Feature Article

Examining the extent of environmental contributions toward DNA methylation and phenotypic variation

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Implications

- Methylation is stable and heritable, and it can have transgenerational impacts.
- Methylation is associated with an extensive variety of livestock phenotypes.
- Dietary nutrients impact DNA methylation and are associated with phenotypic changes.
- Environmental stress impacts DNA methylation.

Key words: DNA methylation, epigenetics, livestock, phenotype

Introduction to Epigenetics in Livestock

Epigenetic modifications allow for flexibility in gene expression without altering DNA sequence. Both transcribed regions of genes and regulatory regions harbor important epigenetic modifications that vary by tissue, with regulatory regions being of particular interest. Davenport et al. (2021) demonstrate the prevalence of diverse epigenetic modifications in regulatory regions in three tissues that have been used to characterize economically important traits in sheep. Chromatin immunoprecipitation with sequencing (ChIP-seq) and whole-genome bisulfite sequencing (**WGBS**) in the spleen, liver, and cerebellum assess CCCTC-binding factor (**CTCF**) binding, DNA methylation, and histone modifications relevant to chromatin state (Davenport et al., 2021). Hypomethylation was mainly identified at active enhancers in all three tissues, whereas hypermethylation was discovered at CTCF-binding sites in the liver and poised enhancer H3K4me1 in the spleen and cerebellum. Methylation sites varied by tissue, but hypo- and hyper-methylation sites were mostly similar between the liver and cerebellum (Davenport et al., 2021).

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Specifically, DNA methylation alters gene transcription through altered transcription factor binding (Figure 1). The presence of methylation may sterically hinder the binding of transcription factors (Figure 1A), directly preventing transcription. Methyl-binding domain proteins can bind to DNA methylation sites, which actively prevent the binding of transcription machinery (Figure 1C). The presence of methylation can also induce a compact nucleosome structure, further hindering transcription (Figure 1D). Finally, methylation can adjust transcription factor binding sites. Rather than simply preventing transcription, the presence or absence of methylation may influence alternative sequence recognition binding sites for transcription factors (Figure 1B). Epigenetic modifications, such as DNA methylation, influence transcription in different ways, allowing for diverse regulation of gene expression.

Methylation interacts directly with the DNA base pairs through the addition of a methyl group to the 5′ position of a cytosine, creating 5-methylcytosine (**5-mC**) (Figure 2). Methyl groups are typically added to cytosines by DNA methyltransferase (**DNMT**) proteins and tend to be removed from cytosines via the demethylation pathway where Ten–eleven translocation (**TET**) proteins oxidize the methyl group (Figure 2). The first step in the demethylation pathway yields 5-hydroxymethylcytosine (**5-hmC**), a stable epigenetic marker that can impact phenotype, and further studies are called for to deduce the role of 5-hmC in livestock. The demethylation pathway continues to oxidize the hydroxy group and eventually return the base to a standard cytosine. The other intermediates in the demethylation pathway, 5-formylcytosine and 5-carboxylcytosine, are not stable and therefore are not considered viable epigenetic markers. The methylation and demethylation pathways yield two stable epigenetic marks, 5-mC and 5-hmC, which have been used to investigate the association of epigenetic modifications with economically important traits (Skvortsova et al., 2017; Wang et al., 2020).

Epigenetic marks are mitotically stable and are maintained through cell divisions and across generations (Skinner et al., 2010). The presence of DNMTs is much lower in post-mitotic cells compared with cells undergoing mitosis, which suggests that DNA methylation is stable in post-mitotic cells (Moore et al., 2013). Still, certain tissues do exhibit distinct measures of variable methylation (Cantrell et al., 2019). One example

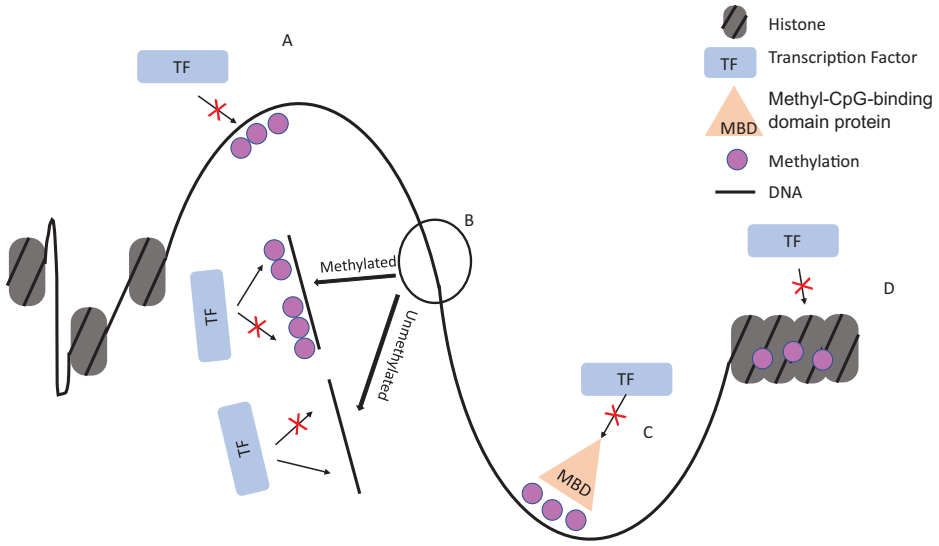

Figure 1. Impacts of DNA methylation on transcription. Methylation can impact transcription in a variety of ways. (A) DNA methylation can sterically hinder the binding of transcription factors, resulting in repressed transcription (Singal and Ginder, 1999). (B) Transcription factors bind to certain sequences only when they are unmethylated and bind to other sequences when methylation is present (Zhu et al., 2016). (C) Methyl-binding proteins may bind to DNA methylation and inhibit the binding of transcription factors and actively repressing transcription (Singal and Ginder, 1999). (D) DNA methylation can lead to a more compact structure of chromatin, which represses transcription (Choy et al., 2010).

is brains, where neurons are particularly unique and DNMTs continue to be present at substantial levels in post-mitotic cells, suggesting a unique role of methylation in the brain (Moore et al., 2013). Methylation in the brain has been associated with learning and memory, which may explain the need for increased dynamic changes in methylation in the brain compared with other tissues.

Comprehending the stability of epigenetic marks merits a brief discussion of the epigenetic reprogramming of a developing zygote that occurs within the maternal environment (Zhu et al., 2021). More specifically, epigenetic reprogramming of a developing zygote occurs within the maternal environment. The contributing maternal genomic content is demethylated passively through progressive loss of methylation occurring at each cell division, and the parental genome is demethylated rapidly and actively at fertilization. Nevertheless, some genomic regions do escape post-fertilization demethylation. For example, imprinted genomic regions that are differentially methylated escape demethylation, and these parental allele-specific methylation sites are transmitted to the next generation resulting in allele-specific expression of associated imprinted genes (Murdoch et al., 2016).

Heritability of methylation through cell generations suggests the consistent role that these modifications play across the lifetime of an animal and permit our molecular understanding of inheritance from parent to offspring (Trerotola et al., 2015). DNA methylation is consistent across cell generations for the lifetime of the animal and can be transgenerationally passed from parent to offspring (Moore et al., 2013; Trerotola et al., 2015). Known genetic factors alone often do not account for the total heritability of many traits from parent to offspring (Manolio et al., 2009). Height is a classic complex trait where identified genetic loci associated with height account for only 5% out of the total 80% heritability (Visscher, 2008). The "missing heritability" here, and in many other traits, is thought to be at least partially comprised of heritable epigenetic modifications (Triantaphyllopoulos et al., 2016).

Susceptibility of DNA methylation to modification from environmental stimuli permits flexibility in the regulation of gene expression (Massicotte and Angers, 2012; Moore et al., 2013). In fact, changes in epigenetics during a mammal's early life can continue to impact the animal even after the environmental component that influenced the modification has been removed (Tiffon, 2018). Honeybees rely on nutritional impacts on epigenetics in early life to differentiate between castes, which impacts the bee for the rest of its life. The queen bee is fed jelly during its larvae stage that affects DNA methylation of key genes, resulting in a lifespan up to 20 times longer than other bees (Tiffon, 2018). The honeybee demonstrates the impact of environmental effects in early life on epigenetics that impact the animals throughout their lifetime.

Environment and Epigenetics Connection

Dietary folate and betaine are substrates in the folate and methionine cycles, which produce methionine, a crucial substrate for S-adenosylmethionine (**SAM**) synthesis. As shown in Figure 2, SAM is the methyl donor used by DNMTs to methylate cytosine. Dietary cofactors, such as B vitamins, can also impact methylation by DNMTs (Murdoch et al., 2016). Diets deficient in methyl donors and DNMT cofactors unsurprisingly lead to global DNA hypomethylation. In a multigenerational study, Scottish blackface ewes were fed a diet deficient in vitamin B_{12}, folate, and methionine

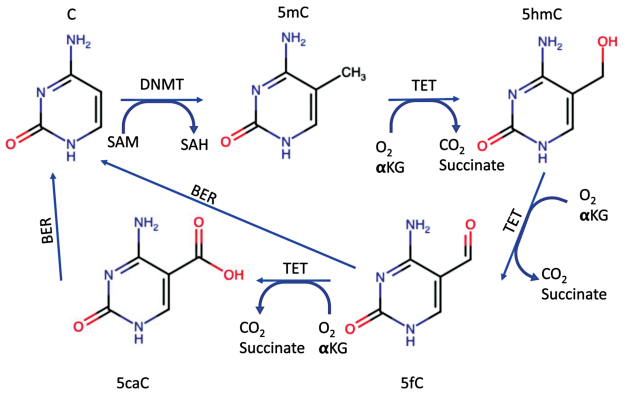

Figure 2. Methylation and demethylation of cytosine. Methylation is performed by DNMTs adding a methyl group onto the 5′ position of cytosine through a reaction using SAM as the methyl donor. Demethylation is performed in a series of steps by TET enzymes using oxygen and alpha-ketoglutarate to oxidize the methyl group until thymine DNA glycosylase removes the base and a new cytosine replaces the previously methylated cytosine through the base excision repair (BER) pathway.

(Sinclair et al., 2007). Hypomethylated and unmethylated 5′-Cytosine-phosphate-Guanine-3′ (CpG) islands identified in their offspring were associated with the methyl-deficient diet (Sinclair et al., 2007). The altered CpG islands differed based on sex, with 53% of loci specific to male offspring and 12% of loci specific to female offspring. The male offspring also presented with an increase in body fat at 22 mo of age, associated with the methyl-deficient diet. Alternatively, diets high in methyl donors predictably associate with global DNA hypermethylation. Newly hatched chicks that were fed a betaine-supplemented diet were found to have increased global DNA methylation and increased levels of the DNMT1 protein (Hu et al., 2015). Although DNA hypermethylation was observed globally, some genes were found to be hypomethylated, including the promoter region of adenosine triphosphate (ATP) binding cassette sub-family A member 1 (*ABCA1*) (Hu et al., 2015). Dietary nutrients are key components of the cellular pathways that generate methylation substrates. Therefore, adding or removing those components from the diet influences DNA methylation levels and potentiates altered gene expression.

Environmental factors can be both physical and situational. Specifically, stress has been shown to significantly impact methylation. In dairy cattle, cortisol levels in the milk were associated with CpG DNA methylation changes (Del Corvo et al., 2020). Dairy cattle with extreme measures of milk-cortisol concentrations, a stress marker in dairy cattle, underwent reduced representation bisulfite sequencing (**RRBS**) and 248 differentially methylated genes (**DMG**s) were identified. Key DMGs were found to be associated with cellular defense and stress response. In pigs, stress has previously been associated with poor meat quality and has now been associated with changes in DNA methylation (Hao et al., 2016). An 8 °C temperature increase has been associated with a change in DNA methylation patterns in pigs, suggesting that heat stress may potentiate epigenetic changes (Hao et al., 2016). Differential methylation was found between heat-stressed and control pigs in both CpG and non-CpG sites. These DMGs were predominantly associated with lipid metabolism, cellular defense and stress responses, and calcium signaling pathways in the *longissimus dorsi* muscle (Hao et al., 2016). These changes are subsequently associated with changes in pig overall meat quality and muscle development following heat stress (Hao et al., 2016).

Transportation stress can also influence DNA methylation, as demonstrated in cattle. The effect of prenatal transportation stress in Brahman cattle was evaluated using RRBS on the offspring of pregnant cows which were transported for 2 h trips at multiple times during pregnancy, compared with offspring from cows which were not transported during pregnancy (Littlejohn et al., 2018). DNA derived from the white blood cells of both sets of offspring was sequenced using RRBS, and differential methylation between prenatally stressed and control groups was identified in 16,128 CpG sites. Differentially methylated sites in promoter regions were linked to 113 pathways, including stress response, metabolism, immune function, and cell signaling (Littlejohn et al., 2018). These findings suggest influence from the environment in utero on epigenetic programming. In both cattle and pigs, stress has been associated with differential methylation in CpG and non-CpG context. This work further emphasizes the effect of environment on DNA methylation and economically important traits in livestock.

Epigenetics and Phenotype Connection

There are a wide range of phenotypes that producers must consider to maximize the health and production of their livestock, and DNA methylation has been shown to influence many of those phenotypes. DNA methylation has been associated with mastitis in dairy cattle (Ju et al., 2020; Wang et al., 2020), the rate of egg laying in chickens (Omer et al., 2020), beef tenderness in cattle (Zhao et al., 2020), wool fiber production in goats (Xiao et al., 2020), fat deposition in swine (Zhang et al., 2016), and milk production in dairy cattle (Liu et al., 2017) (Figure 3). The diversity of traits associated with DNA methylation patterns in a variety of species highlights the significance of methylation in determining phenotype.

Methylation-dependent restriction site-associated DNA sequencing (**Methyl-RAD Seq**) is an enzyme-based sequencing method that reduces the genome size by utilizing methylation-dependent restriction enzymes to narrow analysis to methylation-rich regions of the genome. A common target site for Methyl-RAD seq enzymes is $C^{m}CGG$, which consists of a methylated cytosine followed by an unmethylated cytosine and two guanine bases. This technique has been used to study the association between mastitis and DNA methylation. A study of mastitis in dairy cattle identified differential methylation between cattle with extreme measures of *Staphylococcus aureus*, a common cause of chronic mastitis (Wang et al., 2020). Genome-wide DNA methylation sequencing was performed using the Methyl-RAD Seq method, and 363 DMGs were identified in the $C^{m}CGG$ context. The identified DMGs that were also differentially expressed between groups were enriched with genes associated with immune response, including interleukin

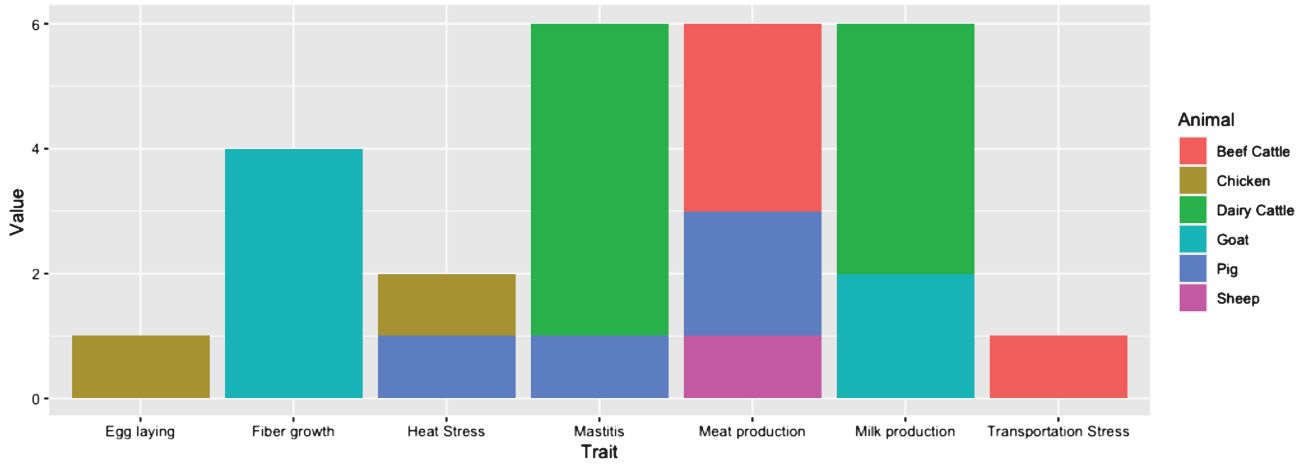

Figure 3. Summary of manuscripts identifying differential DNA methylation in traits of interest in livestock. A PubMed search was performed to identify papers directly studying the association between DNA methylation and traits in livestock. Mastitis, meat production, and milk production had the highest number of manuscripts identifying an association with DNA methylation. Manuscripts represented in this figure can be found at: https://www.ncbi.nlm.nih.gov/sites/myncbi/emily.stassen.1/bibliography/public/.

6 receptor (*IL6R*), tumor necrosis factor (*TNF*), Bruton tyrosine kinase (*BTK*), interleukin 1 receptor type 2 (*IL1R2*), and TNF superfamily member 8 (*TNFSF8*) (Wang et al., 2020). Differential methylation was also identified between cattle with extreme measures of *Escherichia coli* mastitis in blood neutrophils (Ju et al., 2020). Neutrophils are crucial first responders to *E. coli* infection and are triggered by mastitis. RRBS of neutrophil DNA identified 494 differentially methylated regions (**DMR**s) between groups, the majority of which were hypomethylated in infected animals. Transcriptome sequencing revealed a corresponding pattern of gene expression, with the majority of differentially expressed genes upregulated in infected animals. Validation of transcription and methylation with bisulfite sequencing polymerase chain reaction (PCR) and quantitative real-time PCR confirmed methylation of the promoter region in Cbp/p300-interacting transactivator 2 (*CITED2*) and Solute Carrier Family 40 Member 1 (*SLC40A1*) genes in infected animals decreased and expression subsequently increased. Additionally, higher methylation in exon 5 of Leucine-rich repeat-containing G-protein coupled receptor 4 (*LGR4*) gene influenced alternative splicing in healthy cows (Ju et al., 2020). This study indicates that these three genes, *CITED2*, *SLC40A1*, and *LGR4*, are possible candidate genes for increasing resistance to *E. coli* (Ju et al., 2020).

Betaine supplementation has been associated with an increased rate of egg laying in hens, and DNA methylation has been implicated as the cause (Omer et al., 2020). Fatty acid synthesis is a crucial component of yolk creation, and therefore genes and proteins involved in lipid synthesis are of particular interest in the study of egg production. Dietary betaine supplementation upregulated genes involved in lipid synthesis at the mRNA level and also enhanced laying production (Omer et al., 2020). Western blots revealed increased protein levels, confirming upregulation of genes such as sterol regulatory element-binding protein 1 (*SREBP-1*) and stearoyl-CoA desaturase 1 (*SCD1*). Omer et al. (2020) then utilized methylated DNA immunoprecipitation (**MeDIP**) to compare methylomes of livers from the hens fed with diets containing variable amounts of betaine. Hypomethylation was found in the promoter regions of the above genes associated with lipid production, which implies a relationship between dietary betaine and DNA methylation leading to an increase in synthesis of yolk precursor elements.

Beef tenderness has been of interest to producers and consumers, but previous research has predominantly focused on physiological mechanisms of tenderness. A new study delved into the DNA methylation differences of Angus beef with divergent tenderness, based on measurements of Warner–Bratzler shear force, crude fat, fatty acid contents, and cooking loss (Zhao et al., 2020). Methylated DNA-binding domain sequencing was used to measure DNA methylation in tender beef and tough beef, and 7, 215 DMRs were identified between groups of divergent tenderness. Identified DMRs were found in pathways related to beef tenderness, including regulation of guanosine triphosphatases activity, ion transport, and anion transport. Gene expression was then measured using 4 × 44K Bovine Gene Expression Microarrays and compared with DNA methylation to identify candidate genes for beef tenderness biomarkers: myosin heavy chain 8 (*MYH8*), *N*-acetylated alpha-linked acidic dipeptidase 2 (*NAALAD2*), phospholipase A2 group IVA (*PLA2G4A*), and ubiquitin-like with PHD and ring finger domains 1 (*UHRF1*) (Zhao et al., 2020). This study demonstrated, for the first time, the impact of DNA methylation on beef tenderness.

Chinese Zhongwei goats are known for their unique curly-white pelts that mysteriously disappear around 2 mo of age, prompting research into epigenetic mechanisms associated with the curly fleece (Xiao et al., 2020). Xiao et al. (2020) utilized WGBS of skin tissues from when the goats are producing curly pelts (45 d old) and skin tissues after the goats no longer produce curly pelts (108 d old) to compare DNA methylation in the skin between time points. Among the 3,379 DMRs that

were identified, 1,250 of those were found in annotated genes that were mainly involved in intercellular communication and the cytoskeleton, including factors for wool fiber development. The platelet-derived growth factor C (*PDGFC*) gene was specifically identified due to its role in hair follicle cell growth and validated in vitro human hair inner root sheath cells (**HHIRSC**s). Decreased methylation of *PDGFC* was associated with increased expression at the mRNA and protein levels and led to increased cell migration and proliferation in HHIRSCs (Xiao et al., 2020). Increased expression of the PDGFC protein was also associated with increased expression of a series of other key proteins involved with hair follicle development, suggesting a crucial and broad role for methylation of this gene in wool fiber production (Xiao et al., 2020).

Landrace and Rongchang pigs have been selectively bred for reduced fat content and extreme adipose, respectively (Zhang et al., 2016). The differences in the fat deposits and fatty acid composition between these two breeds of pigs were utilized in an effort to uncover the epigenetic mechanisms contributing toward fat deposition and improve pork quality. DNA extracted from backfat harvested from three randomly chosen female pigs from each breed was subjected to MeDIP-sequencing. Landrace pigs were found to have a higher global DNA methylation levels compared with Rongchang pigs, and expression levels of selected DNMTs were found to be significantly higher in Landrace pigs, thus supporting the theory that differences in global DNA Methylation levels are associated changes in DNMT expression. A total of 15,762 DMRs were found, the substantial majority of which (59%) were found in intergenic regions and 85% of the DMRs were hypermethylated in Landrace compared with Rongchang pigs. The 483 DMRs that were located in promoter regions underwent functional enrichment analysis, and a majority of these DMRs were associated with gene ontologies that include olfactory and sensory activity as well as lipid metabolism and ATPase activity. These findings are reflective of the variable dietary habits, fatty acid composition, and energy metabolism levels of these two pig breeds.

Dairy cattle have developed increased quality and quantity of milk production over generations, associated with genetic selection and changing management of cattle, and recent studies have begun to link DNA methylation to improved production. A previous genetic study utilized genome-wide association studies (**GWAS**) to identify genes associated with milk production traits in dairy cattle, and the methylation of those genes has been more recently explored (Liu et al., 2017). The eukaryotic translation elongation factor 1 delta (*EEF1D*) gene was identified by GWAS as a candidate gene and further characterized by Western blot and bisulfite sequencing (Figure 4). Gene expression of *EEF1D* at the protein level was compared across the heart, liver, mammary gland, ovary, and muscle, with the highest expression in the mammary gland. Methylation levels were also compared across those tissues, confirming tissue-specific methylation of the gene with low methylation in the mammary gland. The methylation of the first CpG island of *EEF1D* (Figure 4) negatively correlated with the change in expression of this gene in the mammary gland compared with other tissues in both dry and lactating periods (Liu et al., 2017). Liu et al. (2017) continued to investigate DNA methylation in the dry period and the early lactation stage in an effort to study how DNA methylation changes in association with lactation. In blood, the *EEF1D* methylation level was decreased during the dry period and the methylation level increased during early lactation, which correlated with increased mRNA in the dry period compared with during early lactation (Liu et al., 2017). These associations elucidate the impact of DNA methylation on milk production.

Use of Epigenetics to Benefit Animal Health and Production

Incorporation of genomics into breeding technology has effectively improved selective breeding in dairy, swine, and poultry, and utilizing epigenomics has the potential to further advance breeding (Rolf et al., 2014). Prior to determining the quantitative impact of DNA methylation on genetic variation, the distribution of DNA methylation must be established in tissues relevant to economically important traits. Toward that end, an innovative porcine study developed methylome atlases of adipose and muscle tissues and found DMRs associated with obesity in approximately 80% of obesity candidate genes that were previously identified in humans. Those DMRs were also associated with approximately 72% of porcine quantitative trait loci regions affecting pork quality and animal fatness (Rolf et al., 2014). An additional study in chickens found 145 genes that were heritably differentially methylated between While Leghorn chickens and their wild counterpart, Red Junglefowl. These DMRs were present mainly in selective

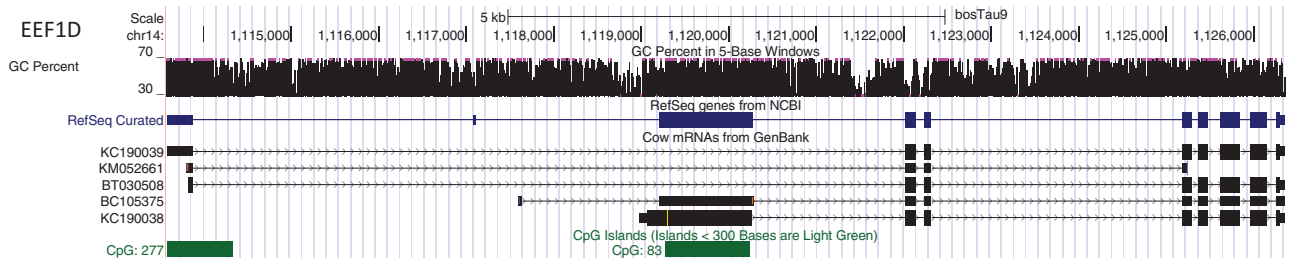

Figure 4. Snapshot of *EEF1D* gene with DNA methylation associated with milk production in dairy cattle. As shown in this figure, this gene contains a high GC content with two CpG islands and produces multiple mRNAs in cattle. Differential methylation at the first CpG island, shown in green, has been linked to differential expression of mRNAs and associated with milk production (Liu et al., 2017). Snapshot was taken from the UCSC genome browser.

sweep regions associated with domestication, implying a role for selection of epialleles in chicken domestication (Rolf et al., 2014). Epigenetic modifications may provide more rapid mechanisms for response to changing environments than genetic selection alone, informing the importance of considering epialleles during breeding. These possibilities call for further research into the stability of epiallele inheritance and enhanced understanding of the mechanisms that produce stable and heritable epigenetic modifications that significantly impact phenotype so that those mechanisms may be harnessed for improved food production (Rolf et al., 2014).

Awareness of epigenetics in breeding was recently demonstrated in a study on the domestication of wild red junglefowl (Bélteky et al., 2018). Two selection lines of red junglefowl were bred for extreme measures of fear of humans over five generations. Briefly, fear levels were defined according to a standardized fear of humans test consisting of an observer rating the fear level of an individual chicken to a human moving around the animal (Bélteky et al., 2018). MeDIP-Seq performed on the hypothalamus tissues from 12 chickens yielded 22 genomic regions that were differentially methylated between the two selection lines in the fifth generation of offspring. Differential methylation was identified in different pathways based on sex: pathways in males included DNA replication, the GABA receptor complex, and chloride channel activity, and in females included transmembrane transporter activity, the synapse part, and neurotransmitter complex (Bélteky et al., 2018). Bélteky et al. (2018) claim that epigenetic effects over five generations contributed to rapid changes in phenotype that could not be caused by classical genetics alone. This study highlights the fascinating potential of using epigenetics for selection in breeding.

Conclusions

Theoretically, awareness of how an animal's environment can impact epigenetic marks of interest has the potential to allow producers to create environments optimal for their desired phenotypes. Utilizing environmental effects to impact DNA methylation and subsequently economically important phenotypes first requires a greater understanding of the distribution of DNA methylation within specific tissues associated with economically important traits (e.g., muscle for growth traits), and a vast improvement of our understanding of the molecular effect of environment on phenotype is critical. In this manuscript, we have described how environmental factors, such as dietary nutrients and stress from heat and transportation, have been shown to impact DNA methylation. Likewise, altered DNA methylation has been shown to impact livestock phenotypes including egg laying, fiber growth, heat stress, mastitis, and meat and milk production (Figure 3). There is now an opportunity for research into the direct links between environment, epigenetics, and phenotype. Ultimately, a thorough understanding of the power of epigenetics to connect the environment with phenotype can provide producers with increased ability to tailor environments to produce their desired phenotypes.

About the Authors

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Brenda Murdoch is an associate professor at the University of Idaho in the Department of Animal, Veterinary and Food Sciences, where she has worked since 2014. She has her PhD and BS in Animal Science from University of Alberta in Edmonton. She also works at Washington State University as an adjunct faculty member in the Center for Reproductive Biology and previously as an assistant research professor in the School of Molecular Biosciences. Her current research focus areas include livestock genomics, genetic improvement, genetic markers, genotyping, gene and quantitative trait mapping, genome-wide association studies, molecular biology, and biotechnology.

Emory Pacht is a current PhD student in the Cellular, Molecular, and Biomedical Sciences program at the University of Vermont working in the Animal and Veterinary Sciences department. She received a BS in Molecular and Cell Biology from the University of Connecticut. Her research has varied from studying adenoviruses, breast cancer, and DNA transcription, and she currently focuses on epigenetics in cattle and sheep. She is a teaching assistant for BioCore classes and primarily teaches cell biology laboratories.

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