Epigenetics: Concepts and applications

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Abstract

Most traits of economic importance in animal production undergo continuous phenotypic variations due to multi gene changes and environmental factors. Numerous quantitative trait loci that impact agronomic traits have been identified, but generally the specific set of genes responsible, are not defined. Studies using genome-wide associations have shown that genetic variation only partially explains the variability of complex traits and that, it is not only the DNA sequence that affects phenotype, but that epigenetic changes that can be transmitted from one generation to the next are also involved.

Non DNA mediated changes have been identified in mammals and birds. Nutritional impacts that appear to elicit "epigenetic" changes have been documented. Published results will be discussed on epigenetic effects associated with protein concentration changes in the maternal line changing protein usage in offspring's. Effects seen associated with neonatal nutritional changes impacting the ability of broilers to growl when fed deficient calcium and phosphorus containing diets that normally would decrease growth will also be discussed.

Keywords: epigenetics, neonatal nutrition, DNA, broilers

Introduction

As we look ahead at technologies with the potential to deliver exponential improvements in our ability to produce animal with lower inputs and production costs as well as with less impact on the environment, application of epigenetic principles is potentially one of the ones with most potential. What the correct term for this effect is and how to define it are still in debate. Early on conditioning was used but currently metabolic imprinting, programming and epigenetics are the terms most used.

But what is epigenetics? Numerous and somewhat divergent definitions for epigenetics can be found in the literature and numerous terms are applied to the same "apparent" mechanism. There is no complete agreement on what the term "epigenetics" refers to and this leads to numerous definitions (HOLLIDAY, 2006; BIRD, 2007; FRESARD *et al.*, 2013). Epigenetics comes from the words "epi" greek for over or above and genetics. It involves modifications of the phenotype without changes in DNA sequence. These modifications can be transmitted to the next generations. BIRD (2007) defined epigenetics as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states". FRESARD *et al.*, (2013) used the definition of FEIL and FRAGA (2012) where epigenetics was defined as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. Recently, DAVID et al (2017) defined epigenetics as "the molecular mechanisms involved in the regulation of gene expression that are reversible and heritable (by mitosis and potentially meiosis) without alteration of the DNA sequence".

Epigenetic changes can be mediated by DNA methylation, chromatin folding and its attachment to the nuclear matrix, packaging of DNA around nucleosomes, non coding RNA and covalent modifications of histone tails to name a few in the growing list of mediating factors. For the purpose of this review the definition of epigenetic changes will involve both phenotypic as well as changes in gene expression that persist through the life of the animal and/or subsequent generations and that are changes that are greater than the normal adaptations that, for example, occur during nutrient deficiency states.

There is great potential for these types of changes to be used as a tool to improve productivity in animals while decreasing the need for costly inputs as well as decreasing environmental impacts. Extensive information on epigenetic changes in wild mammals, humans and wild birds is available. In poultry production, work has been done trying to harness this potential both for poultry management and nutrition. The potential has been demonstrated under controlled conditions but application under commercial conduits lags behind. Full understanding of when these "environmental influences" should occur to maximize positive effects later in life or in future generations has not been fully elucidated. In the area of nutrition, work is just beginning to demonstrate the effects but full understanding of mechanisms and of how to maximize positive effects is lacking.

Background on Epigenetic Regulation

One of the fundamental assumptions of Mendelian genetics is that a specific allele behaves in the same way no matter from where (which parent) it originates from. But as with many assumptions in science this rule does not always hold true. Initial observations in mice looking for the effects of paternal or maternal impacts using genomic translocations indicated that identical chromosomal regions were not equivalent and acted differently in embryo development between maternal and paternal sources (CATTANACH and KIRK, 1985).

The growing incidence of metabolic diseases in humans, such as obesity, diabetes, and cardio-vascular disease has sparked interest and research efforts into both their genetic and environmental (nutritional among others) basis. Fetal programming as it was initially referred to in humans (HALES and BARKER, 1992; BARKER, 2004), encompasses the role of developmental plasticity in response to environmental

signals, including nutrition, during early life and its potential adverse consequences (risk of cardiovascular, metabolic and behavioral diseases) in later life. The first studies in this field highlighted an association between poor fetal growth and chronic adult diseases. Adverse long-term effects reflect a mismatch between early (fetal and neonatal) environmental conditions and the conditions that the individual will confront later in life.

Adverse nutritional conditions during fetal development lead to adaptive changes in metabolism that lead to a 'thrifty phenotype' in the offspring (HALES and BARKER, 1992). For example, poor nutrition in early life produces permanent changes in glucose-insulin metabolism, including a reduced capacity for insulin secretion and insulin resistance (HALES and BARKER, 2001). The initiating factor(s) in the case of nutrients for fetal programming may be nutrient(s) interacting directly with genes and their regulatory elements at the cellular level, altering patterns of growth and gene expression. Embryonic and fetal cells have a complex system to integrate nutritional signals from their environment and adapt their development accordingly to ensure survival (FRESARD *et al.*, 2013).

In chickens there are reports where conditioning in early-life imparts long-term effects. The first report of this type of response was to thermal stress. High temperatures during the first week of life were reported to modulate the response to thermal stress later in life (YAHAV and MCMURTRY, 2001). By simply increasing the brooding temperature from 30ºC to 37.5ºC for 24 hr within the first 5 days post-hatch, broiler chickens were able to tolerate an acute exposure to a temperature of 35ºC for 6 hr at 42 days of age, while "unconditioned" birds were unable to acclimate. The mechanism for this conditioned response was not elucidated then. More recently studies have shown the gene expression changes occur as a result of a heat stimulus in the neonatal period (YOSSIFOFF *et al.*, 2008; KISLIOUK and MERI, 2009; KISLIOUK *et al.*, 2010, 2011). These researchers reported that the expression of brain-derived neurotrophic factor, a key regulator of thermo tolerance acquisition in the hypothalamus of the chick, was different between birds grown under normal temperature and those subjected to high temperatures within the first 3 days of life. These changes were associated with increased methylation of CpG sites in the promoter of the brain-derived neurotrophic factor gene.

Although little is known about the underlying molecular mechanisms, there is evidence that feed stress may alter gene expression, in part through epigenetic changes (LI *et al.*, 2017). For example, XU *et al.* (2012) found that when 3-day old chicks were subjected to a 24-hour fasting, there were methylation changes in histone H3 in the anterior hypothalamus, the area in the brain that controls of body temperature and food intake. Feed stressors such as feeding calcium (Ca) and phosphorus (P) deficient diets in the starter (YAN *et al.*, 2005) or during the first 90 hr post placement (ASHWELL and ANGEL, 2010) change the ability of broilers to utilize diets deficient in Ca and P later in life. These changes were shown to be greater than those associated with normal adaptations that occur when deficient diets are fed. These changes were partially mediated through increased methylation of the NaP cotransporter (ASHWELL and ANGEL, 2010).

There are other examples of potential epigenetic effects mediated by changes in the embryonic or neonatal period of poultry development. For example, exposure of the embryo to green monochromatic LED light appears to improve growth in broilers and turkeys (HALEVY *et al.*, 2006; ZHANG *et al.*, 2012). This can be partially explained by the enhancement that was seen in the differentiation and proliferation of myoblasts (HALEVY *et al.*, 2006).

Effect of Early Nutrition

Most of our knowledge on the effects of nutrients on gene expression has been acquired in animal models, many examples of which can be found in the overview of the mechanisms by which nutrients interact with their molecular targets to modify gene expression (MULLER and KERSTEN, 2003). In avian species nutrients deposited into the egg by the hen are the only source of nutrients available to the embryo, and this may be the last direct chemical means by which the hen may transfer an epigenetic message to its offspring.

A means of manipulating the nutrients deposited in the egg, beyond those that can be changed through maternal nutrition, is to inject nutrients into the egg at a time when the embryo is most sensitive to epigenetic programming. This timing has not been fully elucidated and may differ for different system. Injecting an isotonic in ovo feeding (IOF) solution into the embryonic amnion, the embryo can naturally consume supplemental nutrients orally before hatching and thus, nutritional manipulations can be implemented inside the egg. In ovo feeding "jump-starts" the ability of the animal to adjust to external feeding. In addition to the increased body weights typically observed at hatch, the positive effects of in ovo feeding may include increased hatchability (UNI *et al.*, 2005); advanced morphometric development of the intestinal tract (TAKO *et al.*, 2004) and mucin barrier; enhanced expression of genes for brush boarder enzymes (sucrase-isomaltase, leucine aminopeptidase) and their biological activities, along with enhanced expression of nutrient transporters, SGLT-1, PEPT-1, and NaK ATPase (TAKO *et al.*, 2005; FOYE *et al.*, 2007); increased liver glycogen status (UNI *et al.*, 2005; TAKO *et al.*, 2004) and increased breast muscle size at hatch (UNI *et al.*, 2005; Foye *et al.*, 2006). In ovo feeding clearly advances the digestive capacity, energy status, and development of critical tissues of the neonate by about 2 days at the time of hatch.

Early Dietary Adaptation

Adaptation to low nutrient diets has been long recognized. Animals respond to nutrient restriction in general by increasing absorption rates and utilization efficiency, which decreases excretion of the restricted nutrients. Although several reports demonstrate the ability to program chicks through early dietary manipulation to improve Ca and P utilization later on in life, there has been little work reported in the literature applying this concept with dietary protein utilization.

A study by RAO *et al.* (2009) where the effect of feeding a low protein diet (100 g/kg) versus a normal breeder diet (150 g/kg) to females of an inbred indigenous Chinese line (Langshan). Chicks from eggs laid by hens fed the low protein diet were lighter (5%) and had

less pectoralis muscle weight (16 %) at hatch as compared to chicks from eggs laid by hens fed the normal breeder diet. By 28 days of age these chicks from eggs laid by low protein fed breeders had greater body weight (6%) and pectoralis mayor muscle weight (51%) then those of for birds hatched from eggs laid by hens fed the normal protein diet. These changes in growth rate were associated with changes in patterns of gene expression in the yolk sac membrane, hypothalamus and muscle in the embryo. While these authors did not look at the persistence of these gene expression changes or the mechanisms that caused them, they clearly demonstrated a long term effect of maternal nutrition challenges.

Adaptation to P and Ca restricted diets has been reported in chickens. BLAHOS *et al.* (1987) reported an increase in duodenal and ileal P absorption in broiler chickens fed a low Ca diet for two weeks and a smaller, but still significant increase in duodenal but not ileal P absorption in chicks fed a low P diet. The adaptation to P or Ca restriction has been associated with an increased level of circulating 1,25-(OH)2-D3 (BLAHOS *et al.*, 1987) and duodenal calbindin content (MONTECUCCOLI *et al.*, 1977). When duodenal calbindin concentrations and changes with age for 1991 and 2001 strains of broilers, BAR *et al.* (2003) concluded that modern broilers exhibit higher adaptation capacity to P or Ca deficiency and this capacity remains high for the whole growth period.

However, the authors could find no published work that evaluate the long-term effects of neonatal P or Ca restriction on growth performance, bone mineralization, and Ca and P absorption in poultry and whether "epigenetic" type of impacts had been documented. Thus, a study was done to determine whether we could elicit a positive epigenetic response to short term neonatal deficiencies in Ca and P. We defined epigenetic responses (phenotypic and in the expression of specific genes) as effects that were significantly above those that occur at the time the animal is exposed to a nutrient deficiency and that persisted through the productive life of the broiler chicken. In the first study, we evaluated the ability of the broiler chicken to adapt to a moderate early life deficiency in P and Ca and characterized this adaptation changes by examining the impact of the previous P and Ca status (starter phase, hatch to 18 days of age) on performance, bone characteristics, and nutrients absorption of broilers in the grower phase (19 to 32 days of age) (YAN et al, 2005).

Broilers fed a diet moderately deficient in P and Ca from hatch to 18 days of age demonstrated the ability to adapt to the deficiency, shown in the increased ileal absorption of P and Ca, the increased PP disappearance, greater growth and bone mineralization (tibia ash, tibia and shank bone mineral density and bone mineral content) in a later growth phase (18 to 32 days of age) in broilers fed the deficient diets early on as compared to those fed diets that met requirements (YAN et al, 2005).

The second experiment (ANGEL and ASHWELL, 2008) was done to determine if feeding deficient diets for a shorter time after hatch could elicit positive effects on performance and change expression of the chicken intestinal sodium phosphate co-transporter (NaPcoT). Ross 308 male chicks were fed either a prestarter control diet (C) consisting of 11.1 g/kg Ca and 5.0 g/kg non phytate P (nPP) which met or exceeded NRC (1994) recommendations or a deficient or low diet (L) containing 5.9 g/kg Ca and 2.5 g/kg nPP from hatch to 90 hr. Between 90 h and 8 days of age, all birds were fed the prestarter C diet and from 9 to 22 days of age a starter C diet (8 g/kg Ca and 4.5 g/kg nPP). From day 23 to 38 of age, broilers were either maintained on a grower C diet containing 7.0 g/kg Ca and 3.0 g/kg nPP or a grower L diet containing 4.0 g/kg Ca and 1.2 g/kg P. The three treatments, C- C-C, C-C-L, and L-C-L met or exceeded all other nutrient recommendations (NRC,1994) and were formulated to meet average USA industry use for all nutrients except Ca and nPP. Data collected for each phase included body weight and feed intake, and at the end of each phase all birds in preselected pens were sampled and toe and tibias removed to determine bone ash and ileal content for apparent Ca and P digestibility determinations as well as duodenal, jejunal and ileal mucosa for gene expression determinations. Data for performance, bone ash and digestibility are presented in Table 1 and for gene expression in Table 2.

Broilers fed the prestarter L diet from placement to 90 hr were better able to handle a P deficiency in the grower phase (22 to 38 days of age) than those fed the C diet in the first 90 hr (non conditioned birds) and then fed deficient diets (L) in the grower phase (Table 1). Broilers fed the L grower diet from hatch to 90 hr were heavier, had better feed conversion, greater toe ash and P digestibility at 38 days of age, as compared to the none conditioned birds when these two groups of birds were fed the same deficient diet in the grower phase. The improved digestibility was greater than that seen simply by feeding a defiant diet in the grower phase. At 38 days of age, P digestibility was lowest in broilers fed the C diets from hatch to 38 days, a significantly greater P digestibility was seen in broilers fed the L diet from 23 to 38 days and that had been fed the C diets prior to 23 days of age, and greatest for the conditions birds (fed the L diet for the first 90 hr after placement) and the L diet from 23 t0 38 d of age. This establishes that there was a "conditioning or imprinting" in these birds meaning that modifications are occurring in these birds that are long term and that allow for improved P utilization when P deficient diets are fed in the grower/finisher phases. Despite this improved ability to handle deficiencies in Ca and P in latter growth phases, these "conditioned" birds were not able to catch up performance wise and in bone ash with those fed the C diets throughout all growth phases.

These data suggest that, in broilers, during the period immediately post hatch there is a phenomenon occurring related to Ca and P digestibilities that allows for changes to be made that can alter the bird's long term ability to handle Ca and P deficiencies. But are these phenotypic changes associated to long term changes in gene expression. The chicken sodium phosphate co transporter (NaPcoT) gene expression was determined using real-time quantitative PCR. The differences in CT (expression) values for gene expression were determined to be different using a student's t-test and relative differences in expression caused by the dietary treatment were determined by correcting for the efficiency of the PCR as calculated by the standard curves of dilutions of pooled cDNA across the experiment (ASHWELL and ANGEL, 2008). Expression levels and N-fold expression changes (changes relative to the control) are reported in Table 2. Feeding the prestarter L diet from hatch to 90 hr had a significant effect on the expression of the NaPcoT as seen by the average n-fold increase of 2.8 in the mRNA levels in the small intestine. Nearly identical stimulatory effects were seen across all segments of the intestine with relative expression decreasing from duodenum to ileum.

These apparent epigenetic changes appear to be mediated through changes in methylation. When methylation determinations were done on the small intestine samples obtained at 90 hr, apparent methylation was observed at 29 positions within the 52 predicted CpGs in the

PCR products in samples fed the C diets while 19 CpGs were methylated in the DNAs extracted from the programmed or condition birds fed the L diet from hatch to 90 h (ASHWELL and ANGEL, 2008). This 43% reduction in methylation of cytosines in this region may be involved in the increased gene expression observed in the programmed birds relative to controls. Further characterization of the differential methylation patterns is needed both post programming as well as later in life to verify these observations.

This work clearly establishes that "imprinting" or modifications are occurring in the animal that are long term and that allow for improved P utilization when P deficient diets are fed in the grower/finisher phases (ANGEL AND ASHWELL, 2007; ASHWELL AND ANGEL, 2007). These long term effects on performance and gene expression as a result of nutritional neonatal programming in the chick are strong evidence for the role of epigenetics in the regulation of these phenomena.

The challenge now is how to combine diets that promote neonatal programming while minimizing any unwanted negative effects on early development. For example, skeletal growth rate is highest in broilers during the first 8 to 10 days post hatch but neonatal programming Ca and P deficient diet concentrations appears to be required for at least 90 hr post hatch. This feeding of low nutrient concentration diets during the neonatal period goes against the long held "dogma" for prestarter diets where nutrient density tends to be high for nutrients that are in high demand during early post-hatch development. Herein lies the challenge and also the rational for neonatal programming. Research must identify "the window of opportunity" and the extent of the deficiency needed to exert these epigenetic changes where short term early life changes in nutrients fed can elicit long term changes in gene expression while minimizing the deleterious effects of feeding a deficient diet on productivity.

Table 1. Impact of early dietary deficiencies of phosphorus (P) and calcium (Ca) on performance and apparent digestibility (AD) of P (ANGEL and ASHWELL, 2008)

Treatment [1]	C-C-C	C-C-L	L-C-L	SEM	P values
Hatch to 90 hour					
Weight gain, g	41.2[2]		36.2[2]	0.775	<0.001
Feed to gain ratio	0.83		0.88	0.016	0.028
Toe ash, g/kg	133.7		111.7	0.57	<0.001
90 hour AD P, g/kg P	576.2		663.9	6.67	<0.001
91 hr to 8 days of age					
Body weight, g (8 days)	150.2		141.8	0.248	<0.001
Weight gain, g	63.5		64.5	1.222	0.557
Feed to gain ratio	1.15		1.12	0.027	0.367
9 to 22 days of age					
22 days Body weight, g	904.9		884.7	6.530	0.025
Weight gain, g	754.2		742.2	6.605	0.179
Feed to gain ratio	1.40		1.40	0.010	0.967
22 day AD P, g/kg P	514.6		529.4	16.12	0.513
23 to 38 days of age[4]					
38 days Body weight	2345.9[a]	2235.4[c]	2285.6[b]	20.158	0.001
Gain, g	1450.9[a]	1333.0[c]	1390.6[b]	15.766	0.03
Feed to gain ratio	1.82[ab]	1.89[a]	1.76[b]	0.033	0.013
38 day Toe ash, g/kg	126.2[a]	105.3[c]	118.4[b]	1.71	<0.001
38 day AD P, g/kg P	453.9[c]	515.4[b]	601.1[a]	1.562	<0.001

[1]Treatments are: Control (C) – C –C (fed in all phases) diets that met or exceeded NRC (1994) nutrient recommendations (including those for Ca and P); C-C- Low (C-C-L) fed the C diets from hatch to 22 d of age (prestarter from hatch to 8 days and starter from 9 to 22 days) and then the grower L diet from 22 to 38 d of age; L-C-L fed a prestarter L diet from hatch to 90 hr, a C diet from 90 hr to 22 d (prestarter C from 90 hr to 8 days and starter from 9 to 22 days) and the grower L diet from 22 to 38 d. The L diet met or exceeded all NRC (1994) nutrient recommendations except for those of Ca and P. From hatch to 90 hr the L diet contained 5.9 g/kg Ca and 2.5 g/kg non phytate P (nPP) and the C diet 11.1 g/kg Ca and 5.0 g/kg nPP. From 22 to 38 d of age the L diet contained 4.0 g/kg Ca and 1.1 g/kg nPP and the C diet 7.0 g/kg and 3.0 g/kg nPP.

[2] Up to 22 days of age, treatment C was replicated 16 times and treatment L eight times. Between 22 and 38 days of age all treatments were replicated 8 times.

[3] Means within row with different superscript letter

Table 2 - Neonatal phosphorus (P) and calcium (Ca) deficiencies on sodium phosphate co-transporter (NaPcoT) gene expression (ASHWELL and ANGEL, 2008)

Dietary treatment	Tissue	Gene expression[1]		n-fold[2] change
		18s rRNA	NaPcoT	
90 hr				
Control	Duodenum	15.8±0.2a	23.2±0.2a	1.0
	Jejunum	16.1±0.3[a]	24.4±0.3[a]	1.0
	Ileum	16.2±0.3[a]	25.3±0.4[a]	1.0
Low P	Duodenum	16.0±0.3[a]	24.5±0.3[b]	3.1
	Jejunum	16.2±0.3[a]	26.1±0.4[b]	2.9
	Ileum	16.2±0.4[a]	26.9±0.2[b]	2.5
d 38				
Control	Duodenum	16.5±0.4a	24.1±0.5a	1.0
	Jejunum	16.2±0.3[a]	23.7±0.4[a]	1.0
	Ileum	15.8±0.4[a]	24.9±0.4[a]	1.0
Low P	Duodenum	16.6±0.4[a]	25.4±0.4[b]	2.5
	Jejunum	16.1±0.5[a]	25.3±0.6[b]	2.7
	Ileum	16.8±0.5[a]	26.7±0.4[b]	1.8

[1] Expression values are presented as the average ± the Std Dev (n=8).

[2] n-fold change in gene expression was calculated by the 11 11 Ct method of PFAFFL (2001) including the amplification efficiency for both genes. 18s amplified with 94% and Na/Pi IIb amplified with 91% efficiencies respectively as determined by the standard curves of diluted cDNA. Expression levels for the control diet were set as 1.0 for fold change effects within a tissue and within an age.

[ab] Means within a column and age with common superscript letter do not differ (P<0.05).

Conclusions

Nutrition is constantly varying and composed of a very large amount of known and unknown bioactive compounds. Furthermore, nutrition touches the core of metabolism by supplying the vast majority of nutrients (both macro- and micronutrients) for maintaining metabolic homeostasis. This homeostasis stretches from gene expression to lipid metabolism and from signaling molecules to enzyme cofactors. Thus, nutrition by its nature needs to be studied in an integrated way. So far, most of the tools for this integration have been lacking, thus maintaining an unbridgeable gap between classical nutrition (studying physiology with a focus on biochemical pathways) and biomedical sciences (determination of disease-related molecular mechanisms). In applying Systems Biology to nutritional sciences, these paradoxical extremes are bridged and the complexity of the relationship between nutrition and health can be met in an integrated approach. Many hurdles need to be overcome, before this research area reaches maturity.

The data included in this review demonstrating long term effects on performance and gene expression as a result of nutritional manipulation in the chick are evidence for the role of epigenetics in the regulation of these phenomena. Further work must be conducted to elucidate the specifics of neonatal programming in the chicken and the extent of its effects on DNA methylation or other mechanisms that

mediate epigenetic changes. Demonstrating these epigenetic effects in the chicken will contribute to the understanding of the impact of the environment during key development phases in the embryo and neonate on the regulation of the genome and start to elucidate the potential mechanisms that will allow for improving performance and economics of poultry production beyond the incremental improvements that have been seen up to now. Harnessing the potential benefits of these effects for productive purposes will be the challenge.

Nutrition research in the future will increasingly focus on the ways in which genes are affected by what we feed breeders, how these breeders are managed, how eggs are incubated and how we manage and feed the neonatal. If these effects can be better understood and implemented, then dietary "conditioning" offers opportunities for exponential improvements in animal productivity.

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