# Potential Role of DNA Methylation as a Driver of Plastic Responses to the Environment Across Cells, Organisms, and Populations

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## Abstract

There is great interest in exploring epigenetic modifications as drivers of adaptive organismal responses to environmental change. Extending this hypothesis to populations, epigenetically driven plasticity could influence phenotypic changes across environments. The canonical model posits that epigenetic modifications alter gene regulation and subsequently impact phenotypes. We first discuss origins of epigenetic variation in nature, which may arise from genetic variation, spontaneous epimutations, epigenetic drift, or variation in epigenetic capacitors. We then review and synthesize literature addressing three facets of the aforementioned model: (i) causal effects of epigenetic modifications on phenotypic plasticity at the organismal level, (ii) divergence of epigenetic patterns in natural populations distributed across environmental gradients, and (iii) the relationship between environmentally induced epigenetic changes and gene expression at the molecular level. We focus on DNA methylation, the most extensively studied epigenetic modification. We find support for environmentally associated epigenetic structure in populations and selection on stable epigenetic variants, and that inhibition of epigenetic enzymes frequently bears causal effects on plasticity. However, there are pervasive confounding issues in the literature. Effects of chromatin-modifying enzymes on phenotype may be independent of epigenetic marks, alternatively resulting from functions and protein interactions extrinsic of epigenetics. Associations between environmentally induced changes in DNA methylation and expression are strong in plants and mammals but notably absent in invertebrates and nonmammalian vertebrates. Given these challenges, we describe emerging approaches to better investigate how epigenetic modifications affect gene regulation, phenotypic plasticity, and divergence among populations.

**Key words:** acclimation, DNA methylation, environmental stress, epigenetics, gene expression, phenotypic plasticity.

## Significance

Epigenetic sources of phenotypic variation are increasingly invoked in ecological and evolutionary studies, often in the context of environmental stress associated with global change. However, summary and synthesis of the causal evidence in support of epigenetically driven phenotypic plasticity in environmental performance is limited. Here we evaluated the biological and evolutionary significance of this hypothesis by reviewing evidence from cellular-to-population level processes.

## Introduction

The emergence of multicellularity is a major transition during evolution (Szathmáry and Smith 1995). The advent of multicellularity enabled differentiation of highly specialized cell types in a single organism. Epigenetic mechanisms are necessary for developmental differentiation in many multicellular organisms (Waddington 1957; Lister et al. 2013; Chen and Dent 2014; Atlasi and Stunnenberg 2017; Kawakatsu et al. 2017). This is well conceptualized by the so-called "epigenetic landscape" by Waddington (1957) and demonstrated across molecular biology (Chen and Dent 2014; Atlasi and Stunnenberg 2017). Multicellular organisms can often further alter their phenotypes in response to biotic and abiotic environmental cues, a process termed phenotypic plasticity (West-Eberhard 2008). It is hypothesized that epigenetic modifications to DNA and chromatin are one suite of mechanisms regulating phenotypic plasticity in response to the environment (Duncan et al. 2014; Loughland et al. 2021).

Traditionally, epigenetic mechanisms were defined as heritable information not encoded by DNA sequence (Waddington 1957). A more modern definition of epigenetic mechanism is inspired by emerging studies of chromatin organization and concerns how genomic DNA is modified and structured to shape physical interactions between regulatory machineries and genomic regions underlying differences within and between cells (Feil and Fraga 2012; Yi 2017). Epigenetic mechanisms can encompass, but are not necessarily limited to, DNA methylation, posttranslational modifications to histone tails, transposable elements, and noncoding RNAs. Among these mechanisms, DNA methylation is the most widely studied, especially in non-model organisms. Data on other epigenetic modifications are sparse compared to DNA methylation. For this reason, the findings we discuss in this review concern DNA methylation unless otherwise specified.

The potential impacts of epigenetic modifications on ecology and evolution are increasingly appreciated (Feil and Fraga 2012; Verhoeven et al. 2016). Recent studies have investigated the roles epigenetic modifications can play in the regulation of phenotypic plasticity. Aside from some mammalian and plant model species, the impact of epigenetic processes on phenotypic plasticity and evolution is unclear across most eukaryotes. Uncovering the extent to which phenotypic plasticity is explained by epigenetic modifications and their interactions with genetic variation will shed light on the basis of phenotypic plasticity and the epigenetic and genomic material that drives its evolution (Jablonka and Lamb 2005).

In this review, we discuss current knowledge regarding several key components connecting epigenetic mechanisms, phenotypic plasticity, and evolution. Our primary focus is phenotypic plasticity associated with environmental change, a critical component of environmental adaptation with ample literature for synthesis. We begin by introducing models of epigenetic variations that can arise in nature. We then summarize and synthesize studies across the following scales (Fig. 1). First, we review studies that link epigenetic processes to phenotypic variation at the organismal level. We start with this scope because phenotypic plasticity is most often appreciated as an organismal process. By first reviewing studies of epigenetically driven plasticity at a phenotypic and organismal level, we address the level of support for this hypothesis before delving into its potential effects in natural populations and underlying mechanisms. We then discuss epigenetic variation associated with the environment among wild populations in studies addressing epigenetic contributions to plastic phenotypic variation in nature. Finally, we review current literature evaluating the links between epigenetic variation and phenotypic plasticity at a molecular and cellular level, posing several key open questions that reframe observations at organismal and population levels.

Because strong phylogenetic variation in epigenomic patterns and their regulation exists across taxa, we discuss research pertaining to plasticity at each level of biological organization across a diversity of species, including many non-model systems. We discuss and contrast literature in plants and animals, with greater emphasis on animals in areas where findings are less clear than those in plants. We have refrained from discussing research in fungi only because it is outside our expertise. We close our review by discussing how we can synthesize these findings across different scales to improve our understanding on the role of epigenetic variation on the manifestation and evolution of phenotypic plasticity. Illustrations by Samuel N. Bogan.

## Sources of Epigenetic Variation

### Genetic Variants Causing Epigenetic Differences

One of the strongest factors that influence inter-individual epigenetic variation is genetic variation (Mendizabal et al. 2014; Villicaña and Bell 2021). Early analyses of DNA methylation and genetic relatedness from humans reported that additive genetic effects (narrow-sense heritability, $h^2$) explaining variation in DNA methylation were substantial, ranging between 0.09 and 0.50 (Boks et al. 2009; Kaminsky et al. 2009; Bell et al. 2012; Gordon et al. 2012; Grundberg et al. 2013). It should be noted that the exact amount of heritability can be influenced by how DNA methylation was measured and how much genetic variability was included in each study. Nevertheless, these metrics indicated substantial influence of genetic variation on DNA methylation. Subsequent studies furthered this premise by identifying genetic variants that explain variation of DNA methylation, or "DNA methylation quantitative trait loci" (also referred to as "mQTLs"). In humans, mQTLs have

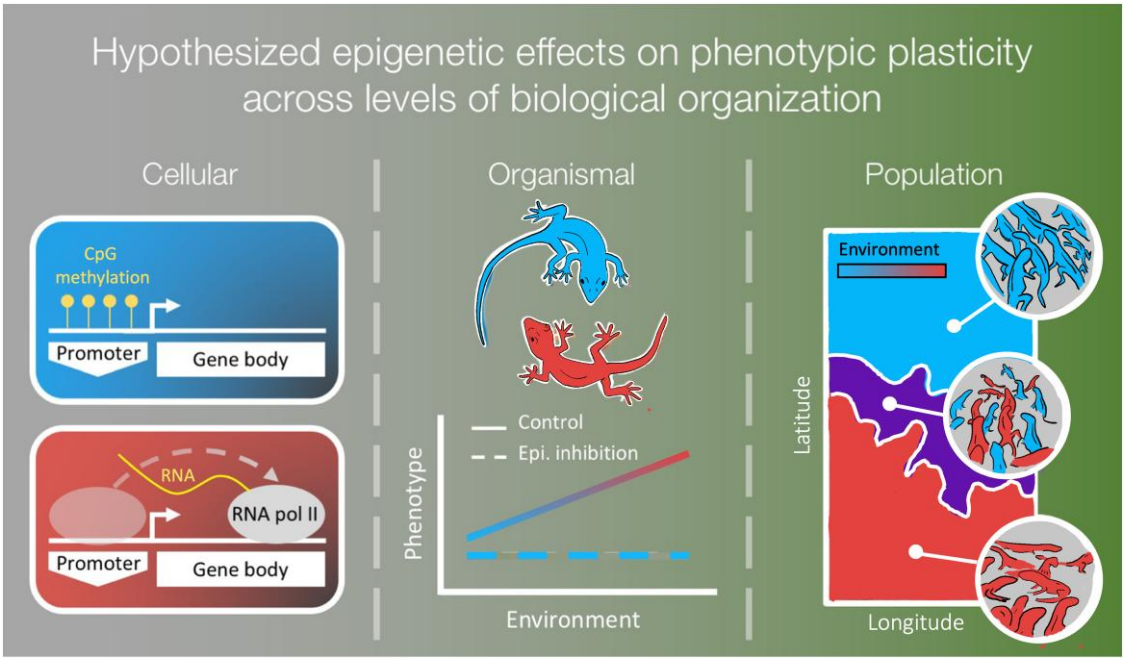


**Fig. 1.**—In this review, we examine evidence for or against epigenetic regulation of phenotypically plastic responses across cellular, organismal, and population levels. We explore this hypothesis across levels of biological organization by reviewing literature regarding (cellular) associations between differential methylation and expression induced by environmental variation (left panel), (organismal) causal tests of chromatin-modifying enzymes' effects on phenotypic plasticity (middle panel), and (population) environmentally associated epigenomic variation across natural populations (right panel). We depict a continuous environmental variable corresponding to gene regulatory and phenotype states. The canonical model posits that an environmental state (for example, one of the two colors) may affect gene transcription via changes in DNA methylation (left panel) which may adjust phenotype toward an optimum in that environment (i.e. specific color phenotype in a specifc color environment). This mechanism can be tested by modifying epigenetic factors in organisms and measuring the extent of their phenotypic change between levels of the environmental variable (middle panel). Assuming this model, associations between environment, methylation, and phenotype should arise across natural landscapes and their populations (right panel).

been associated with a substantial fraction of DNA methylation variation across the genome (van der Graaf et al. 2015). For example, 15% to 17% of the additive genetic variance of DNA methylation in blood could be explained by mQTLs in a study of 32,851 participants (Min et al. 2021).

Studies in other species, which are much rarer than in humans, also indicate substantial impacts of genetic variation on epigenetic variation. For example, in a study of maize (*Zea mays*), nearly half of all differentially methylated regions among 20 maize inbred lines were associated with a specific SNP in *-cis* (Eichten et al. 2013). In *Arabidopsis thaliana*, it was demonstrated that a large amount of CHH methylation (where H stands for nucleotides other than G) variation across climates was linked to a mQTL in CMT2, a DNA methyltransferase (Dubin et al. 2015). As more data are generated from diverse species, a solid picture that genetic variation is a significant component of epigenetic variation is emerging (Gáspár et al. 2019; Wang et al. 2016b). On the other hand, the extent to which genetics influence epigenetic variation differs between studies. It should be noted that the molecular and statistical methods to detect genetic and epigenetic variations of non-model species vary widely, which may become an additional source of variation in comparing these data (Roessler et al. 2016; Huh et al. 2019).

There are several takeaways from genetic studies of epigenetic variation. First, our understanding of the mechanism of how genetic variation affects epigenetic variation is still at its infancy. Some examples are produced from well controlled studies in mammalian model systems, but information from the vast majority of species is lacking. Currently, variation in transcription factor binding motifs, targets of DNA methylation such as CpGs, and coding regions of chromatin-modifying enzymes generally appear as potential genetic factors influencing methylation (Hu et al. 2013; O'Malley et al. 2016; Zhu et al. 2016; Yi 2017; Yin et al. 2017; Klughammer et al. 2023). Importantly, local genetic variation has a significant influence on proximal epigenetic variation. Studies seeking to measure DNA methylation without an explicit focus on genetic variation should still take it into account. In addition, studies using sequencing of bisulfite-converted DNA are susceptible to genetic variation as the method relies on the presence of C/T and G/A SNPs to call DNA methylation. In cases when genetic variants are known, C/T and G/A SNPs should be masked to avoid confounding effects of

genetic variation (Mendizabal et al. 2019; Wu et al. 2020). Many studies however, including those cited in this review, do not consider this variation and are subject to the issue of confounding polymorphism (Laine et al. 2023).

## Epimutations

Epigenetic variation may arise due to stochastic changes in molecular pathways that affect epigenetic marks (Becker et al. 2011; Johannes and Schmitz 2019). Much like genetic mutations, such "epimutations" can become a source of heritable variation associated with phenotypes. Currently, the most extensive data on epimutations are from *A. thaliana* DNA methylation. One of the most salient findings in *A. thaliana* studies is that the rate of epimutation is orders of magnitude higher than that of genetic mutation rate (van der Graaf et al. 2015). Environmental changes are known to influence epimutation rates in *A. thaliana*. For example, in a high salinity environment, nearly 50% more epimutations were reported than in ambient conditions (Jiang et al. 2014). It should be noted that this was in addition to a 100% increase in genetic mutations (Jiang et al. 2014).

Studies of epimutations in other species are relatively sparse. A recent study of small RNAs in *Caenorhabditis elegans* (Beltran et al. 2020) suggested substantial rates of epimutations. Investigating fold changes of 22-nucleotide-long RNAs (22G-RNAs) in mutation accumulation lines of *C. elegans*, they (Beltran et al. 2020) reported that epimutations occurred more frequently than point mutations (mean 23.5 epimutations/generation vs. 0.2 to 1 single nucleotide substitutions per generation). Therefore, spontaneous changes of epigenetic marks may occur frequently in these species. Some of those changes may be facilitated by environmental shifts (Beltran et al. 2020).

## Epigenetic Drift

DNA methylation of a specific genomic position is not a fixed trait, but one that changes over the lifetime of an organism. The term "epigenetic drift" is often used to refer to this phenomenon (Cooney 1993; Egger et al. 2004; Teschendorff et al. 2013; Sun and Yi 2015). "Drift" refers to stochastic changes in the frequency of existing variants over evolutionary time. Epigenetic drift was initially used to describe stochastic changes in DNA methylation. However, studies in model species indicate that there is some "direction" for the epigenetic drift, which is consistent with gradual dysregulation of epigenetic pathways as organisms age. Consequently, epigenetic drift may be linked to aging. The direction of changes in DNA methylation at specific positions has been used to predict the chronological age of organisms with accuracy (Horvath 2013; Horvath and Raj 2018). Epigenetic drift due to aging is observed in diverse species such as mammals, birds, other vertebrates, and some invertebrates (Fairfield et al. 2021; Sun et al. 2021; Mayne et al. 2022; Brink et al. 2023). If epigenomic studies of populations include individuals of different ages, epigenetic drift can present itself as variation within or between populations, independent of genetic variation. The effect of epigenetic drift in studies of natural population is seldom considered and should be addressed in systems where age can be reasonably determined.

## "(Epi)Genetic Capacitor" Hypothesis

One source of epigenetic variation that is little explored could be attributed to chaperone proteins, such as heat shock proteins (hsps). Hsps prevent misfolding of nascent proteins, chaperone denatured proteins to the proteasome (Hartl 1996), and are upregulated in response to cellular stress including but not limited to exposure to cold, UV light, tissue regeneration, diseases, and heat (Benjamin and McMillan 1998; Jarosz 2016). The heat shock protein hsp90 can "buffer" cryptic genetic variation and canalize phenotypes. This is achieved by hsp90's attenuation of *trans* regulatory elements and its direct effect on chromatin's accessibility to such elements (Morcillo et al. 1993). Under oxidative stress, hsp90 is directed toward chaperoning denatured proteins and releases attenuated regulatory elements which can then interact with mutations within accessible *cis*- regulatory sites (Rutherford and Lindquist 1998; Ruden et al. 2003). When hsp90 activity is genetically or pharmacologically inhibited, mirroring loss of attenuation during oxidative stress, wide-ranging phenotypic variation that was previously "masked" can emerge (Rutherford and Lindquist 1998; Queitsch et al. 2002).

Hsp90 can also affect chromatin state indirectly via interactions with chromatin regulators such as the Polycomb group (PcG) and Trithorax Group (TrxG) (Ruden and Lu 2008; Tariq et al. 2009). Ruden et al. have demonstrated that Hsp90 stores epigenetic potential for morphological variation during development and disease progression (Ruden et al. 2003; Ruden and Lu 2008). Other chromatin modifiers can also buffer genetic and epigenetic variations. For example, deletion of multiple histone modifying enzymes, but not metabolic enzymes, increased interspecific variation in gene expression between yeasts (Tirosh et al. 2010). This suggests that differential regulation of Hsps and/or other chromatin modifiers due to environmental factors may release cryptic genetic and/or epigenetic variants influencing gene expression. Buffering of cryptic genetic variants by epimutations was discussed by O'Dea et al. (2016) as a mechanism of epigenetically driven plasticity and an evolutionary process that maintains genetic diversity and affects rates of evolution. The phrase "epigenetic capacitor" was first used by Sollars et al. (2003) to describe heritably altered chromatin states and morphological diversity following reductions in hsp90 function. We propose that the epigenetic capacitor hypothesis extends beyond

hsp90 and its cofactors. It stands as a broader model of epigenetically driven plasticity by which environmentally dependent release or activation of epigenetic regulatory factors promotes otherwise cryptic epigenetic variation. This cryptic epigenetic variation arises from context-dependent release of *trans*-acting epigenetic factors. However, it can also be affected by coding variants within these factors or mutations in *cis*- elements such as SNPs proximal to methylated CpGs. Environmental stressors such as increasing temperature therefore affect the regulation and expression of these factors, impacting downstream pathways and causing epigenetic variation that may drive phenotypic plasticity.

## Observations at the Organismal Level—Causal Tests of Epigenetic Effects on Phenotypic Plasticity

Many studies have experimentally tested causal effects of epigenetic variation on phenotypic plasticity, often by rearing control and epigenetically manipulated organisms under different conditions and phenotyping organisms from those treatments. A causal effect of epigenetic manipulation on the level of plasticity between conditions should imply that epigenetic modifications at least partially explain observed phenotypic plasticity. To perform epigenetic modifications, studies often employ chemical inhibition of chromatin-modifying enzymes (Table 1). Such pharmacological agents are known to have genome-wide impacts on epigenetic marks and often yield considerable side effects (Christman 2002; Zhou et al. 2002). Epigenetic enzymes can also be interrogated using CRISPR knockout lines (Loughland et al. 2021) and RNA interference or RNAi (Dai et al. 2018). Epigenetic editing at targeted loci can be achieved by TALEN or CRISPR-Cas9 (Maeder et al. 2013; Xu et al. 2016b). In addition, epigenetic recombinant inbred lines (epiRILs) (Johannes et al. 2009; Reinders et al. 2009) have enabled tests of phenotypic differences between epigenetic variants with nearly identical genomes (Table 1). Integrating these approaches with ecophysiological experiments, we can assess epigenetic contributions to plasticity.

### Effects of DNA Methylation on Plasticity in Animals

Studies in animals provide both evidence for and against epigenetic regulation of plasticity in environmental performance, while supportive evidence has been more frequently reported. Traits spanning distinct levels of biological organization such as whole-organism and pathway-specific phenotypes can differ in the strength or existence of regulation by epigenetic modifications. A recent study in zebrafish tested the effect of DNA methylation on thermal acclimation. Using CRISPR-Cas9, Loughland et al. (2021) generated single de novo methyltransferase A knockout lines (DNMT3aa−/−) and double knockouts (DNMT3aa−/−ab−/−) and examined their acclimation to ambient or cold temperature. DNMT3A and DNMT3B are essential for the establishment and maintenance of DNA methylation (Okano et al. 1998, 1999). The authors found DNMT3 knockouts exhibited reduced survival under cold stress, an organism-level fitness trait. Organismal performance measured via respiration rate worsened under cold when both DNMT3 isoforms were knocked out. The plasticity of citrate synthase activity, a proxy for aerobic metabolism linked to the citric acid cycle, was unaffected by knockout (Loughland et al. 2021). DNMT3 possessed weak effects on singular cellular processes such as citrate synthase activity, but potentially additive effects across multiple pathways yielding an impact on the plasticity of organismal performance.

In invertebrates, studies employing chemical and RNAi inhibition of DNMTs have reported reduced plasticity of thermal tolerance, developmental rate, and acclimation in responses to thermal stress (Dai et al. 2018; Wang et al. 2020; Agwunobi et al. 2021; McCaw et al. 2021). For example, McCaw et al. (2021) reported that the DNMT inhibitor Zebularine reduced the negative effect of thermal stress on fecundity in the seed beetle *Callosobruchus maculatus*, suggesting that DNMTs may mediate life history tradeoffs between fecundity and investment-per-offspring across temperature. Fuchs et al. (2014) determined that strobilation of the moon jelly *Aurelia aurita* during metamorphosis is regulated by seasonal exposure to low temperature and inhibited by 5-Azacytidine. On the other hand, some studies report negligible effects of epigenetic inhibition on plasticity. For example, Gegner et al. (2020) quantified temperature-induced wing pattern polyphenism in the beetle *Harmonia axyridis* when treated with RNAi of DNMT1 associated protein 1 (DMAP1) and found no influence of DMAP1 on thermal plasticity of wing pattern.

This line of study has moved beyond single taxa toward species interactions. Resolving how environmental variation influences species interactions is one of the greatest challenges to predicting biodiversity's responses to environmental change (Tylianakis et al. 2008). Animal studies have measured the impact of DNA methylation on phenotypic plasticity in the context of species interactions including infectious disease and altered predator–prey dynamics (Wilmers et al. 2007; Altizer et al. 2013; Laws 2017). Two studies in arthropods found that DNMT inhibition decreased viral replication and the severity of experimentally introduced viral and bacterial infections (Baradaran et al. 2019; Huang et al. 2019). Studying the toad *Rhinella marina*, Sarma et al. (2020) found no effect of DNMT inhibition by Zebularine on the reaction norm of cortisol response to a predator cue despite significant differential methylation in response to cue exposure.

**Table 1**
Strengths and weaknesses of different epigenetic manipulations with emphasis on inhibiting DNA methyltransferase or TET enzyme activity

| Method | Description | Strengths | Weaknesses | References |
|---|---|---|---|---|
| Site-directed CRISPR-TET1 5-mC mutagenesis | Targeted demethylation achieved via CRISPR vector containing guiding RNAs and TET1 demethylase | • Controls for effect of nonspecific enzymatic interactions on phenotype<br>• Can target single loci | • Challenges to in vivo delivery | Liu et al. (2019) |
| CRISPR-Cas9 DNMT/TET knockout | Deletion or functional removal of all or portion of enzyme | • Highly targeted<br>• Heterozygous knockout | • Challenges to in vivo delivery | Loughland et al. (2021) and Nakamura et al. (2021) |
| CRISPRi DNMT/TET knockdown | siRNA expressed by CRISPR-Cas9 vector | • Targeted<br>• Efficient introduction of siRNAs | • Nonreversible<br>• Challenges to in vivo delivery | Nakamura et al. (2021) |
| RNAi DNMT/TET knockdown | siRNA expressed by traditional viral or nonviral vectors | • Targeted<br>• Reversible | • Incomplete inhibition | Xu et al. (2016a) |
| RG108 DNMT inhibition | Targets DNMT via non-nucleoside small molecule inhibitor | • Low cytotoxicity | • Challenges to in vivo delivery | Lucidi et al. (2019) |
| 5-Azacytidine DNMT inhibition | Chemical inhibition of DNA methylation enzyme | • Ease of delivery | • High cytotoxicity; confounding effect<br>• Low inhibition | Griffin et al. (2016) |
| Zebularine DNMT inhibition | Chemical inhibition of DNA methylation enzyme | • Ease of delivery | • Cytotoxicity<br>• Less inhibition than 5-Azacytidine | Griffin et al. (2016) |
| Epigenetic recombinant inbred lines (epiRILs) | Crossing experimentally demethylated lines with wild types before repeated selfing | • Directly targets chromatin/DNA modifications rather than enzymes | • Global, nonspecific effects<br>• Infeasible in certain systems | Zhang et al. (2013) |

We focus on these enzymes as they are frequently studied to interrogate the maintenance and regulation of DNA methylation. The "References" column includes papers describing, comparing, and/or validating these methods in the context of manipulating epigenetic variation.

## Effects of DNA Methylation on Plasticity in Plants

There are abundant causal tests of DNA methylation's effect on plasticity in plants. More than a decade of research interrogating *A. thaliana* DNA methylation using chemical DNMT inhibitors or epiRILs have demonstrated effects on the plasticity of growth, morphometrics, and flowering time across nitrogen, salinity, and nutrition levels (Bossdorf et al. 2010; Boyko et al. 2010; Zhang et al. 2013; Jiang et al. 2023). Similar experiments in non-model plants including angiosperms and bryophytes have detected significant effects of DNA methyltransferase inhibition on plastic responses to intraspecific competition (Puy et al. 2018), salinity (Arya et al. 2016), transplantation (Sammarco et al. 2022), and iron deficiency (Sun et al. 2021). Moreover, inhibition of histone deacetylases has shown to increase survival under salinity stress in some plants (Roca Paixão et al. 2019). Inhibition of DNA methylation has generally increased the plasticity of flowering time while decreasing the plasticity of growth and morphometric traits (Bossdorf et al. 2010; Kottler et al. 2018). Related to hsp90 and the (epi)genetic capacitor hypothesis, inhibition of hsp90 has shown to increase the thermal plasticity of multiple phenotypes in plants (Sangster et al. 2007). These observations underscore trends observed in animals: in terms of the impacts of DNA methylation on plasticity, their strength and direction are dependent on trait type such that methyltransferase activity is necessary for the plasticity of higher order organismal traits such as growth and survival, while bearing weaker or opposing effects on the plasticity of specific pathways or non-fitness correlating traits.

## Caveats to Causal Tests of Epigenetically Driven Plasticity

We note that at least two confounding effects are pervasive across aforementioned causal tests of epigenetic effects on plasticity: (i) enzymatic inhibition may shape phenotype via nonspecific protein–protein interactions and (ii) causal effects of enzymatic inhibition on phenotype are often not partitioned from chemicals' cytotoxic effects. For example, Loughland et al. (2021) observed a strong effect of DNMT3A knockout on survivorship, but there was little differential methylation genome-wide, which indicates that changes in DNA methylation may have had little to do with observed phenotypic change. Three methods exist for

controlling against nonspecific effects of chromatin-modifying enzyme inhibition: (i) cytotoxic or nucleosidal effects (e.g. DNA damage) of chemical inhibitors can be measured and controlled for via inclusion as parameters in statistical analysis, (ii) multiple regions of an enzyme such as its active site and interacting domains can be knocked out so that their subsequent phenotypic effects can be compared, or (iii) site-directed 5-mC manipulation via CRISPR can be used to modify DNA methylation at singular bases, avoiding nonspecific effects of enzyme inhibition (Liu et al. 2019). In addition, the effects of DNA methylation may be highly cell-type specific (e.g. Mendizabal et al. 2019), making it difficult to accurately track functional DNA methylation interrogations in the absence of cell-type specific information.

## Epigenetic Variation Across Natural Populations

Studies measuring epigenetic variation across natural populations address another facet of the epigenetic plasticity hypothesis: if epigenetic processes regulate plasticity, to what extent do these mechanisms drive plasticity in nature? These studies often utilize next-generation sequencing to analyze samples collected from populations in situ and/or laboratory experiments sourced from different populations. Numerous examples in plants and animals have observed environmentally associated epigenetic structure between genetically unstructured or nearly identical populations that possess phenotypic differences. However, only a handful of studies have directly controlled for the effects of local genetic variants on DNA methylation at associated bases, which may confound interpopulation variation in DNA methylation that appears to covary with environment. This is not to say that methylated or epigenetically modified mutations are unimportant for plastic responses to in situ environmental variation. Under the (epi)genetic capacitor hypothesis, described above, epigenetic release of an otherwise buffered mutation may induce phenotypic change in response to environmental variation. Accounting for genetic variation in population epigenomic studies enables detecting environmentally driven epigenetic divergence.

### Environmentally Associated Epigenetic Variation Between Populations

Epigenomic variation within and across populations appears to be abundant in plants (Moler et al. 2019) and animals (Hu and Barrett 2017). Epigenetic divergence can be driven by environmental differences between habitats (Rey et al. 2020). A growing body of literature addresses the extent to which epigenetic divergence between environments in the wild is attributed to genotype-environment associations and genetically linked epigenetic states. Some studies suggest associations between environment and DNA methylation independent of genetic variation. Studying eight populations of the lizard *Anolis cristatellus*, Wogan et al. (2020) analyzed >8,000 SNPs and DNA methylation variants derived from reduced representation bisulfite sequencing in conjunction with spatial and environmental data. They showed that populations exhibited significant genetic isolation by distance and environment. Population pairs exhibiting high genetic divergence ($F_{ST}$) also exhibited high epigenetic divergence ($\Phi_{ST}$), and epigenetic variation also correlated with environment. After controlling for genetic distance, residual epigenetic isolation across distance was lost while residual epigenetic isolation by environment was held, supporting environment-epigenetic associations independent of genetic background (Wogan et al. 2020). In three studies of fishes comparing wild populations or domesticated populations, intergenerationally stable epigenetic variation was observed between populations, either in the absence of interpopulation genetic variation or after controlling for it (Anastasiadi and Piferrer 2019; Konstantinidis et al. 2020; Vernaz et al. 2022).

Population-level metrics of genetic structure are useful for evaluating genetic and epigenetic variations at microevolutionary scales, but to robustly detect environmentally induced, epigenetic variants, we must control for the direct contribution of mutations to methylation at proximal CpGs. Of the four aforementioned studies on vertebrate populations, none directly controlled for effects of genetic variants to DNA methylation at proximal CpGs. This same issue is present in most common garden studies of vertebrate populations, where stable differential methylation between populations has been detected across multiple generations of acclimation to a common environment (Heckwolf et al. 2020; Kelley et al. 2021). By contrast, environmental studies in invertebrate populations have made some progress in controlling genetic contributions to methylation.

Among invertebrates with large dispersal distances, $F_{ST}$ and $\Phi_{ST}$ are frequently uncorrelated and epigenetic divergence exceeds genetic distance (Johnson and Kelly 2020; Liew et al. 2020; Silliman et al. 2023). Silliman et al. recently measured the contribution of genetic background to intraspecific variation in DNA methylation at a nucleotide resolution in the oyster *Ostrea lurida*. They identified 3,963 differentially methylated loci between two populations of *O. lurida* exhibiting low $F_{ST}$ and high $\Phi_{ST}$. Between-individual $F_{ST}$ and $\Phi_{ST}$ did not correlate, but 27% of intraspecific DNA methylation variation was explained by genetic variation via mQTL analysis (Silliman et al. 2023). However, mQTL detection was likely underpowered due to small sample size. Thus, the effect of genetic contributions to methylation may have been underestimated. Nevertheless, the studies described suggest that substantial epigenome differences can arise between connected populations that do not necessarily exhibit genomic divergence. Some studies are revealing significant epigenetic variation between clonal individuals inhabiting different

environments despite their identical or nearly identical genomes (Thorson et al. 2019; Tönges et al. 2021). Among studies on animal populations to date, environmental variation bears nearly ubiquitous effects on DNA methylation that may be independent of genetic variation, but this latter point requires further investigation.

Studies of natural or large-scale, experimental plant populations have observed more mixed environmental effects on DNA methylation. De Kort et al. (2020) and van Moorsel et al. (2019) found that demography and genetic divergence, rather than environmental variance, drove stable differential methylation between plant populations and communities. Studying wild and domesticated strawberries, Shen et al. (2018) found a contrasting result: of 5,412 regions differentially methylated between populations, only 22.54% were directly attributed to genetic variants local to methylation sites. These studies and their designs have great potential to resolve environmental versus genetic contributions to epigenetic variation among populations and epigenetics' role in plastic responses to natural environments.

### Extending Population Epigenomics to Selection on Metastable Epialleles

Tracing back to Waddington's framework for the canalization of acquired characters (Waddington 1942), stable epigenetic variation between populations may not only be linked to induction by the environment, but natural selection acting on and maintaining heritable epigenetic variants often called metastable epialleles (Kalisz and Purugganan 2004; Jablonka 2013). Confirming selection on metastable epialleles requires demonstrating (i) inheritance independent of underlying genetic variation and (ii) fitness effects. As discussed earlier, several studies in plants and animals have demonstrated multi-generation metastability of DNA methylation epialleles that vary between natural populations (Heckwolf et al. 2020; Kelley et al. 2021). Recently, Sammarco et al. (2024) studied the heritability of DNA methylation markers that varied between 21 wild strawberry populations in association with climate and found metastability of methylation variants independent of genetic effects. Though methodologically difficult, it remains crucial to study the fitness effects of epigenetic variants (Stajic and Jansen 2021). Integrating fitness studies with the analysis of metastable epialleles that vary between environments will help determine the role of epigenetically driven plasticity in evolution.

## Epigenetic Regulation of Molecular Responses to the Environment

In this section, we discuss studies that address a key facet of the epigenetic model of plasticity—namely, molecular and cellular mechanisms by which epigenetic variation can influence gene expression, and consequently, phenotype. The most tested and discussed model of epigenetically mediated plasticity is that environmentally induced changes to chromatin modifications such as DNA methylation and histone tails cause differential expression or alternative splicing of transcripts that underpin a phenotypic effect (Law and Jacobsen 2010; Duncan et al. 2014). Below we summarize and synthesize evidence that associations between differential gene regulation and differential methylation in response to abiotic stress is apparent in some plants but limited in almost all studies in nonmammalian vertebrates and invertebrates (Fig. 2).

In many examples, we will discuss gene body DNA methylation. Even though DNA methylation is phylogenetically widespread, there are some general differences between invertebrate versus vertebrate DNA methylation (Suzuki et al. 2007; Elango et al. 2009; Keller et al. 2016). While DNA methylation of vertebrate genomes typically is "global", meaning that most of the genome is methylated in any given tissue and developmental time point, invertebrate genomes are generally (but not always) sparsely methylated except in some regions that encode transcriptional units. Intragenic DNA methylation is referred to as "gene body methylation" (GBM). Phylogenetic surveys of DNA methylation have established that GBM is the ancestral form of DNA methylation in animal genomes, and global DNA methylation arose during vertebrate evolution (Tweedie et al. 1997; Yi 2012).

The functional roles of GBM are still contested. In well-studied mammalian examples, DNA methylation of promoters, especially those harboring clusters of lowly methylated CpGs or "CpG islands", often leads to the down-regulation of associated genes (Weber et al. 2007; Elango et al. 2009). On the other hand, GBM does not necessarily silence gene expression. Rather, positive, negative, as well as "bell-shaped" correlations between GBM and gene expression have been observed across diverse taxa (Zeng and Yi 2010; Bonasio et al. 2012; Dixon et al. 2018; Gatzmann et al. 2018; Jeong et al. 2018; Li et al. 2018; Downey-Wall et al. 2020; Strader et al. 2020; Johnson et al. 2022). In at least some metazoans, intragenic methylation is bound by methyl-DNA-binding domain protein 2/3, which recruits acetyltransferases promoting H3K27 acetylation and elongation of transcription (Xu et al. 2021). Alternatively, GBM is implicated in reducing transcriptional noise in many species (Hunt et al. 2010; Huh et al. 2013; Gatzmann et al. 2018; Li et al. 2018; Wu et al. 2020). GBM may also correlate with gene expression because it supports sequence conservation. Methylated gene bodies are less accessible in at least some metazoans (Gatzmann et al. 2018; Bogan et al. 2023), which could suppress mutations (Shi et al. 2016). Consistently, methylated genes tend to exhibit reduced evolutionary rates (Hunt et al. 2010; Park et al. 2011; Sarda et al. 2012; Dixon et al. 2016).

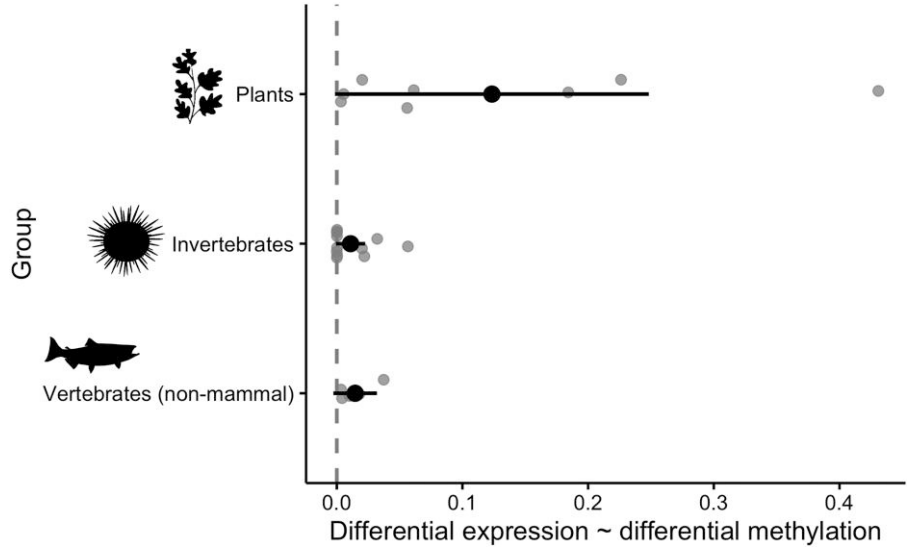


**Fig. 2.**—Variance in environmentally induced differential DNA methylation (DM) associated with differential expression (DE) across 23 datasets in plants, invertebrates, and vertebrates. Values either represent overlap in genes exhibiting DE and DM as a proportion of DM sites exhibiting DE or correlation coefficients. Small gray points represent singular pairwise contrasts used to estimate DE and DM (e.g. control vs. stress treatments). Large black points depict means ± 95% CI. Fidelity was given to the data as they were described in publication and thus, genomic methods and reporting statistics are not standardized.

## Limited Associations Between DNA Methylation and Differential Expression Across Environments

Whether and how epigenetic variation contributes to phenotypic plasticity and environmental acclimation is obscured by increasing evidence that, transcriptome-wide, environmentally induced differential DNA methylation (referred to as "DM" henceforth) and differential expression (DE) frequently do not necessarily correlate. A notable example is found in a study of stony corals by Dixon et al. (2018). The authors transplanted coral fragments between populations inhabiting two distinct coastal environments. Fragments then acclimatized before being sampled for RNA-seq and methyl-binding domain sequencing. DE between populations inhabiting the distinct environments correlated with interpopulation DM. DE and DM associated with plastic responses to transplantation did not correlate (Dixon et al. 2018). Saban et al. found that DM attributed to divergence between plant populations experiencing different $CO_2$ levels affected DE 11.6× more than plastic responses to experimental increases in $CO_2$. However, plastic DM and DE did correlate (Saban et al. 2020). We find that differences in the strength of associations between DE and DM attributed to genetically fixed versus plastic effects is broadly observable in metazoans while, in some cases, plants have shown stronger correlations between environmentally induced changes in methylation and expression (Fig. 2).

The general lack of association between DE and DM is particularly evident in invertebrates, as summarized in earlier reviews largely focused on insects (Glastad et al. 2019; Oldroyd and Yagound 2021; Duncan et al. 2022). Here we list some examples, as many are described in the above reviews and other papers. Dixon and Matz performed a reanalysis of environmental acclimation studies including WGBS and RNA-seq libraries from eight species of arthropods and cnidarians. They found either no associations between DM and DE or at most an extremely weak effect (Dixon and Matz 2022). Similarly, DM between environments does not correlate with gene expression in molluscs (Downey-Wall et al. 2020; Johnson et al. 2022) and echinoderms (Strader et al. 2020).

It should be noted that despite a common narrative in the scientific literature that vertebrate promoter methylation affects gene expression in response to environmental signals, this association is not universal. Rather, it is often limited to a subset of genes. Studies in fishes have frequently shown weak associations (Wang et al. 2016; Skjærven et al. 2018; Lai et al. 2019; Anastasiadi et al. 2021; Jones and Griffitt 2022). Other vertebrates such as birds and reptiles also exhibit weak relationships between promoter DNA methylation and gene expression in response to the environment (Lindner et al. 2021; Ruhr et al. 2021). The role of promoter methylation in regulating gene expression may need revision as data from diverse taxa accumulate, especially in the context of phenotypic response to environmental signals.

Complex relationships between DNA methylation and other epigenetic processes may conceal associations between differential GBM and gene expression. For example, the relationship between DE and DM may be dependent upon chromatin state (Lea et al. 2018; Bogan et al. 2023). Genetic manipulation of DNA methylation machinery can provide useful information in this

regard. To date, several groups performed RNAi mediated knockdown experiments to causally test the gene regulatory role of DNA methylation in insects (Bewick et al. 2019; Ventós-Alfonso et al. 2020; Arsala et al. 2022; Ivasyk et al. 2023). While these studies affirm critical roles of DNA methylation in development, their results varied with respect to the relationship between DNA methylation and gene expression. For example, knockdown of DNMT1 in the milkweed bug *Oncopeltus fasciatus* by Bewick et al. led to widespread failure in egg laying and embryo development and reduction of DNA methylation in ovaries. However, reduced levels of DNA methylation did not lead to reduction of gene expression (Bewick et al. 2019). On the other hand, a recent knockdown study of DNMT1a in *Nasonia vitripennis* exhibited strong reduction of DNA methylation and down-regulation of gene expression (Arsala et al. 2022). Interestingly, a recent CRISPR/Cas9 study in clonal raider ants that did not address gene expression observed that loss-of-function DNMT1 mutants reduced DNA methylation and fertility but did not affect development (Ivasyk et al. 2023). In addition to the confounding pleiotropic effects of DNMTs, effects of DNA methylation may be highly cell-type specific, and may be masked in some experimental designs. Further studies are needed to elucidate causative relationship between GBM and gene expression.

The relationship between DE and DM appears more robust in plants. On macroevolutionary time scales, plant genes that changed DNA methylation status between species tend to show weak yet significant, changes of gene expression (Seymour and Gaut 2020). Studies within species further demonstrate that changes to plant methylomes across abiotic environments are more frequently associated with gene expression relative to animals. For example, 20% to 26% of differentially expressed genes were found near differentially methylated positions by Saban et al. (2020). The proportion of DM associated with DE genes ranges between 5% and 26% across studies of grasses, mangroves, and mulberries in response to draught, desiccation, nutrient deprivation, and transplantation (Li et al. 2020; Rajkumar et al. 2020; Sun et al. 2021; Zhao et al. 2021; Miryeganeh et al. 2022). These relationships starkly contrast those in animals (Fig. 2). Figure 2 was generated from the results of 16 environmental studies in plants (Li et al. 2020; Rajkumar et al. 2020; Saban et al. 2020; Sun et al. 2021; Zhao et al. 2021; Miryeganeh et al. 2022), invertebrates (Arsenault et al. 2018; Downey-Wall et al. 2020; Strader et al. 2020; Dixon and Matz 2022; Johnson et al. 2022), and nonmammalian vertebrates (Skjærven et al. 2018; Anastasiadi et al. 2021; Lindner et al. 2021; Ruhr et al. 2021; Jones and Griffitt 2022) integrating RNA-seq and BS-seq.

## Differences in Addition and Removal of DNA Methylation by Plants and Animals

Divergence in the molecular pathways regulating addition, maintenance, and removal of 5-methylcytosines between plants and animals may offer explanations for differences in the association between DM and DE across environments. The RNA-directed DNA methylation pathway in plants responds to biotic and abiotic stressors by guiding establishment of DNA methylation specific to loci transcribed by RNA Pol V via small RNAs (Erdmann and Picard 2020; Miryeganeh et al. 2022). Demethylation in plants is regulated by direct base excision repair (BER) carried out by DNA glycosylases, which are able to directly excise 5-mC before it is replaced with an unmethylated base (Zhu 2009). Several DNA glycosylases involved in demethylation fall within the REPRESSOR OF SILICING 1/DEMETER-LIKE protein families, which are unique to plants (Roldán-Arjona et al. 2019) and can target specific loci rather than inducing untargeted demethylation (Zhou et al. 2016). BER also drives active demethylation in animals, but 5-mC is not yet known to be directly excised. Rather, DNA glycosylases remove target cytosines after 5-mC is glycosylated or aminated (Zhang and Zhu 2012). BER is sensitive to stress in both plants and mammals (Zhou et al. 2016; Liu and Lang 2020). It is possible that the site-specific targeting of de novo methylation and active demethylation by pathways derived in plants, and potentially mammals, contributes to their stronger associations between changes in DNA methylation and expression across environments. This hypothesis could be tested by comparative studies measuring the specificity of environmentally induced DM to methylation-sensitive *cis*- acting elements in plants and animals in conjunction with RNA-seq and/or interrogation of BER.

## Roles for DNA Methylation Alternative to Gene Regulation

Beyond regulatory differences between plants and animals, there are numerous explanations for the lack of a clear relationship between DNA methylation and gene expression across environments (Bewick and Schmitz 2017; Muyle et al. 2022) For example, DNA methylation is not singularly predictive of gene expression, and evidence of this is particularly strong in animals. Across human cell types for example, the relationship between intercellular DM and DE depends on the chromatin accessibility of associated *cis*-regulatory elements (Lea et al. 2018; Rizzardi et al. 2019). In the purple sea urchin, it was found that associations between DM and DE and alternative splicing induced by ecologically relevant stress depended on chromatin accessibility at transcriptional start sites and intragenic regions (Bogan et al. 2023). Secondly, GBM may prevent highly expressed and conserved genes from generating spurious transcripts rather than exerting a direct influence

**Table 2**
Conclusions and areas for future research regarding hypothesized epigenetic regulation of phenotypic plasticity across cellular, organismal, and populations levels of biological organization

| Level of organization | Conclusions | Future directions |
|---|---|---|
| Cellular | • Plants and mammals exhibit correlations between differential methylation and expression induced by the environment. This relationship is largely absent in nonmammalian vertebrates and invertebrates. | • Experiments and modeling approaches determining whether differential methylation precedes or follows differential expression. |
| | • Interactions between DNA methylation and other chromatin modifications may cause context-dependent associations between methylation and expression. | • Integration of BS-seq or enzymatic methyl-seq and additional epigenomic sequencing approaches (e.g. ATAC-seq and ChIP-seq). |
| Organismal | • Inhibition of DNA methyltransferases and demethylases yields effects on environmental plasticity of performance-level traits, but rarely specific pathways. | • Increased application of inhibition methods with reduced cytotoxicity. |
| | • Whether this causal effect is mediated by DNA methylation versus other molecular mechanisms such as protein–protein interactions is unclear. | • Experimental control against effect of nonspecific interactions rather than DNA methylation on phenotypic plasticity.<br>• Tests across cell types in addition to whole organisms. |
| Population | • Populations inhabiting distinct environments frequently exhibit environmentally associated epigenetic structure in the absence of genetic structure. | • Increased application of controls against genetic effects on methylation in order to unconfound detection of metastable epialleles. |
| | • In rare examples directly controlling for effects of local genetic variants on DNA methylation, interpopulation variation in methylation was driven by environmental differences after controlling against genetic effects. | • Increased integration of phenotypic data with population epigenomics. |
| | • Population epigenomic studies infrequently integrate phenotypic measures of plasticity. | • Integration of epigenomic data in genomic predictions of fitness across populations. |

on gene expression level (Hunt et al. 2010; Park et al. 2011; Sarda et al. 2012; Dixon et al. 2016; Wu et al. 2020). There is some evidence that DNA methylation also canalizes transcription and reduces transcriptional noise, serving an additional function extrinsic of regulating gene expression level or splicing (Neri et al. 2017; Li et al. 2018). Lastly, DM may follow DE rather than initiating it, consistent with a role in canalizing induced transcriptional changes or priming secondary transcriptional responses (Secco et al. 2015; Pacis et al. 2019).

## Conclusions and Future Directions

Here we have reviewed literature addressing the hypothesis that epigenetic modifications to DNA and chromatin drive phenotypic plasticity across abiotic environments, primarily focusing on DNA methylation. We conducted this review across levels of biological organization, specifically addressing three levels of the hypothesis: that epigenetic processes initiate phenotypic plasticity via changes in gene expression (cellular level) that manifest to drive plasticity (organismal level) and shape phenotypic variance and evolution across environmentally distributed metapopulations (population level). We described two primary avenues of support for epigenetically driven plasticity: (i) that inhibition of chromatin-modifying enzymes such as DNMTs and TETs yields significantly reduced phenotypic plasticity and acclimation across environments in plants and animals and (ii) that metapopulations inhabiting distinct environments frequently exhibit environmentally associated differential methylation in the absence of genetic structure or when controlling for the effect of genetic variation on DNA methylation (Table 2). Together, these results suggest that the regulation of DNA methylation affects phenotypic plasticity and its manifestation in nature. However, multiple equivocal findings must be further studied in order to support this conclusion.

At the cellular level, it appears that associations between environmentally induced changes in DNA methylation share little to no association with gene expression in nonmammalian vertebrates and invertebrates. By contrast, this relationship is much stronger in plants (Fig. 2). The weak relationship between differential methylation and differential expression across environments among animals connects to a weakness in causal tests of DNA methylation's effect on phenotypic plasticity. Rarely are such studies able to demonstrate that the phenotypic effect of methyltransferase inhibition is indeed mediated by DNA methylation rather than nonspecific interactions of the enzyme. If differential methylation bears no effect on gene expression in most animals and some plants, does it remain plausible that DNA methylation affects phenotype? In

Table 2 below, we (i) lay out areas for future research that address this question at cellular, organismal, and population scales in addition to needs for overcoming other confounding effects and (ii) summarize results prevalent across the literature at these scales.

In addressing these issues, the field of evolutionary epigenomics arrives at a fork in the road whereby resolving the aforementioned confounding effects will reveal either causal effects of epigenetic variation or demonstrate that DNA methylation's effects on biological processes are extrinsic to direct impacts on phenotype. If future research supports the former, quantifying the heritability of epigenetic variants independent of underlying mutations is necessary to understand the evolutionary significance of epigenetically driven phenotypic plasticity, as such studies remain in their infancy within ecology and evolution. Ultimately, the fields of ecological and evolutionary epigenomics have immediate potential to confirm or rule out the hypothesis that environments may shape heritable phenotypic variation through nongenetic means. This pursuit should expand beyond DNA methylation to determine the roles of noncoding RNAs, histone modifications, and transposable elements in plasticity to determine whether other epigenetic mechanisms adhere to principles we describe here. For example, (i) whether epigenetic modifications regulate the plasticity of specific pathways versus a ubiquitous mode of regulation across the genome and (ii) whether plasticity arises directly from epigenetic change or interactions between epigenetic and genetic variants.

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## Author Contributions

S.N.B. and S.V.Y. conceived of the aim and scope of the review and contributed equally to its writing. S.N.B. produced figures and wrote code associated with Fig. 2. Both authors edited and approved the final manuscript.

## Conflict of Interest

The authors declare no competing interests.

## Data Availability

All data and code associated with this review pertaining to Fig. 2 can be obtained at the following GitHub repository: https://github.com/snbogan/EpiRev. This repository has been archived on Zenodo at https://zenodo.org/records/10574247.

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