher's discovery that (in modern

terminology) the evolutionary fate of a new mutation is controlled by the product of the effective population size (N_e) and the intensity of selection that it experiences [8]. A selection intensity of the order of the reciprocal of N_e can prevent a harmful mutation from spreading, or allow selection to promote the spread of a beneficial mutation. Even when selection is weak, it is therefore likely to dominate over drift and mutation pressure for most traits, except in species with very small population sizes.

There has, however, been a long history of proposed alternatives to the MS, including Goldschmidt's saltational theory of evolution by 'macromutations' creating coordinated adaptive phenotypes with multiple differences from their progenitors [9], and the Lysenkoist advocacy of the inheritance of acquired characters that dominated biology in the Soviet Union and its satellites for many years [10, 11]. In the 1970s and 1980s, advocates of punctuated equilibria, developmental constraints and molecular drive again challenged the MS [3], and claims for the Lamarckian inheritance of acquired characters were renewed [12]. These challenges were quickly shown not to raise serious difficulties, and the appearance of inheritance of acquired characters in immune responses was explained in terms of other processes [12]. Recently, however, several challenges to the MS have again been made, resurrecting some of these old criticisms and adding new ones. It is claimed that neo-Darwinism has overlooked important evolutionary factors, and must be supplemented by a self-proclaimed 'Extended Evolutionary Synthesis' (EES) [13-15], which "*is not just an extension of the MS, but a distinctively different framework for understanding evolution*" [14]. Some even propose that the MS needs to be replaced, e.g. [16].

In the present review, we evaluate one aspect of such claims: the central question of the source of the variability involved in adaptive evolution. Other aspects have been studied within the framework of the MS, and therefore do not seriously challenge neo-Darwinism. These include the roles of developmental constraints and phenotypic plasticity in evolution, and interactions of organisms with their environment in ways that influence their subsequent evolution, 'niche construction' [3, 4, 17, 18]. We therefore focus on empirical evidence relevant to the claim that natural selection acting on 'random' mutations is inadequate to explain adaptive evolution [14-16, 19-21] (see also the website *www.thethirdwayofevolution.com*). To avoid circularity, we define an adaptation as a trait that appears to be designed to fulfil an organismal purpose.

We critically examine the current status of evidence for proposed alternative mechanisms for generating adaptively useful variation, especially the inheritance of acquired adaptive characters and directed mutation. Our motivation for focussing on this topic is that neo-Darwinian evolution requires the transformation of a population over time as a result of natural selection. If variants tended systematically to arise when they are adaptive, many or all individuals in a population could acquire adaptations without the need for selection; this would indeed constitute a serious challenge to the MS. As John Maynard Smith once said "*.. the question of the origin of hereditary variation remains central to evolutionary biology, if only because Lamarck's theory is the only alternative to Darwinism that has been suggested*" [22, p.91].

Overall, based on recent research papers and reviews that exhaustively examine the proposed alternative processes generating variation, we find no evidence to support such a challenge. Indeed, modern research in population genomics is providing ever-stronger evidence for the footprints of natural selection [23-25].

2. Unconventional inheritance systems and adaptive evolution

"Before we rewrite the textbooks, divert funding initiatives, refocus our disease intervention strategies, or alter our view of neo-Darwinian biology, it is our obligation to attempt these simple tests to assure ourselves that we are not chasing a ghost" [26].

The EES and other recent critiques of neo-Darwinism claim that new discoveries undermine its core premise that random mutations are the source of the variation on which natural selection acts. Specifically, it is proposed that 'unconventional' modes of inheritance such as 'epigenetic' inheritance permit the transmission of acquired, adaptive characters [19, 21]. Point (vi) of Table 3 in [15] states that "*in addition to selection, adaptive variants are propagated through repeated environmental induction, non-genetic inheritance, learning and cultural transmission*"; point (vii) proposes that the induction of functional variants may help explain rapid phenotypic evolution.

We will not discuss cultural transmission, since this way of passing information between generations does not involve heritable processes as normally understood in biology, although of course cultural practices may affect biological

148 evolution in the small minority of species with advanced social behaviour [4]. Instead,
149 we focus on mechanisms that might allow adaptive phenotypic traits to become
150 expressed by all or most members of populations, without a neo-Darwinian
151 evolutionary process.
152
153 **(a) Classical genetics and inheritance**
154 The MS was based on the rules of inheritance discovered by classical genetics, which
155 apply to any stably inherited type of variant associated with a chromosome, whether
156 or not it involves a DNA sequence change. Early 20th century genetics showed that
157 most genetic variants associated with major phenotypic differences in animals, plants
158 and fungi are stably and biparentally inherited (Mendelian inheritance), and
159 chromosomally located, as was eloquently summarised by H.J. Muller [27]. It was
160 subsequently shown that inheritance in bacteria and viruses obeys fundamentally
161 similar rules [28]. Matrilineal inheritance also occurs, involving the transmission of
162 variants in plastid and mitochondrial genomes [29], or of cytoplasmic endosymbionts
163 such as *Wolbachia* [30]. The multifactorial theory of quantitative trait variability, and
164 its experimental validation, showed that Mendelian variants with small phenotypic
165 effects underlie heritable quantitative trait variation, acting together with non-genetic
166 factors [31]. These discoveries allowed population geneticists to model evolutionary
167 changes within populations; their results convinced biologists that natural selection
168 was highly effective as an evolutionary mechanism, contrary to other views that had
169 prevailed into the 1930s [31].
170 Some rare cases of unstable inheritance of mutant phenotypes, however,
171 initially remained puzzling. It is now known that these are often caused by disruptions
172 of gene function by insertions of transposable elements (TEs), whose excision can
173 sometimes restore the wild-type allele [32]. Because most TE insertions excise very
174 rarely, such mutations mostly follow Mendel's laws – indeed, many of the classical
175 mutations in *Drosophila* genetics [33], and in the sweet peas studied by Mendel,
176 involved TE insertions [34].
177 In recent years, the term 'genetic inheritance' has come to mean the
178 transmission of alterations in the DNA sequence (or RNA sequence, in the case of
179 some viral genomes), as distinct from a heterogeneous set of phenomena that do not
180 involve such alterations. In the next sections, we outline current knowledge about
181 these other processes, which have come to be called 'epigenetic' inheritance, and

consider their implications for the validity of the MS (see [35] and [36] for earlier discussions of this issue).

(b) Epigenetic inheritance processes

We define epigenetic inheritance as the transmission of epigenetic information between generations, distinguishing between two types of processes. The first (type 1) includes variants (epialleles) involving chromatin marks such as methylation of DNA basepairs and histones. Epialleles are defined as 'marked' allelic forms whose phenotypic effects (if any) depend on their epigenetic states, rather than on DNA sequence differences. Type 2 involves changes associated with regulatory molecules such as small interfering RNAs, which can be transmitted through the gametes, resulting in non-Mendelian inheritance. Both types can be associated with phenotypic effects, and could potentially allow characteristics acquired during the life of an individual to be inherited by its descendants, in the absence of any DNA sequence variants [19, 21].

In examining the role of type 1 epigenetic inheritance in evolution, we distinguish meiotically heritable but potentially reversible chromatin alterations at a site, without associated DNA sequence differences, from alterations controlled by sequence variants, either at the site or elsewhere in the genome. It can be difficult to determine whether epigenetic marks are transmitted across generations independently of DNA sequence differences [37, 38].

Several situations that are sometimes regarded as epigenetic inheritance do not involve transmission of informational macromolecules across generations, so that part of the controversy about the importance of epigenetic inheritance is semantic [26]. Here, we exclude phenomena such as direct effects of parental condition on the offspring in organisms like mammals, and maternal effects mediated through provisioning of the egg cytoplasm. Chemical treatments can pass from maternal parents and affect the progeny while they are developing, including the germ lines of both male and female progeny, so that effects can occur two or even three generations after exposure [39]. Both genetically and environmentally caused maternal effects have long been included in models of evolutionary processes [40, 41], and do not challenge neo-Darwinism.

There are, however, several questions concerning the evolutionary significance of epigenetic inheritance, some of which remain to be answered by future research.

- For how many generations do inherited epigenetic marks persist, and are they stable enough to affect evolutionary processes? For example, if advantageous to individuals, can they spread through a population and become almost fixed, or do they change back to the unmarked state too frequently for these marks to maintain adaptation? In evolutionary terms, what are the forward and backward mutation rates?
- What kinds of sequences in genomes are affected by these phenomena, and what fraction of the genome do they represent? Specifically, are the 'core genes' of organisms affected, or are epigenetic modifications largely confined to transposable element sequences or to other types of repetitive sequences? Are these effects due to processes that evolved to defend genomes against selfish 'genomic parasites' (particularly in the germ line)?
- Do epiallelic variants affect phenotypes?
- Does epigenetic inheritance contribute to variability in quantitative characters of evolutionary importance?
- Are epigenetically inherited changes an important source of adaptive change, compared to DNA sequence change?

In the following sections of the paper, we discuss several phenomena that are relevant to these questions.

3. Experimental evidence for epigenetic inheritance

(a) Epigenetic systems in defence against transposable elements and viruses

An initially very puzzling exception to Mendelian inheritance was provided by the phenomenon of hybrid dysgenesis, discovered in *Drosophila melanogaster* in the late 1970s, and which is now known to involve high rates of movement of certain types of transposable elements (TEs) [42, 43]. TEs can cause harmful effects on their hosts when they insert into coding or regulatory sequences. Other effects include

chromosome breakage when TEs insert or excise, and the production of chromosome rearrangements by recombination between homologous TEs in different genome locations. These harmful fitness effects of TEs often keep their frequencies at potential insertion sites low in natural populations, and generate selection on their hosts to suppress their movement [43, 44].

Hybrid dysgenesis occurs when a male that carries members of certain TE families is crossed with a female that lacks them [42, 43]. In the eggs of such mothers, the defence system in the cytoplasm fails to inactivate the TEs introduced from the father, which therefore transpose very actively in the offspring, causing sterility. Susceptibility to hybrid dysgenesis can be transmitted through the maternal lineage over several generations. The system whose failure causes hybrid dysgenesis involves elaborate molecular mechanisms that have evolved to defend genomes against TEs in both plants and animals [43, 45, 46], involving small interfering RNAs that are produced in response to the presence of TEs in the genome. The great diversity of sequences and genomic locations in which they can be inserted means that the mobility of TEs is their only common distinguishing feature; this is their 'Achilles' heel' that allows cells to detect them [46].

In animals, the RNAs involved in TE silencing belong to a class called piRNAs. In *Drosophila*, maternal TE-derived piRNAs are incorporated into the egg before fertilization, resulting in a form of epigenetic inheritance. However, the maintenance of effective TE suppression requires the presence in the DNA of genomic clusters of TE insertions, providing a 'memory' of previously active elements, like the immune memory systems that defend cells against previously encountered pathogens. Once acquired, these clusters of TE-derived sequences prime the resistance pathways anew each generation through a self-perpetuating amplification process called 'ping-pong', whereby the piRNAs produced by the clusters interact with those from active TEs to repress transposition [47, 48]. When maternally-derived piRNAs from TEs are not generated, there may be insufficient piRNA for repression, explaining the maternal inheritance associated with hybrid dysgenesis.

This intricate system is a biological marvel, which represents the outcome of natural selection to overcome the harmful effects of TE mobilization. Hybrid dysgenesis is simply a product of the temporary failure of this system; it is a transient, pathological phenomenon, and occurs in nature only when a new TE type is

introduced into a population, as is currently happening with the *P* element in *D. simulans* [43].

The non-nuclear transmission of small interfering RNAs provides, however, a potential mechanism for the inheritance of an adaptively useful trait acquired in response to an environmental treatment [47]. An example has been described in *Caenorhabditis elegans*, where small interfering RNAs derived from an RNA virus, conferring protection against infection, can be transmitted through the cytoplasm over several generations of self-fertilisation [49]. It remains to be determined how frequently such processes occur in nature.

(b) Paramutation

Another exception to Mendelian inheritance is paramutation [50, 51], whose discovery in maize involved puzzling interactions between two alleles at a single locus, in which a paramutagenic allele induced a heritable change in the expression of another (paramutable) allele, without changing its DNA sequence; the paramutated allele may itself become paramutagenic. Although paramutation looks like a form of directed mutation (see below), and the paramutated state can persist for many generations, the change is usually impermanent, decaying over time. Paramutation is now known to occur in fungi, animals and plants [51].

Genetic analyses have revealed that paramutation has similarities with silencing of transposons by small RNAs. Reactivation of an inactive piRNA-producing cluster in *Drosophila* can be induced by interactions with a different, but partially homologous, cluster within a genome to produce active, paramutated versions that can silence new TE sequences that insert into old or new clusters [51, 52]. This may explain the progressive establishment over several generations of repressive capacity after hybrid dysgenesis-producing *I*- or *P*-elements are introduced by paternal inheritance into a cytoplasm without *I*- or *P*-homologous piRNAs [52]. There is no firm evidence as yet that paramutation plays a role in adaptive evolution, although it could act like a type of meiotic drive [53], with the paramutated allele increasing in frequency in the population by propagating new copies of itself at the expense of alternative alleles. Rather, it appears to reflect a process that evolved in response to threats to genome integrity, and is strongly associated with the presence of repetitive DNA sequences [51].

(c) Stability of transmission of epigenetic marks across generations

Epigenetic marks such as DNA or histone methylation can undoubtedly be transmitted across cell divisions in unicellular organisms. Early in the history of genetics, it was recognised that transmission across cell divisions of phenotypic changes induced by environmental conditions could occur in protists, but tended to revert after several divisions. The best-studied example of such *Dauermodifikationen* [54] is serotype switching in *Paramecium*, in which temperature can affect which gene is expressed out of a large set that control surface antigens [55]. The functional significance of this plastic response is still unclear.

In multicellular organisms, the role of epigenetic chromatin modification in stable cell differentiation during multicellular development is also, of course, well established [26]. The crucial question for evolutionary biology is how often such marks are transmitted between generations via sexual reproduction, independently of any causal DNA sequence differences. For the development of a fertilised egg into an adult, it is important for the zygote to be totipotent, suggesting that epigenetic marks affecting gene regulation should normally be erased during germ cell production. This is indeed usually the case in animals, apart from some exceptions such as imprinted genes in mammals, where either paternally- or maternally-derived genes are inactive [26, 35, 39]. The most convincing cases of trans-generational inheritance of epigenetic marks in animals are associated with repetitive sequences, and it has been proposed that selection in favour of mechanisms that maintain repression of their expression has been responsible for the ability to transmit these marks across generations [56].

In plants, however, resetting of epigenetic marks such as methylation is less efficient that in animals, and there is evidence from crossing experiments for transmission of methylation states across generations [57] especially methylation of C at CpG dinucleotide sites [57-59]. The methylation status of such C sites is, however, quite unstable, with a higher frequency of losses than gains, and overall ‘mutation’ rates around 10^{-4} per basepair per generation, 5,000 times higher than those for DNA nucleotide changes. Despite this instability, such epiallelic variants could have a role in evolution [58]: with reversion at a rate of 10^{-4}, a selective advantage of 1% in heterozygotes would allow an advantageous epiallele to spread to an equilibrium frequency of 99% [60]. However, mutations to deleterious alleles create a genetic

load. In large populations, the load depends strongly on the mutation rate [60]. If CG dinucleotide methylation were often functionally significant, such a load would select for a lower epimutation rate [61]. The high rate that is observed thus suggests that the sites involved are mostly irrelevant to fitness. Indeed, a recent population study capable of detecting very weak selection suggests that CG epimutations outside TE insertions are close to neutral, and thus probably not relevant to adaptive evolution [59].

(d) Contributions of epiallelic variation to discrete trait variation

While many major mutations have been found to be associated with DNA sequence changes and TE insertions, there is little evidence that stable epiallelic variants without associated DNA sequence variants are abundant among spontaneous mutations. A much-cited exception is the peloric flower phenotype in the toadflax *Linaria*, which appears to arise frequently despite causing almost complete sterility of the affected flowers [62]. RNA expression of the gene involved, *cycloidea*, is completely silenced in peloric flowers, due to hypermethylation. However, silencing maps to a single nucleotide polymorphism in an unmethylated region 308 basepairs downstream of the stop codon [63]. It affects only the rarer *cyc308G* allele, and not the *CYC308A* allele. Silencing is recessive, and all plants with peloric flowers are GG homozygotes, with both copies silenced. This genotype also often has wild-type flowers, and the degree of *cycloidea* methylation correlates with the strength of the phenotypic effect. This demonstrates epigenetic control of peloric flowers, with incomplete penetrance, when the DNA sequence variant is present. There is no evidence that peloric mutations are evoked by environmental challenges, contrary what is sometimes claimed [21]. Some other examples of epiallelic mutant phenotypes in plants are described in [57].

(e) Contributions of epiallelic variation to quantitative trait variation

If epialleles were to contribute to variability in a trait subject to stabilizing selection, standard evolutionary models of the interaction between stabilizing selection and mutation [64] imply that the high epiallelic mutation rate mentioned above could potentially contribute substantially to genetic variance, and hence to responses to selection if the phenotypic optimum changes. The numerous measurements of both mutational and standing variability in quantitative traits [64, 65] include any potential

contributions from epiallelic variants. Finding that epigenetic variation plays a significant role in quantitative trait variability would thus not radically change our understanding of how populations respond to selection.

Nonetheless, the question of the extent to which epiallelic variants contribute to natural quantitative trait variability is of great interest, where critical evidence is currently lacking. Experiments using a strain of *A. thaliana* that had been stripped of its methylation, and then allowed to remethylate, suggest that variability in methylation amongst genetically identical progeny is associated with heritable variability in quantitative traits [57]. This shows that quantitative traits can be affected by epiallelic variability. However, it remains unclear to what extent natural trait variation is caused in this way. For one trait, gene expression levels in *A. thaliana*, the contribution of epialleles has been estimated [66]. In this highly self-fertilising plant, populations are strongly spatially isolated. DNA methylation variants are therefore correlated with sequence variants in the DNA, complicating the analyses. Indeed, genome-wide differences in SNPs can explain the overall expression results just as well as DNA methylation differences, and *vice versa*. To take population structure into account, genome-wide association (GWAS) analyses were done using SNP-based kinship estimates. For *cis*-acting methylation variants (the majority of the effects detected), only 63 significant methylation associations were found without an accompanying SNP association. Thus, fewer epigenetic loci appear to affect gene expression than SNPs; their effects are also smaller than those of SNPs. Of course, there may be detection biases against methylation variants that are not associated with SNPs at the sites in question, and further research is clearly desirable.

(f) Does epigenetic inheritance contribute to the transmission of adaptive acquired characters?

If epigenetic changes producing *adaptive* changes in phenotypes induced by external circumstances were often transmitted to the offspring, this would involve a major change in outlook. The so-called 'Central Dogma' of molecular biology, e.g. Chap. 4 in [67], states that information flows from nucleic acid sequence to protein sequence, and not vice versa. More generally, there is no known mechanism for systematically generating adaptive and heritable DNA sequence variation (see the discussion of 'directed mutation' in section 5 below).

As described above, mechanisms have evolved by which specific kinds of

adaptive responses can potentially be transmitted across one or more generations, involving epigenetic marks or the production of small RNA molecules that are transmitted through the germ cells. If these changes could produce stable adaptive traits in the offspring, and if they occurred sufficiently frequently, such 'Lamarckian' inheritance could play a significant role in phenotypic variation and evolution [19, 21]. However, as noted long ago by Haldane [5] and Muller [27], such a process is unlikely to be of general importance, because a large body of genetic experiments has established the ineffectiveness of selection on homozygous lines, which lack genetic variation but still show phenotypic variation. In striking contrast, family selection, with no exposure of the selected individuals to the environment in which the trait is favoured, is highly effective [68]. One of the most spectacular examples of non-genetic phenotypic differences is provided by the sterile worker castes of social insects. Darwin himself pointed out that these could not possibly have evolved by a Lamarckian mechanism, but must be the product of selection on the genotypes of the reproductive individuals to produce workers with phenotypes adapted to different tasks [68]. There is therefore a long-standing and strong empirical basis for rejecting the inheritance of acquired characters as a frequent phenomenon (see also the discussion of directed mutation in section 5).

Epigenetic marks certainly change in response to environmental factors, e.g. vernalisation in flowering plants [69]. However, when consistent epigenetic changes are seen in response to specific treatments or environments, transmission to the next generation is rarely tested, and it is often not known whether these change any phenotype or affect gene expression [70, 71]. A thorough review of the evidence on mammals concluded that evidence for "*widespread transgenerational epigenetic inheritance is lacking to date"*, and that *"the concept of transgenerational epigenetic inheritance in humans remains equivocal*" [39].

A convincing, but artificial, case has been described in *C. elegans*, in which heat-induced expression of a multicopy array of the gene coding for the heat-shock protein Hsp90 was transmitted for 14 generations, through both eggs and sperm, due to loss of histone HK3K9 methylation from the array [72]. No such transmission was, however, found with the normal situation of a single copy of the gene. Statistical concerns have been raised about many other published claims of multigeneration transmission of acquired traits [73, 74]. Overall, the evidence that such transmission is a common phenomenon is weak [75], even in plants where the germline is not sharply

distinct from the soma [57, 76].

Another situation that has been claimed to involve the inheritance of acquired characters [20] involves the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) defence mechanism that protects prokaryote genomes from transmissible genetic elements such as bacteriophages and conjugative plasmids. These systems have similarities to the defences against TEs described above, in that 'naïve' cells acquire the ability to recognise new infections. Again, this represents a change elicited by a specific environmental factor (invasion), which is heritable by a cell's descendants (a 'mutation'). In these systems, short pieces of foreign DNAs that enter a cell are cut out at 2-5 bp sequence motifs (called "Protospacer Adjacent Motifs" or PAMs) and integrated into a repeat-containing CRISPR locus in the host cell, which thus becomes interleaved with 'spacer' sequences that match specific sequences of foreign origin [77]. These sequences provide a 'memory' of foreign sequences that the cell has received. Complementarity between CRISPR-expressed RNAs and sequence in invading DNA ('proto-spacer' sequence) allows cells to detect the corresponding sequence (e.g. phage) during subsequent infections, and target it for destruction, similarly to the RNA interference mechanism that inhibits gene expression in eukaryotes [1, 77].

Importantly, however, the system includes no function to ensure that the 'mutations' (new spacers in a CRISPR array) benefit the cell, rather than harming it. Elements with the required sequence signatures can generate the targeting outcome, whether or not they target a sequence that forms part of something that is harmful to the cell. Indeed, a plasmid carrying a gene whose loss reduces cells survival can be destroyed. Some spacers target the cells' own DNA, which is clearly maladaptive and can cause cell death. This system, like other mutational processes, generates mutations irrespective of their benefits, and cell lineages that are lucky enough to gain suitable spacers will tend to increase, while ones that produce damaging ones, or cell death, are eliminated [1].

(g) Lateral gene transfer

A substantial proportion of some prokaryotes' genomes can consist of horizontally acquired sequences, whereas horizontal transmission appears to be much less prevalent in eukaryotes [78]. The acquired sequences may sometimes be adaptive in

their new organismal environment, but need not be. In any organism where such gene transfers may occur, a gene-centred perspective is necessary, in which the genes (or sequences) are the replicators that are subject to natural selection, and other components of the genome are part of their environment. The acquisition of selectively favourable DNA sequences by lateral gene transfer in prokaryotes is thus entirely consistent with neo-Darwinism [1], and labelling it as 'quasi-Lamarckian' [20] is misleading.

4. Sequence versus epigenetic changes in phenotypic evolution

Modern molecular genetic methods allow evolutionary biologists to detect selection from DNA sequence data. Many such studies have directly detected selection acting on DNA sequence variants in either protein sequences or regulatory non-coding sequences, using analyses of substitutions along evolutionary lineages [79], polymorphisms within natural populations [24], or a combination of the two [23]. In many cases, however, the basis for inferring selection is indirect, often coming simply from a 'footprint of selection' such as an observation of reduced variability in a small region of the genome [24, 25], suggesting that the spread of an initially rare variant (at an unknown selected site) has caused the 'hitchhiking' of variants at closely linked neutral or nearly neutral variants. In such cases, the selected variant could be either a DNA sequence variant or an epiallele.

(a) The causes of new mutations

At least two approaches can help to test the extent to which DNA sequence versus epigenetic variants contribute to adaptive evolution. First, one can assess the contributions of different types of variants to components of *de novo* mutational variation in traits of potential evolutionary significance. Innumerable molecular genetic analyses have shown that new mutations with detectable phenotypic effects, tabulated in databases such as *OMIM* (mutations causing human genetic diseases), *Flybase* and *Wormbook,* frequently involve DNA sequence changes. There may, however, be a bias towards detecting sequence changes, due to the difficulty of characterising epigenetic changes.

Systematic, unbiased surveys of the causes of mutations causing specific phenotypes are currently scarce, because such work became technically possible only

517 recently. However, an analysis of mutations that suppress the harmful fitness effects
518 of 251 deletion mutations in yeast genes identified sequence mutations in 86% of
519 cases; as the effects of some sequence mutations must have been undetectable (false
520 negatives), this leaves little scope for epigenetic variants [80]. A screen of exome
521 sequences of 4,923 human families ascertained through an offspring with a severe
522 developmental disorder detected coding sequence mutations in 42% of cases [80].
523 This study was not designed to detect either regulatory mutations in non-coding
524 sequence or major chromosomal rearrangements, two further important sources of
525 harmful mutations, so that there is probably only a narrow margin for epigenetic
526 variants.
527
528 **(b) The causes of phenotypic variants**
529 An approach that is more directly relevant to evolution is to assess the extent to which
530 epigenetic versus genetic variants have caused phenotypes involved in putatively
531 adaptive phenotypic change or variation. Martin and Orgogozo [81] tabulated 252
532 examples of phenotypic differences within natural populations, or between closely
533 related species, where linkage mapping localized genetic factors to a small region;
534 245 further examples involve domesticated animals or plants. Only one of the natural
535 cases is a potentially epigenetic variant, the *Drosophila* zygotic lethal male rescue
536 factor, a change associated with repetitive DNA in heterochromatin (this compilation
537 also included the *Linaria* peloric mutation; however, as discussed above, this is
538 associated with a sequence change). In 184 cases of natural phenotype differences,
539 associated DNA sequence variants were found, while in 67 (26.6% of the total) no
540 associations of any kind were detected. In many of the cases where sequence
541 differences were detected, these were nonsynonymous mutations or
542 insertions/deletions in coding sequences. Such variants are usually kept at low
543 frequencies by selection; they are thus plausible candidates for causing the phenotypic
544 differences, as it is unlikely that they could hitchhike to high frequencies along with
545 an advantageous epiallele.
546 Ideally, manipulation of DNA in transgenic experiments, where epigenetic
547 marks are necessarily removed, should be used to determine whether candidate causal
548 sequence variants have functionally relevant effects. Such tests are possible only for
549 variants with large phenotypic effects, but provide a guide to what is likely to be the
550 case more generally. A pioneering study of this kind examined the *Alcohol*

551 *dehydrogenase* (*Adh*) electrophoretic polymorphism of *D. melanogaster*, where fast
552 electrophoretic alleles are associated with higher ADH protein production than slow
553 alleles. This difference was mainly due to an insertion of several base pairs in the first
554 intron of the fast allele, together with several other regulatory sequence variants [82].
555 Stern and Orgogozo [83] listed 46 successful functional studies among their
556 'restricted' dataset of 162 phenotypic differences associated with DNA sequence
557 differences. Given the technical difficulties of this type of experiment, this is an
558 impressive rate of success. A more recent survey of this kind [81] did not record
559 transgenic experiments; however, none out of 100 later papers that cited it indicated
560 any role for epigenetic variants. Nine of these described transgenic experiments, all of
561 which identified sequence changes that caused naturally occurring phenotypic
562 differences in yeast, plants, and animals.
563 With the increasing use of CRISPR technology for genetic manipulation, we
564 anticipate a rapid increase in such tests. Strategies for extending these approaches to
565 differences among taxa that cannot interbreed, and hence are inaccessible to genetic
566 mapping, are also being developed. A notable example is the analysis of the effect of
567 the *Fzd8* enhancer in promoting larger brain size in humans compared with
568 chimpanzees [84]. This enhancer was identified as a candidate by screening
569 noncoding sequences that have enhancer roles in neocortex development, and were
570 highly conserved in most mammals but evolved rapidly in the human lineage.
571 Transgenic experiments in mice revealed that the human enhancer sequence caused
572 larger brain size than the chimpanzee sequence.
573
574 **(c) Some general implications**
575 Genetic studies of adaptive phenotypes have yielded several further important
576 conclusions. First, there are now many examples of phenotypic differences within and
577 between species whose genetic control maps to a small region, but with multiple
578 nucleotide differences within the region being causally involved [85]. This supports
579 Darwin's and Fisher's view that adaptive phenotypes are usually built up by a series
580 of relatively small changes, which has been challenged by proponents of the EES [15,
581 19].
582 Second, phenotypes that show plastic responses to environmental conditions
583 also often show considerable genetic variation in these responses, and DNA sequence
584 variants associated with these heritable differences have been identified, supporting

585 the view that plasticity has evolved in a neo-Darwinian fashion [4]. For example,
586 vernalisation responses in flowering plants involve a period of exposure to cold that is
587 required for seed germination. (This was the basis for the notorious Lamarckian
588 theories of T.D. Lysenko, which seriously damaged Soviet agriculture [10, 11]).
589 Vernalisation is under the control of a complex epigenetic regulatory system, which is
590 reset each generation [57, 69]. Natural vernalisation response differences are
591 controlled by DNA sequence variation in cis-acting regulatory sequences [86, 87].
592 In contrast to the rigorous empirical evidence for the role of DNA sequence
593 variants in adaptive evolution that we have outlined, there is currently little evidence
594 for effects of epigenetic changes, although more data are required. Recent claims for
595 such effects have been based on evidence that changes affecting the methylome are
596 more numerous than some types of sequence variants in evolving lineages of
597 Darwin's finches [88] and darter fish [89]. Such comparisons, however, provide no
598 evidence that the epigenetic variants in question had any role in phenotypic evolution.
599 Several theoretical studies show that the general framework of population and
600 quantitative genetics applies to epigenetic inheritance [90, 91]; indeed, the basic
601 theory was developed half a century before the molecular basis of inheritance was
602 determined. Combining modes of inheritance that differ in their mutation rates and
603 transmission patterns can alter the outcome of selection in complex ways – similar to
604 the complexities possible with maternal effects on quantitative traits mentioned in
605 section 3.e [40, 41]. However, this is not of fundamental significance as far as the
606 general properties of evolutionary dynamics are concerned. Even if new alleles
607 affecting a trait are induced by a specific environment, they can contribute to
608 adaptation only if transmission is fairly stable and the environment is quite
609 predictable, so that the new allele remains advantageous in future environments [92,
610 93].
611 Finally, we note that demonstrating a causal role for epialleles in an adaptive
612 phenotype is a necessary, but not sufficient, condition for radical changes to the neo-
613 Darwinian theory of adaptive evolution. To support a neo-Lamarckian mode of
614 evolution, evidence would be needed that (i) a given environmental treatment tends
615 systematically to induce heritable, adaptive epiallelic variants (ii) natural selection is
616 not involved in the spread of such variants through populations (iii) the variants in
617 question can be stably transmitted for many generations in the absence of the
618 treatment. If the claim is instead that variation is systematically biased towards

generation of adaptive variants, which are then picked up by selection, then one has to show that this bias has a significant effect on the outcome, beyond what would have been produced by selection on random variation. In view of the vast body of evidence for neo-Darwinian mechanisms, the principle that 'extraordinary claims require extraordinary evidence' [12, 94] implies that such stringent criteria must be met before we should consider abandoning or substantially modifying neo-Darwinism. The case of 'directed mutation' that we discuss next brings out the importance of experimental rigour in dealing with these problems.

5. Directed mutation

The concept of 'directed mutation' proposes that organisms might respond to an environmental challenge by an increased mutation rate in a target DNA sequence that specifically results in mutants with higher fitness in the new environment [95]. This concept is similar to the inheritance of acquired characters, but differs from it because it involves changes in the genetic material without a prior change in the phenotype. It traces its origin back to studies of rapid adaptive responses by bacteria to new laboratory environments, which revealed astonishing speeds of bacterial adaptation. For example, naturally occurring *lac*$^{-}$ strains of *Escherichia coli*, known as *E. coli mutabile*, are normally unable to ferment lactose, but can acquire the ability to do so a day or two after transfer to lactose as a carbon source [96], and maintain it when grown in a lactose-free medium.

Until the 1940s, it was widely believed that exposure to the new environment directly induced these adaptive, heritable changes, and bacteriology was "the last stronghold of Lamarckism" [97]. But this ended when bacterial inheritance became understood. Brilliant genetic and biochemical studies developed and verified a straightforward, neo-Darwinian interpretation for these observations [88]: if rare mutations producing the adaptive phenotype constantly arise independently of the state of the environment, they would have a selective advantage and quickly replace their less fit competitors when grown in the new environment [28]. The vast numbers of cells in bacterial cultures, and the short times between cell divisions in cultures of dividing cells, make this inevitable. The Lamarckian alternative hypothesis can be tested by asking whether the mutant bacteria are already present in the population *before* exposure to the selective agent (which then merely reveals their presence — the neo-Darwinian interpretation). Several experimental tests were devised, starting

653 with the 'fluctuation test' [98]. By the mid-1950s, the evidence overwhelmingly
654 supported the neo-Darwinian interpretation.
655 The universality of this conclusion was later challenged by results from
656 bacteria and yeast [95, 99]. However, as reviewed by Maisnier-Patin and Roth [99], a
657 neo-Darwinian explanation exists for findings that apparently suggested the
658 involvement of mutations that specifically conferred an adaptive phenotype.
659 Experiments involving *E. coli* with leaky mutations in a *lac* operon gene found that
660 growth on medium with lactose as the carbon source is severely impaired, but that,
661 over time, colonies appeared, indicating that growth was occurring. Moreover,
662 mutants conferring the ability to grow on lactose appeared only in the presence of
663 lactose [95, 99]. Inability to grow on lactose is due to a frameshift mutation in the
664 *lacZ* member of the *lac* operon carried on a plasmid present in low copy number. 90%
665 of revertants regaining the ability to grow on lactose had a stable compensating
666 mutation in the *lacZ* gene, while 10% had unstable tandemly amplified copies of the
667 mutant gene. About 100 times more mutations occurred than would be expected based
668 on mutation rates under non-selective conditions. 10% showed a 100-fold increase in
669 the mutation rate, affecting all genes tested, probably attributable to the stressful
670 conditions experienced by the bacteria. But the critical question is: what is the source
671 of the 90% of revertants with no increased mutation rate? These appear be targeted at
672 the *lacZ* gene to specifically produce beneficial revertants.
673 It turns out that the observations do not require directed mutations, and that a
674 neo-Darwinian explanation is more likely, once the intricate experiments are
675 understood in detail [99]. This explanation proposes that spontaneous fluctuations
676 sometimes produce cells with increased numbers of the plasmid carrying the (mutant)
677 *lacZ* gene. This would allow a non-dividing cell to use lactose to provide sufficient
678 energy to copy the plasmids, increasing the probability of occurrence of *lac*+
679 revertants, which then permit the cell to divide. Descendant cells' plasmids carry
680 revertant genes, making it appear that mutations were targeted to the site involved in
681 the reversion. Having multiple copies of the plasmid may also increase the mutation
682 rate, because the plasmid carries an error-prone DNA polymerase gene. Natural
683 selection can thus produce the appearance of directed mutagenesis. This model, while
684 not fully confirmed experimentally, is consistent with all currently available data. As
685 Maisnier-Patin and Roth [99] comment "*it is important to remember that natural*
686 *selection sees almost everything and is always watching*".

6. Is there an evolvability problem?

a) Genetic variation and evolvability

It is sometimes stated that standard modes of generating mutational variability are inadequate to explain the speed of adaptive evolution, and that additional processes are thus needed to ensure the 'evolvability' of a species, a concept discussed from a neo-Darwinian perspective in [100]. For example, Laland *et al.* [14] state that "*Inclusive models help to explain a wide range of puzzling phenomena, such as the rapid colonization of North America by the house finch, the adaptive potential of invasive plants with low genetic diversity, and how reproductive isolation is established*". However, a vast literature on artificial selection [65] and experimental evolution [101] shows that selection can change almost any trait over a very short timescale, implying that there is usually ample heritable variation on which selection can act. As Darwin emphasised in Chapter 1 of *The Origin of Species* [102], examples such as dogs and domestic pigeons demonstrate the power of artificial selection to alter phenotypes, often resulting in changes as great as those distinguishing different genera.

These observations provide strong evidence that selection can quickly take a population towards a nearby fitness optimum, without any need for special mechanisms generating new variability. Even in humans, with their relatively small population size over most of our history, the mutation to sickle-cell haemoglobin that confers resistance to malaria has spread independently at least four times, in different populations, and hundreds of other polymorphisms for mutations conferring malaria resistance are known [103]. Rates of long-term evolution are thus probably largely controlled by environmental changes, and not by the supply of mutations. This conclusion was reached by the founders of the MS, and many recent studies support it [104].

However, some situations involve evolution to new 'adaptive peaks' that can only be reached by crossing a 'valley' of phenotypes with reduced fitness, especially when a coordinated complex of characters changes. Goldschmidt suggested that such phenotypic changes require complex macromutations, which, in a single step, produce beneficial multi-trait combinations [9]. This proposal has been thoroughly tested by genetic analyses in the case of mimicry, and rejected in favour of the process of

721 stepwise improvement proposed by Fisher [8], whereby a mutation with a relatively
722 large effect on one aspect of mimetic resemblance produces an adequate, but
723 imperfect, mimic, with the subsequent accumulation of more minor changes that
724 improve mimicry [105, 106, Chap.3]. While mutations with major effects on
725 individual traits can certainly contribute to adaptive evolution (see section 4 above),
726 as was well-known to the founders of the MS [5], there is no evidence for a role for
727 macromutations of the type postulated by Goldschmidt and his followers [3].
728 As we have seen, however, critics of neo-Darwinism often argue that more
729 attention should be paid to the availability of adaptive variation. If we discard the
730 possibility that induced adaptive variability is at all common, as argued above, there
731 are only two well-established processes whose rates of occurrence significantly affect
732 the amount of variability available for adaptive evolution – mutation and genetic
733 recombination. Analysing the evolution of these genome properties has been central
734 in evolutionary biology, starting with work by Fisher at the beginning of the MS [8].
735
736 **b) The evolution of mutation rates, sex and genetic recombination**
737 Selection on variants that alter the mutation rate has been intensively studied, both
738 theoretically and experimentally [61, 107, 108], with the aim of understanding the
739 outcome of the conflict between the potential advantage of producing beneficial
740 mutations, and the fact that most mutations that affect fitness are deleterious [27, 61].
741 In largely asexually reproducing populations, an allele that causes an increased
742 mutation rate (a 'mutator') can remain linked to any beneficial mutations that it
743 induces, and hence increase in frequency by 'hitchhiking' [100]. Adaptation in
744 microbial populations indeed often leads to evolution of mutator strains whose DNA
745 repair is defective, and which produce beneficial mutations more frequently than non-
746 mutators, resulting (often temporarily) in an increased mutation rate [107] In sexual
747 populations, however, recombination quickly disassociates mutator alleles from any
748 beneficial mutations, and their increased frequency of deleterious mutations favours
749 alleles conferring lower mutation rates [61, 108].
750 The elaborate molecular machinery for correcting errors in DNA replication
751 strongly suggests that natural selection has generally favoured reduced mutation rates
752 [61]. However, there are examples where special mechanisms have evolved to
753 generate variability in situations where there is intense selection for rapid change, as
754 in pathogenic microbes whose surface antigens are targeted by the host immune

system [100]. A particularly well-studied example is the 'cassette' of *vlsE* genes of the Lyme disease bacterium *Borrelia burgdorferi*, in which there is a group of similar but diverse genes that code for the VlsE antigen, only one of which is expressed at a given time by virtue of its presence at an expression site [109]. Recombination with this site produces expression of different versions of the antigen, and selection favours sequence differences in members of the cassette, partly because of mutation-prone sequences in regions targeted by host antibodies [109].

Work on the evolution of sex and recombination over many decades has built a sophisticated theoretical understanding of how selection acts on genetic variants that modify the rate of genetic recombination or the frequency of sexual reproduction, as described in [106, Chap.3] and [110]. One important conclusion is that genetic recombination can be favoured because it facilitates responses to selection by generating new combinations of favourable alleles, and the frequencies of sex [111] and recombination [112] indeed tend to increase in experimentally selected populations. Crucially, studies of both mutation and recombination show that, although selection may lead to the adaptive modulation of the *amount* of variation, there is no *bias* towards the production of beneficial variants.

c) Canalisation and robustness

While much more empirical work remains to be done, the research just outlined shows how features of the genome that affect evolvability can be understood using the principles of the MS. Similar arguments apply to the 'canalisation' of developmental systems, which buffers them against genetic or environmental perturbations that produce deleterious phenotypes, leading to phenotypic 'robustness' [113]. For example, the Hsp90 heat shock protein is a 'chaperone' that minimises deleterious protein misfolding. When this system is disrupted, phenotypic variants are revealed. Because these might occasionally be beneficial, it has been suggested that Hsp90 is an 'evolutionary capacitor' that evolved *because* its disruption in challenging environments occasionally reveals useful heritable variants [114]. However, systems such as Hsp90 are more likely to have evolved to *minimise* deleterious phenotypic variation; their breakdown is probably maladaptive, occurring when stress impairs normal control systems [113].

The existence of these buffering mechanisms contradicts claims that "*Developmental systems facilitate well-integrated, functional phenotypic responses to*

789 *mutation or environmental induction*" (point (iii) of Table 1 in [15]), as does the
790 overwhelming evidence that most mutations with noticeable phenotypic effects are
791 deleterious [27]. While there are unquestionably many examples of adaptive
792 phenotypic plasticity, there are strong reasons for thinking that these are *evolved*
793 responses to environmental challenges, consistent with the evidence for genetic
794 variation in plasticity described in section 4.c, rather than inherent properties of
795 developmental systems [3, 4]. This also applies to cases where a plastic response can
796 be transmitted over one or more generations [35, 36].
797

798 ## 7. Conclusions

799 We have focussed our discussion on the sources of the variability used in *adaptive*
800 evolution. However, it is important to understand that contemporary evolutionary
801 biology does not take a dogmatically adaptationist or pan-selectionist view of the
802 evolutionary causes of all characteristics of living organisms. This is especially true
803 for properties of the genome itself, many of which must involve interactions between
804 the effects of mutational processes, selection and genetic drift. Some examples are
805 reviewed in [115] and Chap. 10 in [106]. For example, the effectiveness of selection
806 is greatly weakened when genetic recombination is very infrequent, which explains
807 the evolutionary degeneration of Y chromosomes through the accumulation of
808 deleterious mutations (despite the fact that the suppression of crossing over between
809 the ancestors of X and Y chromosomes was originally favoured by selection).
810 Furthermore, selfish genetic elements such as TEs and segregation distorters can
811 promote their own spread within genomes and populations at the expense of the
812 fitness of their hosts [53]. Nevertheless, we finish by re-emphasising the central
813 concept of neo-Darwinism and the MS: allele frequency change caused by natural
814 selection is the only credible process underlying the evolution of adaptive organismal
815 traits.
816

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820

821 ## References

822

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