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Review

Cite this article: Charlesworth D, Barton NH, Charlesworth B. 2017 The sources of adaptive variation. *Proc. R. Soc. B* **284**: 20162864. http://dx.doi.org/10.1098/rspb.2016.2864

Received: 3 January 2017
Accepted: 4 May 2017

Subject Category:
Evolution

Subject Areas:
evolution, genetics

Keywords:
modern synthesis, extended evolutionary synthesis, mutation, natural selection, epigenetic inheritance

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The sources of adaptive variation

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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 summarized 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, p. 567]

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 (MS) of evolution was named by Julian Huxley [2] to emphasize 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 recognized 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.

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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' [15, p. 3]. 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 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 focusing 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, p. 983]

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, p. 10] 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, as 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 evolution in the small minority of species with advanced social behaviour [4]. Instead, we focus on mechanisms that might allow adaptive phenotypic traits to become expressed by all or most members of populations, without a neo-Darwinian evolutionary process.

(a) Classical genetics and inheritance

The MS was based on the rules of inheritance discovered by classical genetics, which apply to any stably inherited type of variant associated with a chromosome, whether or not it involves a DNA sequence change. Early twentieth-century genetics showed that most genetic variants associated with major phenotypic differences in animals, plants and fungi are stably and biparentally inherited (Mendelian inheritance), and chromosomally located, as was eloquently summarized by Muller [27]. It was subsequently shown that inheritance in bacteria and viruses obeys fundamentally similar rules [28]. Matrilineal inheritance also occurs, involving the transmission of variants in plastid and mitochondrial genomes [29], or of cytoplasmic endosymbionts such as *Wolbachia* [30]. The multi-factorial theory of quantitative trait variability, and its experimental validation, showed that Mendelian variants with small phenotypic effects underlie heritable

quantitative trait variation, acting together with non-genetic factors [31]. These discoveries allowed population geneticists to model evolutionary changes within populations; their results convinced biologists that natural selection was highly effective as an evolutionary mechanism, contrary to other views that had prevailed into the 1930s [31].

Some rare cases of unstable inheritance of mutant phenotypes, however, initially remained puzzling. It is now known that these are often caused by disruptions of gene function by insertions of transposable elements (TEs), whose excision can sometimes restore the wild-type allele [32]. Because most TE insertions excise very rarely, such mutations mostly follow Mendel's laws—indeed, many of the classical mutations in *Drosophila* genetics [33], and in the sweet peas studied by Mendel, involved TE insertions [34].

In recent years, the term 'genetic inheritance' has come to mean the transmission of alterations in the DNA sequence (or RNA sequence, in the case of some viral genomes), as distinct from a heterogeneous set of phenomena that do not involve such alterations. In the next sections, we outline current knowledge about these other processes, which have come to be called 'epigenetic' inheritance, and consider their implications for the validity of the MS (see [35,36] 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 TE 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 with 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 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 *Drosophila 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-fertilization [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 recognized 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 multi-cellular organisms, the role of epigenetic chromatin modification in stable cell differentiation during multi-cellular 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 fertilized 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 transgenerational 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 than 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 of around 10^{-4} per basepair per generation, 5000 times higher than those for DNA nucleotide changes. Despite this instability, such epiallelic variants could have a role in evolution [59]: 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 bp 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 *Arabidopsis thaliana* that had been stripped of its methylation, and then allowed to remethylate, suggest that variability in methylation among 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-fertilizing 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 single-nucleotide polymorphisms (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 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. ch. 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 §5).

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 §5).

Epigenetic marks certainly change in response to environmental factors (e.g. vernalization 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, p. 2463].

A convincing, but artificial, case has been described in *C. elegans*, in which heat-induced expression of a multi-copy 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 multi-generation transmission of acquired traits [73,74]. Overall, the evidence that such transmission is a common phenomenon is weak, even in plants where the germline is not sharply distinct from the soma [57,75].

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 'naive' cells acquire the ability to recognize 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 [1,76].

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 [77]. 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 [78], 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 characterizing epigenetic changes.

Systematic, unbiased surveys of the causes of mutations causing specific phenotypes are currently scarce, because such work became technically possible only recently. However, an analysis of mutations that suppress the harmful fitness effects of 251 deletion mutations in yeast genes identified sequence mutations in 86% of cases; as the effects of some sequence mutations must have been undetectable (false negatives), this leaves little scope for epigenetic variants [79]. A screen of exome sequences of 4923 human families ascertained through an offspring with a severe developmental disorder detected coding sequence mutations in 42% of cases [80]. This study was not designed to detect either regulatory mutations in non-coding sequence or major chromosomal rearrangements, two further important sources of harmful mutations, so that there is probably only a narrow margin for epigenetic variants.

(b) The causes of phenotypic variants

An approach that is more directly relevant to evolution is to assess the extent to which epigenetic versus genetic variants have caused phenotypes involved in putatively adaptive phenotypic change or variation. Martin & Orgogozo [81] tabulated 252 examples of phenotypic differences within natural populations, or between closely related species, where linkage mapping localized genetic factors to a small region; 245 further examples involve domesticated animals

or plants. Only one of the natural cases is a potentially epigenetic variant, the *Drosophila* zygotic lethal male rescue factor, a change associated with repetitive DNA in heterochromatin (this compilation also included the *Linaria* peloric mutation; however, as discussed above, this is associated with a sequence change). In 184 cases of natural phenotype differences, associated DNA sequence variants were found, while in 67 (26.6% of the total) no associations of any kind were detected. In many of the cases where sequence differences were detected, these were non-synonymous mutations or insertions/deletions in coding sequences. Such variants are usually kept at low frequencies by selection; they are thus plausible candidates for causing the phenotypic differences, as it is unlikely that they could hitchhike to high frequencies along with an advantageous epiallele.

Ideally, manipulation of DNA in transgenic experiments, where epigenetic marks are necessarily removed, should be used to determine whether candidate causal sequence variants have functionally relevant effects. Such tests are possible only for variants with large phenotypic effects, but provide a guide to what is likely to be the case more generally. A pioneering study of this kind examined the *Alcohol dehydrogenase* (*Adh*) electrophoretic polymorphism of *D. melanogaster*, where fast electrophoretic alleles are associated with higher ADH protein production than slow alleles. This difference was mainly due to an insertion of several base pairs in the first intron of the fast allele, together with several other regulatory sequence variants [82]. Stern & Orgogozo [83] listed 46 successful functional studies among their 'restricted' dataset of 162 phenotypic differences associated with DNA sequence differences. Given the technical difficulties of this type of experiment, this is an impressive rate of success. A more recent survey of this kind [81] did not record transgenic experiments; however, none out of 100 later papers that cited it indicated any role for epigenetic variants. Nine of these described transgenic experiments, all of which identified sequence changes that caused naturally occurring phenotypic differences in yeast, plants and animals.

With the increasing use of CRISPR technology for genetic manipulation, we anticipate a rapid increase in such tests. Strategies for extending these approaches to differences among taxa that cannot interbreed, and hence are inaccessible to genetic mapping, are also being developed. A notable example is the analysis of the effect of the *Fzd8* enhancer in promoting larger brain size in humans compared with chimpanzees [84]. This enhancer was identified as a candidate by screening non-coding sequences that have enhancer roles in neocortex development, and were highly conserved in most mammals but evolved rapidly in the human lineage. Transgenic experiments in mice revealed that the human enhancer sequence caused larger brain size than the chimpanzee sequence.

(c) Some general implications

Genetic studies of adaptive phenotypes have yielded several further important conclusions. First, there are now many examples of phenotypic differences within and between species whose genetic control maps to a small region, but with multiple nucleotide differences within the region being causally involved [85]. This supports Darwin's and Fisher's view that adaptive phenotypes are usually built up by a series of relatively small changes, which has been challenged by proponents of the EES [15,19].

Second, phenotypes that show plastic responses to environmental conditions also often show considerable genetic variation in these responses, and DNA sequence variants associated with these heritable differences have been identified, supporting the view that plasticity has evolved in a neo-Darwinian fashion [4]. For example, vernalization responses in flowering plants involve a period of exposure to cold that is required for seed germination. (This was the basis for the notorious Lamarckian theories of T. D. Lysenko, which seriously damaged Soviet agriculture [10,11].) Vernalization is under the control of a complex epigenetic regulatory system, which is reset each generation [57,69]. Natural vernalization response differences are controlled by DNA sequence variation in *cis*-acting regulatory sequences [86,87].

In contrast with the rigorous empirical evidence for the role of DNA sequence variants in adaptive evolution that we have outlined, there is currently little evidence for effects of epigenetic changes, although more data are required. Recent claims for such effects have been based on evidence that changes affecting the methylome are more numerous than some types of sequence variants in evolving lineages of Darwin's finches [88] and darter fish [89]. Such comparisons, however, provide no evidence that the epigenetic variants in question had any role in phenotypic evolution.

Several theoretical studies show that the general framework of population and quantitative genetics applies to epigenetic inheritance [90,91]; indeed, the basic theory was developed half a century before the molecular basis of inheritance was determined. Combining modes of inheritance that differ in their mutation rates and transmission patterns can alter the outcome of selection in complex ways—similar to the complexities possible with maternal effects on quantitative traits mentioned in §3e [40,41]. However, this is not of fundamental significance as far as the general properties of evolutionary dynamics are concerned. Even if new alleles affecting a trait are induced by a specific environment, they can contribute to adaptation only if transmission is fairly stable and the environment is quite predictable, so that the new allele remains advantageous in future environments [92,93].

Finally, we note that demonstrating a causal role for epialleles in an adaptive phenotype is a necessary, but not sufficient, condition for radical changes to the neo-Darwinian theory of adaptive evolution. To support a neo-Lamarckian mode of evolution, evidence would be needed that (i) a given environmental treatment tends systematically to induce heritable, adaptive epiallelic variants, (ii) natural selection is not involved in the spread of such variants through populations, and (iii) the variants in question can be stably transmitted for many generations in the absence of the 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, p. 1]. But this ended when bacterial inheritance became understood. Brilliant genetic and biochemical studies developed and verified a straightforward, neo-Darwinian interpretation for these observations: 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 with the 'fluctuation test' [98]. By the mid-1950s, the evidence overwhelmingly supported the neo-Darwinian interpretation.

The universality of this conclusion was later challenged by results from bacteria and yeast [95,99]. However, as reviewed by Maisnier-Patin & Roth [99], a neo-Darwinian explanation exists for findings that apparently suggested the involvement of mutations that specifically conferred an adaptive phenotype. Experiments involving *E. coli* with leaky mutations in a *lac* operon gene found that growth on medium with lactose as the carbon source is severely impaired, but that, over time, colonies appeared, indicating that growth was occurring. Moreover, mutants conferring the ability to grow on lactose appeared only in the presence of lactose [95,99]. Inability to grow on lactose is due to a frameshift mutation in the *lacZ* member of the *lac* operon carried on a plasmid present in low copy number. Ninety per cent of revertants regaining the ability to grow on lactose had a stable compensating mutation in the *lacZ* gene, while 10% had unstable tandemly amplified copies of the mutant gene. About 100 times more mutations occurred than would be expected based on mutation rates under non-selective conditions. Ten per cent showed a 100-fold increase in the mutation rate, affecting all genes tested, probably attributable to the stressful conditions experienced by the bacteria. But the critical question is: what is the source of the 90% of revertants with no increased mutation rate? These appear to be targeted at the *lacZ* gene to specifically produce beneficial revertants.

It turns out that the observations do not require directed mutations, and that a neo-Darwinian explanation is more likely, once the intricate experiments are understood in detail [99]. This explanation proposes that spontaneous fluctuations sometimes produce cells with increased numbers of the plasmid carrying the (mutant) *lacZ* gene. This would allow a non-dividing cell to use lactose to provide sufficient energy to copy the plasmids, increasing the probability of occurrence of *lac*+ revertants, which then permit the cell to divide. Descendant cells' plasmids carry revertant genes, making it appear that mutations were targeted to the site involved in the reversion. Having multiple copies of the plasmid may also increase the mutation rate, because the plasmid carries an error-prone DNA polymerase gene. Natural selection can thus produce the appearance of directed mutagenesis. This model, while not fully confirmed experimentally, is consistent with all currently available data. As Maisnier-Patin & Roth [99, p. 2] comment, 'it is important to remember that natural 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 by Sniegowski & Murphy [100]. For example, Laland *et al.* [14, p. 164] 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 time scale, implying that there is usually ample heritable variation on which selection can act. As Darwin emphasized in ch. 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 [9] suggested that such phenotypic changes require complex macromutations, which, in a single step, produce beneficial multi-trait combinations. This proposal has been thoroughly tested by genetic analyses in the case of mimicry, and rejected in favour of the process of stepwise improvement proposed by Fisher [8], whereby a mutation with a relatively large effect on one aspect of mimetic resemblance produces an adequate, but imperfect, mimic, with the subsequent accumulation of more minor changes that improve mimicry [105,106, ch. 3]. While mutations with major effects on individual traits can certainly contribute to adaptive evolution (see §4), as was well-known to the founders of the MS [5], there is no evidence for a role for macromutations of the type postulated by Goldschmidt and his followers [3].

As we have seen, however, critics of neo-Darwinism often argue that more attention should be paid to the availability of adaptive variation. If we discard the possibility that induced adaptive variability is at all common, as argued above, there are only two well-established processes whose rates of occurrence significantly affect the amount of variability available for adaptive evolution—mutation and genetic recombination. Analysing the evolution of these genome properties has been central in evolutionary biology, starting with work by Fisher at the beginning of the MS [8].

(b) The evolution of mutation rates, sex and genetic recombination

Selection on variants that alter the mutation rate has been intensively studied, both theoretically and experimentally [61,107,108], with the aim of understanding the outcome of the conflict between the potential advantage of producing beneficial mutations, and the fact that most mutations that affect fitness are deleterious [27,61]. In largely asexually reproducing populations, an allele that causes an increased mutation rate (a 'mutator') can remain linked to any beneficial mutations that it induces, and hence increase in frequency by 'hitchhiking' [100]. Adaptation in microbial populations indeed often leads to evolution of mutator strains whose DNA repair is defective, and which produce beneficial mutations more frequently than non-mutators, resulting (often temporarily) in an increased mutation rate [107]. In sexual populations, however, recombination quickly disassociates mutator alleles from any beneficial mutations, and their increased frequency of deleterious mutations favours alleles conferring lower mutation rates [61,108].

The elaborate molecular machinery for correcting errors in DNA replication strongly suggests that natural selection has generally favoured reduced mutation rates [61]. However, there are examples where special mechanisms have evolved to generate variability in situations where there is intense selection for rapid change, as 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 ch. 3 of [106] 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) Canalization 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 'canalization' 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 minimizes 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 *minimize* 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 mutation or environmental induction' (point (iii) of table 1 in [15, p. 2]), as does the overwhelming evidence that most mutations with noticeable phenotypic effects are deleterious [27]. While there are unquestionably many examples of adaptive phenotypic plasticity, there are strong reasons for thinking that these are *evolved* responses to environmental challenges, consistent with the evidence for genetic variation in plasticity described in §4c, rather than inherent properties of developmental systems [3,4]. This also applies to cases where a plastic response can be transmitted over one or more generations [35,36].

7. Conclusion

We have focused our discussion on the sources of the variability used in *adaptive* evolution. However, it is important to understand that contemporary evolutionary biology does not take a dogmatically adaptationist or pan-selectionist view of the evolutionary causes of all characteristics of living organisms. This is especially true for properties of the genome itself, many of which must involve interactions between the effects of mutational processes, selection and

genetic drift. Some examples are reviewed in [115] and ch. 10 of [106]. For example, the effectiveness of selection is greatly weakened when genetic recombination is very infrequent, which explains the evolutionary degeneration of Y chromosomes through the accumulation of deleterious mutations (despite the fact that the suppression of crossing over between the ancestors of X and Y chromosomes was originally favoured by selection). Furthermore, selfish genetic elements such as TEs and segregation distorters can promote their own spread within genomes and populations at the expense of the fitness of their hosts [53]. Nevertheless, we finish by re-emphasizing the central concept of neo-Darwinism and the MS: allele frequency change caused by natural selection is the only credible process underlying the evolution of adaptive organismal traits.

Authors' contributions. The authors contributed equally to the design of the paper and to writing §§1 and 7. D.C. and B.C. wrote most of §§2–5, N.H.B. wrote most of §6.

Competing interests. We declare we have no competing interests.

Funding. We received no funding for this study.

Acknowledgements. We thank Douglas Futuyma, Frank Johannes, John Welch and Soojin Yi for their helpful comments on this paper.

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