
# Maternal dietary environment modulates programming responses in avian offspring during embryonic and post-hatch development

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# Abstract

It is well recognised the maternal environment elicits strong programming effects during embryonic development and that these effects are regulated via epigenetic mechanisms. Furthermore, the degree to which these effects are expressed in offspring is further determined by the "predictive adaptive response" (PAR), whereby the combined effect of the maternal and offspring diet dictates if developmental programming phenotypes are expressed or ameliorated during adult life. Due to the various dietary insults imposed on broiler breeder hens, developmental programming and the PAR effect may have strong application in the broiler industry but currently is poorly acknowledged. This thesis presents an overarching hypothesis that the breeder dietary environment elicits epigenetic programming effects, signalled to the developing embryo via mediators present in the yolk. It is hypothesised developmental programming events *in ovo* will have significant long effects on offspring health and performance, dependant on both maternal and offspring environmental conditions.

Findings identify broiler breeder dietary environment causes strong programming effects in offspring, identifiable at various stages of development and in a sex-dependent manner. Differences in hen body weight significantly altered offspring organ weight as early as embryonic day 14 (ED14). Somewhat surprisingly, adult (D42) offspring organ weight (duodenum, jejunum, illium, pancreas and breast muscle) was significantly influenced by maternal body weight, irrespective of any early dietary insults imposed on the chick. It is proposed the observed changes in embryonic and adult organ weight are likely attributed to changes histological and morphology changes in response *in ovo* yolk profile exposure and warrants further investigation.

A PAR effect was also apparent across various dietary conditions. Changing the predominating grain source between generations, irrespective of the grain source, significantly altered progeny body weight and response to immune challenge from 7 days post-hatch to 42 days. Similarly, differences in feed allocation across generations also influenced progeny bodyweight and jejunum weight, specifically in male offspring. These results indicate a PAR effect, hence transgenerational dietary management is an important consideration in poultry production.

Manipulation of the maternal diet altered various biologically active compounds within yolk including hormones, nutrients and miRNA. Altering hen bodyweight and 'stress' response in broiler breeders significantly altered yolk corticosterone concentration, while naturally occurring differences in yolk glucose, insulin, testosterone and thyroxine concentration were found between broiler and layer breeds. Maternal grain source was associated with changes in expression levels of yolk microRNAs. Encouragingly, the miRNAs detected are associated with hen reproductive performance and embryonic development, indicating manipulation of microRNA via the maternal diet may attribute to programming effects *in ovo*. These results highlight the exciting opportunity of epigenetic modulators within the yolk in characterising programming events attributing to embryonic development in avians.

In summary, the current findings provide strong evidence the broiler breeder hen diet provides a mechanism to alter embryonic development and induce long-term effects and the concept of PAR has significant consequences for progeny health and performance. Future investigations should target the *in ovo* environment and potential epigenetic mechanisms for improved health and performance.

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# 1. Preface

## 1.1. Continuing productivity in the broiler industry

Genetic selection for increased growth rate and meat yield and close matching of nutrient requirements with nutrient supply, have dramatically advanced production efficiency in meat birds, and is expected to continue to do so into the future. Consequently, embryonic and neonatal development now accounts for approximately 50% of the productive life of the modern broiler. While genetic selection dictates what genes are inherited across generations, we are becoming increasingly aware of the impact the maternal environment can have on the physiology and health of offspring.

An avian model has previously been employed to identify programming effects, however, in a production context, the consequences of maternal dietary programming on offspring performance has received little attention. Broiler breeder management is heavily focussed on cost-effective dietary management and high reproductive performance. While diets are formulated to provide specific nutrients to improve breeder hen and offspring health and performance, grain sources and dietary management techniques currently employed may invoke underlying transgenerational programming not previously considered. These effects may have positive or negative effects on the offspring. Similarly, research now suggests the extent to which maternal dietary insults are expressed in offspring is influenced by the offspring diet, referred to as the "Predictive Adaptive Response" (PAR). This concept suggests the phenotype of the offspring is heavily influenced by the combined effect of both the maternal diet and offspring diet, whereby dietary environments across the two generations are either 'matched' or 'mismatched'. Therefore, the postnatal environment, particularly during periods of developmental plasticity, is just as important for the adult phenotype. While the effects of maternal diet on hatched and juvenile chicks has previously been identified, the effect of long-term PAR effects in poultry is lacking.

Compounds within the *in utero* environment have previously been associated with developmental programming in both human and mammalian species and is an area of current scientific interest. In avian species, changes in maternally-derived hormones, nutrients and epigenetic regulatory factors in yolk have all been associated with changes in offspring phenotype. As the egg is produced solely by the hen, the impact of the hen's environment during egg production is critical to egg composition. The hen's ability to control deposition of regulatory factors into the egg may significantly impact embryonic development. Consequently, in studying maternal effects on embryonic development it may be more useful to manipulate the hens environment, in comparison to direct *in ovo* injection into the albumen or yolk through the shell, a technique currently employed. Manipulation via the maternal environment reflects the level of transfer naturally occurring and accounts for the potential interactions with other associated yolk factors which may not yet be identified in the literature.

Due to the numerous environmental insults imposed on the chick at hatch, producing a more resilient chick is valuable, hence a greater emphasis is being placed on pre-hatch mechanisms and the application of *in ovo* supplementation to optimise embryonic growth and development. This thesis focusses on the impact of current broiler breeder hen dietary management on embryonic development, the potential role of PAR in poultry production and looks to identify the potential epigenetic mechanisms involved. Manipulation of the broiler breeder hen diet provides an opportunity to further improve productivity and has significant potential for improving the health and welfare of broiler breeder hens and their offspring.

## 1.2. Thesis format

This thesis presents the results of three experiments which focus on identifying the implications of maternal dietary environment on embryonic development and consequences for juvenile and adult phenotype and potential epigenetic regulatory factors.

Chapter 1 provides a background on the current understanding of fetal programming and the potential this has in the context of poultry production. It addresses the primary aims of this thesis and an introduction to the main concepts examined throughout.

Chapter 2 presents a review of the literature summarising effects of manipulation of the maternal diet and the potential PAR effect on embryonic and postnatal development. It emphasises the gaps in knowledge and potential applications for dietary manipulation of the breeder hen to improve offspring health and performance in current poultry production systems.

Chapter 3 investigates the consequences of mismatched dietary environment on offspring performance using two common grain types and the potential epigenetic role of the yolk as a regulatory interface. It also investigates the PAR effect in offspring in a mismatch/match environment due to changes in predominating grain source.

In Chapter 4, the physiological response by the broiler breeder hen to change in body weight associated with severe feed restriction is identified and speculate the yolk is the functional interface between mother and offspring. Consequences for embryonic development at two critical stages (ED14 and ED20) are measured, as well as the PAR effect by feeding offspring diets either *ad libitum* or restricted-fed posthatch.

Chapter 5, entitled “Differences in the nutrient and hormone yolk profile of two genetically-diverse commercial poultry strains” expands on the potential epigenetic role of yolk hormones by identifying differences in specific hormones and nutrients involved in development between broiler and layer phenotypes.

Chapter 6 is the final chapter which draws together the findings of the experimental chapters into coherent and evidence-based conclusions. Conclusions are drawn on the consequences of current broiler breeder management for offspring development and performance. It highlights the potential for the broiler industry to improve offspring performance and health via the maternal diet using yolk as a significant interface for manipulation of the *in ovo* environment. Finally implications of the current work and future directions and considerations relating to developmental programming in the poultry industry are identified.

## 1.3. Project aims

The objective of this thesis was to explore the impacts of the quantity and quality of rations for broiler breeder hens on offspring development as well as the potential role of the PAR effect in poultry production systems. In order to define developmental programming in avian species, the experiments within this thesis were designed to manipulate the maternal dietary environment in a context relevant to current industry practice. This thesis involved a series of experiments designed to elucidate the relationship between breeder hen diet and egg composition, and the growth and performance of the progeny.

The individual projects of this thesis addresses the following research questions:

1) Can dietary grain source induce epigenetic programming in a 'mismatched' environment?
2) Do commercially restrict-fed hens demonstrate physical and hormonal indices of stress and is this reflected in yolk composition?
3) Does feed restriction and changes in breeder hen body weight impact on embryonic and post-hatch development?
4) Does developmental programming produce long-term effects on offspring?
5) Do microRNAs in avian yolk signal embryonic programming and phenotype?

# 2. Review of the Literature: "Adaptive Predictive Response" in commercial broilers: Impact of maternal dietary environment and the role of the egg

## 2.1. Abstract

Changes in the maternal environment during perinatal development can lead to permanent changes in tissue structure, metabolic and endocrine regulation and therefore phenotype of offspring. In particular, the maternal diet alters offspring phenotype, due to fluctuations in nutrient and hormone levels transferred *in utero*, in an attempt to better suit the environment into which it will be born. However when the functional capacity set *in utero* does not match that required postnatally, it can result in abnormal physiological, neuroendocrine and behavioural changes resulting in long-term health risks and pathology. The extent to which this phenomenon exists in avian species and the potential positive and negative implications for poultry production is poorly understood. Intense genetic selection in poultry has resulted in faster post-hatch growth, often at the expense of bird health (ascites and tibial dyschondroplasia). The period of *ovo* development, in comparison to post hatch growth, has remained relatively static, and now attributes approximately 50% of the overall productive life of the modern broiler. Research suggests yolk and albumen composition significantly alters chick phenotype, irrespective of genotype. It is well established that yolk, in particular, contains high levels of hormones and nutrients and, unlike mammalian systems, the presentation of these compounds to the developing chicken is not regulated by a placenta. The composition of the yolk is 'set' during ovogenesis so the developing embryo is exposed to a relatively 'constant' in ovo environment. However the role of the maternal diet in regulating yolk composition, and the consequences of mismatch maternal-offspring environments in poultry production is poorly acknowledged. Management of the broiler breeder hen is heavily focussed on reproductive performance in a cost-effective manner with little consideration to the potential programming effects the maternal diet have on offspring health and performance. Therefore the extent to which the broiler breeder hen diet dictates developmental programming effects in chicks is unknown. This review summarises current literature relating to the manipulation of the maternal

diet and the potential “predictive adaptive response” (PAR) effects during embryonic and postnatal development. It identifies potential applications for breeder hen dietary manipulation, which may result in improvement in health and performance in current poultry production systems.

## 2.2. Introduction

The impact of the maternal environment during embryonic development on offspring physiology and health has been of great interest in human and mammalian species (Barker 1997; Armitage *et al.* 2004). It has been proposed 'programming' events are an evolutionary mechanism, whereby parents transfer information to the offspring regarding the environmental conditions into which they will be born. This allows the fetus to alter developmental trajectory, increasing resilience and robustness. Maternal effects have been associated with a plethora of physiological changes in offspring including mass and/or growth, metabolism, reproduction, neurobiology, immune function, and more recently behaviour (Barker 1997; Fowden *et al.* 2006; Sarkar *et al.* 2008; Gatford *et al.* 2010). While most studies indicate negative programming responses, there is increasing evidence some environmental conditions can have stimulatory or beneficial effects (Costantini *et al.* 2010). Ultimately variability in offspring phenotype is a reflection of deficits from early insults, adaptation to maternal resources or predictive adaptation to the adult environment.

The phenomenon of developmental programming may have significant applications for the broiler industry. Modern broilers currently reach market weight within 32 days with a feed conversion of 1.47, in comparison to birds from 1957 which took 101 days and feed conversion of 4.42. Such dramatic growth rates have been associated with negative health effects such as poor reproductive performance, excessive fatness, increased skeletal abnormalities and ascites (Griffin and Goddard 1994). The mortality rate in broiler strains from 1957 was approximately half that of modern strains, a reflection of intense growth pressure of modern day broilers (Havenstein *et al.* 2003). Continuing to increase productivity via increases in feed conversion efficiency and growth rates raises serious ethical and bird health concerns for the poultry industry.

In comparison, the period of *in ovo* development has remained relatively constant, with little change to the current 21 day incubation period. Consequently, *in ovo* development now contributes up to 40% of the total lifespan of the modern day broiler. Current research highlights the profound effect the *in ovo* environment can have on phenotypic development in avians (Uni *et al.* 2005; Ho

*et al.* 2011; Yair *et al.* 2013). Furthermore, egg composition is 'set' during ovigenesis and not altered as it is in placental mammals, therefore components deposited into the egg are a reflection of the hen and her environment during oviposition and hence has a significant effect on embryonic development (Bellairs 2005). In addition, resulting mismatched environmental cues across generations based on maternal environmental effects and the offspring's environment may induce positive or negative phenotypic effects via the milieu of the egg. Understanding dietary effects in broiler hens and associated changes in egg composition may identify potential applications to be employed or acknowledge in broiler breeder management allowing continued improvement in broiler production by optimising environmental conditions in parental birds which may alter developmental trajectory of offspring.

## 2.3. Developmental programming

Epidemiological studies in humans demonstrate adverse environmental conditions elicited by maternal dietary or placental insufficiency during fetal and infant development may 'programme' susceptibility to postnatal cardiovascular and metabolic disease. The association between adverse intrauterine conditions in middle to late gestation and adult hypertension, insulin resistance and dyslipidaemia and disproportionate fetal growth has led to the 'fetal origins' hypothesis proposed by Barker and colleagues (Barker 1995; Barker 1997; Godfrey *et al.* 1997). Low birthweight has since been linked to other metabolic diseases including; glucose intolerance, insulin resistance, type 2 diabetes, hypercortisolaemia, obesity and reproductive disorders during adulthood (Fowden *et al.* 2006). The suboptimal perinatal conditions causing such effects is commonly referred to as 'intrauterine growth retardation' (IUGR). IUGR has been induced via a range of insults including maternal stress, hypoxia, glucocorticoid administration, dietary manipulation, and placental insufficiency leading to postnatal abnormalities in cardiovascular, metabolic, and endocrine function in a range of species including; rats, guinea pigs, sheep, pigs, horses and primates (Fowden *et al.* 2005; McMillen and Robinson 2005). IUGR in multiparous species is a major determinant of postnatal hypertension, glucose intolerance, and alterations in

the functioning of a number of endocrine axes, including the pancreatic islets, renin-angiotensin system, and hypothalamic-pituitary-adrenal (HPA) axis, resulting in shifts of metabolic set points (Fowden *et al.* 2005). These early insults at critical stages of development lead to permanent changes in tissue structure and function.

### 2.3.1 Environmental perturbations

Fetal growth and development depends primarily on nutrient and oxygen supply, hence associations between low birth weight and adult phenotype have been linked to poor nutrition and oxygenation during embryonic life (Figure 2.1). Since nutrient and $O_2$ availability invariably alters the endocrine environment, the role of hormones as programming signals has been examined (Fowden and Forhead 2004). In general, the main fetal adaption to lack of nutrients or oxygen is to slow the rate of cell division. Cell division shows either as a direct effect of undernutrition on the cell or through altered concentrations of growth factors and hormones (Barker 1997). Consequently, the timing and duration of when these perinatal insults are initiated is important (Armitage *et al.* 2004; Fowden *et al.* 2006). During peri-conception and preimplantation period $O_2$, nutrient, and hormone levels affect oocyte and blastocyst development. Developmental changes arising pre-implantation likely affect cell lineages i.e. exposure of neonate to progesterone and metabolites (i.e. urea) during this period results in increased birth weight in sheep and pigs (McEvoy et al 2001). During organogenesis, environmental perturbations may result in discrete structural defects, permanently reducing the functional capacity of organs. During phases of rapid growth, insults pertaining to supply, uptake and utilisation of nutrients influence tissue growth and induce cell differentiation, altering total cells number. Changes during late development likely alter fetal maturation, a period when tissues undergo structural and functional changes in preparation for extra-uterine environment.

A range of techniques has been used to experimentally alter *in utero* environment, however the two main models involve either; direct intervention of the fetal environment via the placenta or egg, or indirectly via the maternal environment. Such models result in altered nutrient, oxygen

and hormonal exposure to the fetus. Placental restriction results in severe restriction of nutrients (primarily glucose) and partial pressure of oxygen delivered to the foetuses (Gatford *et al.* 2010). In avian species, direct manipulation involves removal of egg components, most commonly albumen and yolk, altering nutrient availability and hormone exposure to the developing chick.

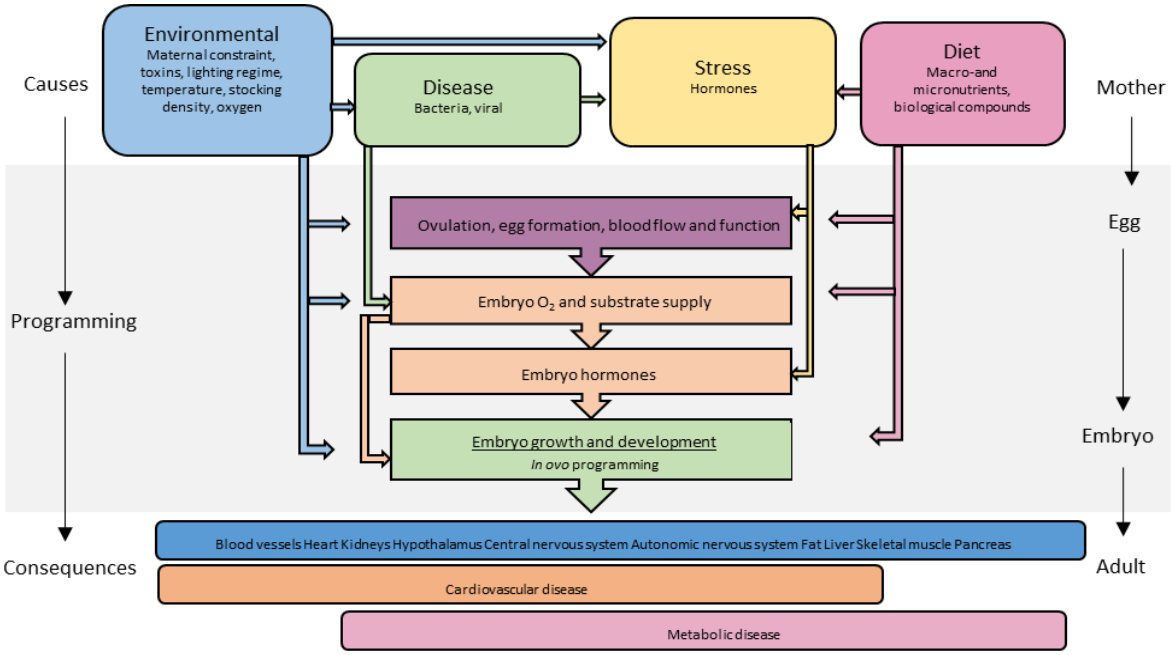


**Figure 2.1 Causes and consequences of intrauterine programming, modified from Fowden *et al.* (2006).**

Prenatal nutritional programming has been demonstrated in a range of species by manipulating the availability of both macro- and micronutrient supply during development. The most common techniques used to reduce fetal availability of nutrients include; restriction of placental and umbilical/uterine blood flow and maternal caloric and protein deprivation. These models all impair fetal development resulting in pre-and postnatal abnormalities in cardiovascular, metabolic, and endocrine function (McMillen and Robinson 2005). Some programming effects of macronutrient restriction can be ameliorated by supplementation of single amino acids and cofactors in the diet (Armitage *et al.* 2004). More recently maternal over nutrition, particularly fat feeding, and dietary manipulation of specific micronutrients, such as minerals (calcium, iron), cofactors (folic acid, taurine), and vitamins (A, D and E) have been reported to alter postnatal physiological function

(Armitage *et al.* 2004). In avian species nutritional manipulation by *in ovo* albumin restriction or enrichment with carbohydrates (CHO) and minerals have strong developmental effects in chicks (Uni and Ferket 2004; Uni *et al.* 2005; Everaert *et al.* 2013; Yair *et al.* 2013). In many circumstances nutrients may contribute to similar metabolic pathways or co-contribute therefore programming effects may in fact be the balance of macro-and micronutrients (Fowden *et al.* 2006).

Mammalian models used to induce fetal undernutrition also reduce fetal $O_2$ supply. Disruption of oxygen and nutrient supply stimulates up-regulation of both maternal and placental cardiovascular and endocrine function that may, in turn, influence fetal response (Owens *et al.* 1987; Jacobs *et al.* 1988; Woodall *et al.* 1996). Hypoxia during pregnancy causes abnormalities in cardiovascular function (Chang *et al.* 1984; de Grauw *et al.* 1986), reduced birth weight and asymmetric growth retardation (Giussani *et al.* 2001; Moore *et al.* 2004). Episodes of hypoxia during fetal development are likely to also cause many neurological disorders (Gadian *et al.* 2000; Vargha-Khadem *et al.* 2003). Avian development provides an excellent model for the study of hypoxia due to the ease of manipulation during incubation. Optimisation of incubation conditions has received considerable attention in the poultry industry due to its effects on hatch rate and chick survival. Both eggshell temperature and $O_2$ concentration during incubation alter embryonic growth and metabolism, likely related to changes in hormone concentrations (Decuypere and Bruggeman 2007; Lourens *et al.* 2007; Oviedo-Rondón *et al.* 2008). Hypoxia in chicks during incubation affects both organ and whole body growth (Camm *et al.* 2001; Dzialowski *et al.* 2002; Miller *et al.* 2002) and is dependent on the severity of insult (from 10 to 15% ambient oxygen concentration) and duration (from 2h to 6 days) of exposure (Altimiras and Phu 2000; Camm *et al.* 2001; Dzialowski *et al.* 2002; Miller *et al.* 2002).

Exposure to fluctuating hormone concentrations *in utero* can lead to programming of key endocrine systems including HPA axis, endocrine pancreas and somatotrophic axis (Fowden *et al.* 2005). Undernutrition, hypoxia and stress can alter both maternal and fetal concentrations of many hormones including growth hormone (GH), insulin-like growth factors (IGFs), insulin,

glucocorticoids (GC), catecholamines, leptin, thyroid hormones (TH), and placental hormones such as eicosanoids, sex steroids, and placental lactogen. In birds, hormone-mediated maternal effects, via androgens, have multiple and long-lasting effects on offspring (see reviews (Groothuis *et al.* 2005; Gil 2008a; Groothuis and Schwabl 2008; Navara and Mendonça 2008; von Engelhardt and Groothuis 2011).

Hormones either cross the placenta or are deposited into the egg and absorbed, therefore fetal endocrine response reflects both maternal and fetal endocrine regulation during uptake and is dependent on the type, duration, severity, and gestation age at onset of insult. In general, nutritional challenges that reduce fetal nutrient availability lower anabolic hormones (Insulin, IGF-1, $T_4$) and increase catabolic hormone concentrations (cortisol, catecholamines, GH), whereas challenges that increase fetal nutrient supply raise anabolic and reduce catabolic hormone levels in fetal circulation (Fowden 1995). Hormones act on fetal growth both directly and indirectly by altering delivery, uptake, and metabolic fate of nutrients in the fetoplacental tissues and can impact at differing levels; gene, cell, tissue and organs and systemic (Fowden 1995; Fowden *et al.* 2006). Specific hormones have been recognised as important for embryonic development due to their ability to regulate metabolic activity and gene expression (De Oliveira *et al.* 2008). In sheep, placental restriction reduces circulating levels and expression of anabolic hormones including IGF-I and –II in the fetus, enhancing fat deposition in early postnatal life, impair insulin secretion and sensitivity and impair β-cell function (Gatford *et al.* 2010).

Of all the hormones, glucocorticoids (GC) are most likely to have widespread programming effects *in utero* (Fowden *et al.* 1998; Seckl 2001). Glucocorticoids are vital for embryonic development, specifically involved in prenatal maturation of organs such as the lungs, liver, kidneys and gut (Fowden *et al.* 1998). Therefore fetal maturation, whereby tissues undergo structural and functional changes, can be activated prematurely by early GC exposure. In rats, guinea pigs, and sheep, fetal overexposure to either exogenous or endogenous GC leads to hypertension, glucose intolerance, and abnormalities in HPA function after birth (Seckl 2004). In avians, negative effects

of GC include altered offspring hatch weight, growth rate, bilateral asymmetry, immune response and HPA-axis activity in a sex-dependant manner (Eriksen *et al.* 2003; Rubolini *et al.* 2005; Hayward *et al.* 2006; Henriksen *et al.* 2011b; Ahmed *et al.* 2014a). *In ovo* exposure to corticosterone has also been associated with neurological and behavioural effects in adult chickens including increases in aggressive behaviour, reduced rate and loudness of late embryonic vocalisation and intensity of begging, impaired memory response and increased fear (Rubolini *et al.* 2005; Janczak *et al.* 2006; Rodricks *et al.* 2006; Ahmed *et al.* 2014a). Elevating corticosterone levels in breeding females affects reproductive performance i.e. egg production, quality and ovary and oviduct weight (Ahmed *et al.* 2014a) and induces significant female-biased hatchling sex ratio (Love *et al.* 2005; Pike and Petrie 2006).

### 2.3.2 Epigenetic mechanisms

Environmental perturbations in early life, particularly nutrition, result in changes in phenotypic expression at birth, referred to in mammals as “fetal programming”. This implies phenotypic expression in the neonate is the direct effect of certain maternally-derived factors (i.e. specific hormones, nutrients). It is now apparent factors, specifically nutrients, induce molecular modifications to DNA and chromatin, and therefore gene expression, without changing the DNA sequence, defined as “epigenetics” (Vickaryous and Whitelaw 2005; Godfrey *et al.* 2007). Epigenetic mechanisms determine how, when and where DNA sequence information is used by suppressing or activating genes. Altering regulation of gene transcription and expression can therefore cause changes in tissue structure and function and alter activities of metabolic pathways and homeostatic control processes. Mechanisms which underlie such changes in gene expression are now beginning to be elucidated (Groothuis and Schwabl 2008). Changes in gene expression can occur via a range of mechanisms. The longest-studied and most well defined epigenetic marker is DNA methylation, whereby an additional methyl group is added to nucleotides, typically silencing gene expression. Another explored mechanism is histone modification, which influences gene expression by changing chromatin structure. Recently, small RNAs, microRNAs and large RNAs have been

identified which act to modulate protein activity via regulation of translation, transcription or protein structure. These conformational changes alter the interaction between DNA and regulatory proteins at the promoter, altering gene expression and suppression (Waterland and Garza 1999).

Epigenetic mechanisms have been the focus of a large body of work in laboratory rodents and sheep, with increasing focus on the role of specific nutrients (Burdge *et al.* 2007). Methyl and acetyl groups are heavily involved in methylation patterns, therefore many changes in gene expression are influenced by maternal dietary effects (Burdge *et al.* 2007). Dietary micronutrients including folate, choline, betaine, B vitamins along with amino acids methionine, cysteine, serine and glycine have been identified as epigentic modulators of DNA methylation (Anderson *et al.* 2012; Lee 2015). During one-carbon metabolism, these substrates catalyse with enzymes to generate S-adenoslymethionine (SAM), the primary methyl donor for DNA methylation and methyltransferase inhibitor S-adenosylhomocystine (SAH) levels (Figure 2.2) (Anderson *et al.* 2012). Other biological substrates such as retinoic acid, resveratrol, curcumin, sulforaphane and tea polyphenols, can alter SAM and SAH levels or regulate DNA methylation and histone modifications in specific genes or at a genome-wide level. Restricting substrates such as folate, methionine and B vitamins alters DNA methylation associated with insulin resistance and elevated blood pressure (Sinclair *et al.* 2007), and gene expression and methylation changes involved in renin-angiotensin system, mitochondrial metabolism and phospholipid homeostasis (Chen *et al.* 2015). Additionally, vitamin B12 and folate deficiencies during gestation and lactation have been associated with decreased birth weight, increased central fat mass, liver steatosis and myocardium hypertrophy (Guéant *et al.* 2013). Choline has been linked to fetal brain development, with maternal choline deficiency shown to modify epigenome of the fetal brain (Mehedint *et al.* 2010).

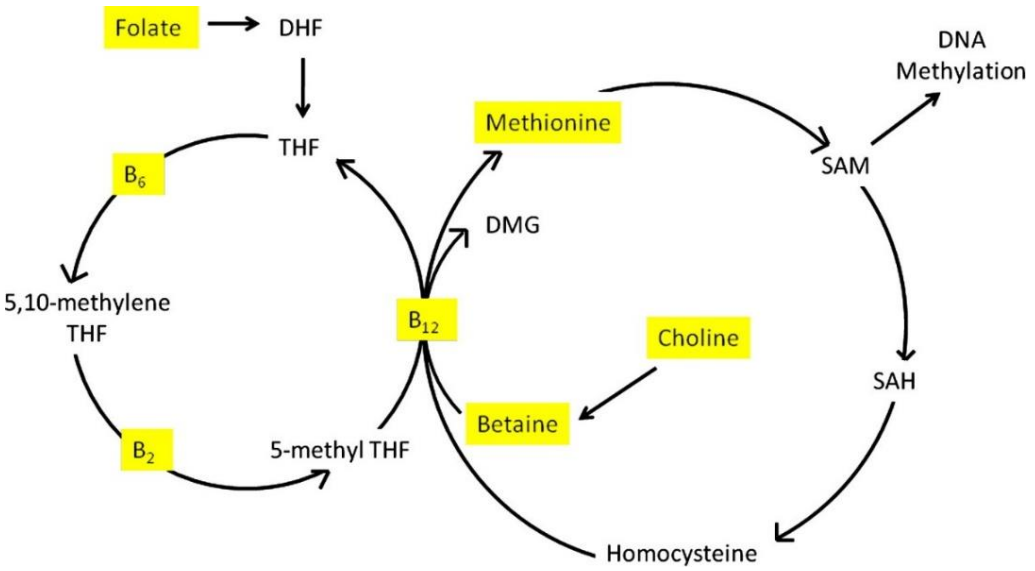


**Figure 2.2 Involvement of dietary micronutrients in one-carbon metabolism. Substrates obtained via diet are highlighted in yellow. Figure abbreviations; Dihydrofolate (DHF), tetrahydrofolate (THF), S-adenoslymethionine (SAM) and S-adenosylhomocystine (SAH) (Anderson *et al.* 2012).**

Currently, the role of miRNA in fetal programming remains largely understudied. Studies are now emerging associating modified miRNA expression profiling with maternal nutritionally-induced epigenetic modifications in offspring and lipid metabolism, insulin resistance and inflammation (Zhang *et al.* 2009). These studies demonstrate maternal dietary nutrient supply are critical to embryonic development and play an important role in fetal programming and associated metabolic disease in adulthood.

## 2.4. Maternal dietary effects

Nutritional imbalances, such as under-and over-nutrition during critical periods of development have been linked to persistent physiological and metabolic alterations in mammals i.e. rodents and sheep. Placental restriction and *in ovo* manipulation models allow the study of specific mechanistic pathways, however do not account for the regulatory effects of the mother and effects of other resulting endocrine and nutritional changes. Undernutrition induced by total caloric restriction and reduced dietary protein content are the predominating models of maternal dietary effects. More recently, altered postnatal physiological function has also been observed after

maternal fat feeding and dietary manipulation of specific micronutrients, such as minerals (calcium, iron), cofactors (folic acid, taurine), and vitamins (A and D). These models result in alterations to physiological, metabolic and endocrine regulation. In particular, dietary perturbations have been associated with changes in hormone levels. Fluctuations in endocrine regulation either directly via hormones regulating feed intake and absorption i.e. insulin, IGF, GC or by association i.e. stress related feed restriction may influence phenotypic expression. More recent studies have identified the molecular basis for changes in metabolic and endocrine pathways, with specific focus on GC activity, carbohydrate and lipid metabolism (Burdge *et al.* 2007; Lillycrop 2011; Chango and Pogribny 2015).

### 2.4.1 Under supply

Both severe and mild undernutrition (in particular total caloric and protein restriction) result in decreased body weight (BW) and/or disproportionate body size at birth (Mellor 1983; Woodall *et al.* 1996; Bertram *et al.* 2001; Whorwood *et al.* 2001; Maloney *et al.* 2003; Vonnahme *et al.* 2003; Fagundes *et al.* 2007; Podaza *et al.* 2015). Zambrano *et al.* (2006) found BW effects of fetal undernutrition are sex-dependent, with only female offspring lower in body weigh at birth. In some instances no significant change in birthweight may occur, however structural changes of specific tissues such as the liver, and endocrine sensitivity in vital organs such as the brain and liver may be apparent (Burns *et al.* 1997; Whorwood *et al.* 2001; Vonnahme *et al.* 2003; Podaza *et al.* 2015). Such changes have been linked to alterations in gene transcription, which in turn alters activities of metabolic pathways and homeostatic control processes (Han *et al.* 2004). In rats, mild maternal protein restriction (PR) increases glucocorticoid receptor (GR) protein and mRNA expression in kidney, liver, lung, and brain and reduces expression of 11β-hydroxysteroid dehydrogenase type 2 in kidney, adrenal and brain in offspring (Bertram *et al.* 2001) and liver when total caloric restriction was imposed (Gluckman *et al.* 2007). Similar results have been reported in sheep with maternal caloric restriction, increasing GR expression in the adrenal, kidney, liver, lung and peri renal adipose tissue and decreased 11β-hydroxysteroid dehydrogenase type 2 in adrenal

and kidney of the offspring (Whorwood *et al.* 2001). Ewes fed a protein restricted resulted in lambs with significantly higher insulin-like growth factor receptor 1 (IGF-1R) mRNA expression towards the end of gestation, however this was only in single-born offspring compared to twins (Brennan *et al.* 2005). Maternal PR is also associated with reduced glucokinase expression in liver of rat offspring, potentially altering glucose uptake (Burns *et al.* 1997; Bogdarina *et al.* 2004). In the liver of newborn piglets, proteins known to play important roles in energy and iron metabolism, protein degradation, and cellular signal transduction were found to be differently expressed when IUGR was triggered (Wang *et al.* 2008).

Maternal dietary restriction alters expression of specific genes related to lipid homeostasis. Rats fed a PR diet during pregnancy and lactation increased genes involved in acetyl-CoA carboxylase and fatty acid synthase in the liver of offspring (Maloney *et al.* 2003). Peroxisomal proliferator-activated receptors (PPARα and γ), important regulators coordinating lipid metabolism between the liver and adipose tissue have been linked to maternal PR. Hepatic PPARα expression is reportedly increased in offspring, accompanied by up-regulation of acyl-CoA oxidase (Burdge *et al.* 2004; Lillycrop *et al.* 2005). Comparatively, Gluckman *et al.* (2007) observed down-regulation in expression of both PPARα and GR in adult offspring born to dams exposed to 30% total caloric restriction. Maternal nutrient restriction during late gestation also decreased adipose GLUT4, responsible for glucose uptake, in adult offspring (Gardner *et al.* 2005). Such changes in gene expression are associated with changes in plasma and hepatic triglyceride, nonesterified fatty acid, cholesterol, glucose, leptin, insulin, IGF-1 and GC concentrations in offspring, and in many circumstances increases visceral fat mass and total body fat (Woodall *et al.* 1996; Maloney *et al.* 2003; Vonnahme *et al.* 2003; Burdge *et al.* 2004; Zambrano *et al.* 2006; Fagundes *et al.* 2007; Gluckman *et al.* 2007; Podaza *et al.* 2015).

Increased fat deposition in offspring due to maternal dietary restriction may not only reflect metabolic and endocrine regulation but also structural changes in skeletal muscle, resulting in poor energy utilisations. Reduced myofibre number in IUGR neonates limits the capacity for postnatal

compensatory growth of skeletal muscle (Hegarty and Allen 1978) while muscle composition and distribution of muscle fibre type, in addition to fat and collagen concentrations, in skeletal muscle is also affected by IUGR (Wigmore and Stickland 1983; Karunaratne *et al.* 2005). IUGR in piglet skeletal muscle changes expression level of 12 proteins related to macronutrient metabolism, immune response, cellular structure, extracellular matrix and antioxidant function (Wang *et al.* 2008).

Abnormalities in intestinal structure, metabolism and function also occur during fetal growth restriction. Reducing maternal dietary protein from 17% to 6% in pregnant rats increased lactate and sucrase activity in small intestine, and mRNA expression in proximal intestine of 3-week-old offspring. In addition sucrase activity and mRNA abundance in duodenum increased in 16-week-old offspring (Pinheiro *et al.* 2013a). In a similar study, PR offspring expressed higher cell proliferation in the small intestine and up-regulated transporter gene expression, specifically sodium-glucose co-transporter 1 (SGLT1), peptide transporter 1 (PEPT1) and glucose transporter 2 (GLUT2) (Pinheiro *et al.* 2013b). Proteomic analysis of piglets exposed to IUGR found 11 differentially expressed proteins related primarily to energy metabolism, immune response, cellular structure, and antioxidant function in the small intestine (Wang *et al.* 2008).

Maternal dietary restriction is associated with changes in glucose (Oliver *et al.* 2005) and hormone i.e. IGF-1, insulin and GC concentrations (Woodall *et al.* 1996; Oliver *et al.* 2005). Fluctuations in maternal hormone concentration may in turn cause changes in placental weight (Woodall *et al.* 1996) and endocrine regulation. Langley-Evans *et al.* (1996) found mild maternal PR attenuated placental 11βHSD activity which may result in overexposure of cortisol to the developing fetus.

### 2.4.2 Over supply

Maternal obesity or related gestational diabetes mellitus (GDM) increases the risk of obesity, impaired glucose tolerance, and other aspects of metabolic syndrome in adult offspring (Parsons *et al.* 2001; Ramsay *et al.* 2002; Gluckman and Hanson 2004; Boney *et al.* 2005; Catalano

and Ehrenberg 2006). Animal models indicate maternal hypernutrition conveys a propensity for offspring hyperphagia, increased adiposity and tissue triglyceride levels, abnormal glucose homeostasis and reduced insulin sensitivity, particularly in male offspring (Armitage *et al.* 2005; Taylor *et al.* 2005; Samuelsson *et al.* 2008; Nivoit *et al.* 2009; Ainge *et al.* 2010; Rooney and Ozanne 2011). Maternal obesity may play a direct role in transmission of obesogenic and diabetogenic traits from generation to generation, with maternal hyperleptinemia, hyperinsulinaemia, hyperglycaemia or maternal inflammation all previously associated with altered offspring phenotype (Weissgerber *et al.* 2006).

Maternal fat supplementation and/or obesity increases adiposity in offspring which can occur with (Chen *et al.* 2008; Nivoit *et al.* 2009; Shelley *et al.* 2009; Tamashiro *et al.* 2009) or without (Buckley *et al.* 2005) absolute increases in body mass. It is likely increased adiposity is the result of decreased glucose tolerance and whole body insulin resistance (Buckley *et al.* 2005; Taylor *et al.* 2005; Chen *et al.* 2008; Samuelsson *et al.* 2008; Nivoit *et al.* 2009). These metabolic effects have been associated with structural changes in pancreatic islets, including reduced β-cell number and cell volume (Cerf *et al.* 2005). While at a molecular level, maternal obesity alters expression of insulin-signalling pathway genes including; insulin receptor substrate 1, glucose transport 2 and glucokinase (Buckley *et al.* 2005; Cerf *et al.* 2005; Shelley *et al.* 2009; Martin-Gronert *et al.* 2010; Oben *et al.* 2010; Yan *et al.* 2011). Additionally, Samuelsson *et al.* (2008) found offspring of obese mice displaying adipocyte hypertrophy had altered mRNA expression of β-adrenoceptor 2 and 3, 11βHSD1 and PPARγ2 in inguinal fat.

Skeletal muscle attributes 40-50% of total body mass and is the principal site for glucose and fatty acid utilisation. Therefore changes in mass and function of skeletal muscle may influence offspring predisposition to obesity and insulin resistance. It is likely elevated body fat percentage is the result of reduced lean mass rather than increased fat mass *per se* (Buckley *et al.* 2005; Samuelsson *et al.* 2008). A recent review by Du *et al.* (2010) suggests changes in fetal skeletal muscle due to maternal obesity are associated with maternal inflammation by inducing a shift in

fetal mesenchymal stem cells from myogenesis to adipogenesis and fibrogenesis. As a result offspring display increased intramuscular fat, triglyceride, collagen content and connective tissue, as well as reduced numbers of muscle fibres and/or diameter (Tong *et al.* 2009; Yan *et al.* 2011). These structural changes are associated with decreased downstream insulin signalling, increased phosphorylation of protein kinase C and insulin receptor substrate 1, increased expression of fatty acid transporters and changes in myogenic markers of fetal semitendinosus muscle including reduced MyoD, myogenin, and desmin and downregulation of Wnt/β-catenin signalling pathway (Tong *et al.* 2009; Yan *et al.* 2011). Effects of maternal obesity on expression of insulin-signalling proteins in offspring appear sex-dependant (Shelley *et al.* 2009). Adult offspring of obsess mice had reduced expression of IRS-1 (initial step in activation of PI3K) and PI3K subunit, p110β, and Akt phosphorylation at Serine 473 in female offspring, while in male offspring there was an increase in the skeletal muscle expression of Akt2 and PKCζ and decrease in mitochondrial-linked complex I-II. Changes in muscle function have also been reported which may attribute to increased adiposity in offspring. Maximal twitch tension and maximal tetanic tension of the semitendinosus were reduced in 10-week-old offspring of junk food fed mothers (Bayol *et al.* 2009) while offspring of obese mice displayed reduced locomotor activity mice at 3 months of age (Samuelsson *et al.* 2008).

Hyperphagia occurs in offspring of high-fat fed mothers and has been associated with decreased plasma leptin levels, increased blood lipid and NEFA levels, and hypothalamic leptin resistance (Férézou-Viala *et al.* 2007; Chang *et al.* 2008; Morris and Chen 2008; Nivoit *et al.* 2009). High fat diet during gestation increases neurogenesis and therefore increased density of peptide-expressing neurons at birth. Consequently an increase in expression of orexigenic peptides in hypothalamus occurs (Chang *et al.* 2008). At birth, offspring of high-fat fed mice had decreased hypothalamic mRNA expression of neuropeptide-Y (NPY), pro-opiomelanocortin (POMC), leptin receptor and signal transducer and activator of transcription3 (STAT3) (Morris and Chen 2008). An amplified and prolonged neonatal leptin surge, accompanied by elevated leptin mRNA expression in abdominal white adipose tissue evident at weaning followed by leptin-induced appetite suppression and phosphorylation of STAT3 detection during adulthood (Kirk *et al.* 2009). Maternal

consumption of high-fat diet can cause DNA hypomethylation changes in association with long-term alteration in brain dopamine and opioid gene expression, and a preference for sucrose and fat diets in offspring (Vucetic *et al.* 2010). The type of fat consumed by the mother influences developmental programming effects. Maternal diets high in unsaturated fatty acids decrease birth and postnatal weight gain, and increased pancreatic islet number without affecting glucose tolerance. In comparison, a diet high in saturated fatty acids reduced number of pancreatic islets and resulting in a faster and higher insulin response in offspring (Siemelink *et al.* 2002).

Long-term effects of high maternal protein intake on neonate development are limited. In rodent models, maternal high protein decreases birth weight, increases fat deposition and alters energy efficiency (Daenzer *et al.* 2002) and may be sex-dependant (Thone-Reineke *et al.* 2006). A recent study by (Hallam and Reimer 2013) also found sex-dependant effects with increased body weight and percentage of body fat in female offspring of high-protein maternal diet which may be the result of impaired muscle growth previously reported by Rehfeldt *et al.* (2013). The difference in body weight of females could partially be due to decreased expression of fatty acid synthase (FAS) observed in offspring of high-protein diet.

### 2.4.3 Dietary Mismatch and the "Predictive Adaptive Response"

The phenomenon of 'fetal programming' heavily focusses on the *in utero* environment as a means to permanently alter neonatal phenotype. More recently the 'Predictive Adaptive Response' (PAR) hypothesis proposes the degree of mismatched interaction between pre-and-postnatal environments is detrimental to offspring phenotype. Therefore, the postnatal environment, particularly during periods of developmental plasticity, is just as important for the adult phenotype. Maternal dietary restriction during the prenatal period results in metabolic adaptions to a low nutritional environment. When the offspring is exposed to a similarly poor postnatal diet, programming of the 'thrifty' phenotype will confer 'predictive adaptive' advantage, biologically prepared to withstand the poor diet, resulting in a 'normal' phenotype. However, if adequate or abundant nutrition is supplied post-natally, a mismatch of metabolic programming and postnatal

environment can cause disease and malfunction (Hales and Barker 2001). This suggests disease only manifests when offspring diet diverts from the 'predicted' maternal dietary programming phenotype. Postnatal environmental changes may therefore ameliorate or exaggerate the morphological and functional changes programmed *in utero*.

Mismatched dietary environmental research has focussed primarily on the effects of 'catch-up' growth on offspring phenotype (Nobili *et al.* 2008). Maternal dietary restriction in combination with postnatal catch-up growth has been associated with obesity, systolic blood pressure and insulin resistance (Huxley *et al.* 2000; Ong *et al.* 2000; Soto *et al.* 2003). In sheep, mismatched pre-and post-natal nutrition increases the predisposition to cardiovascular disease offspring (Cleal *et al.* 2007). The modulating effects of mismatch diet and potential PAR have recently been reported in avians. Chicks from restrict-fed hens significantly lighter at hatch and when fed *ad libitum* posthatch offspring were significantly heavier and had greater abdominal fat at 6 weeks of age than progeny of ad lib hens (van der Waaij *et al.* 2011). At a molecular the combination of maternal dietary restriction and catch-up growth in rodents has been linked to increased adipocyte size, reduced expression of IRS-1, PI3K p110β, Akt phosphorylation and protein levels of Akt-2 (Berends *et al.* 2013).

With the exception of IUGR and catch-up growth, limited research exists on mismatched dietary programming. These studies mostly pertain to maternal high fat environments and demonstrate in some instances positive effects in offspring. Feeding offspring the same fat-based diet as their dams prevented endothelial dysfunction and reduced heart rate, but did not attenuate development of raised blood pressure (Khan *et al.* 2004). In a similar study, matched high-fat feeding of dams and their offspring resulted in smaller body weight and feed efficiency, suggesting programming of increased energy expenditure (Férézou-Viala *et al.* 2007). In pigs, offspring fed a similar diet to parents provided a protective effect from atherosclerosis by preventing development of aortic fatty streaks (Norman and LeVeen 2001).

Programming effects of macronutrient restriction *in utero* can be ameliorated by supplementing the altered maternal diet with single cofactors and amino acids. Supplementation of maternal PR diet with folic acid normalises changes in GR and PPARα promotor methylation and gene expression (Torrens *et al.* 2006). Likewise, maternal supplementation with glycine prevents blood pressure or endothelial dysfunction otherwise induced by maternal PR (Jackson *et al.* 2002; Brawley *et al.* 2004). Of the 1.3% of genes altered by maternal PR, only 0.7% of the hepatic genes were changed with folic acid supplementation (Lillycrop *et al.* 2010). This suggests a balance of micro-and macronutrients may be more important than absolute amounts *per se* and supplementation alone cannot prevent all changes in gene expression related to maternal dietary restriction (Armitage *et al.* 2004; Lillycrop 2011).

## 2.5. Avian model and manipulation of the *in ovo* environment (maternal regulatory control)

Eggs contain all components required for embryonic development and therefore establishment of physiological set points for posthatch phenotype. Unlike mammalian fetal development, whereby nutrient and hormone transfer across the placenta is variable and constantly moderated, deposition of programming 'agents' into the avian egg is determined during yolk and albumen formation only. Embryonic development does not initiate until after the egg is laid and incubation commences, at which point all programming 'agents' are already set. Consequently birds are an extremely useful model to examine maternal dietary effects in context of developmental programming.

*In ovo* development is classified into three stages; establishment of the germ, completion of the embryo form by embryonic day (ED) 14, and preparation for emergence and hatching at ED21 (Moran 2007). Germ establishment is reliant on nutrients the yolk (Speake *et al.* 1998) and albumen (Deeming 1989) absorbed from via the developing vascular system (Ribatti 1995). The biochemical composition of albumen and yolk differs significantly and these components are therefore involved in different functions of embryonic and posthatch development (Carey *et al.* 1980). Albumen is the

primary water and protein source to the embryo (Romanoff 1960). The majority of albumen nutrients are incorporated into the amniotic fluid and swallowed during late-term embryonic development. Albumen nutrient composition has been recognised in improving posthatch intestinal development for example, increased albumen carbohydrate concentration increased villi size and intestinal capacity to digest disaccharides and has a trophic effect on the small intestine and enhanced goblet cell development (Tako *et al.* 2004; Smirnov *et al.* 2006). These studies demonstrate amnionic supplementation during embryonic development, combined with early posthatch feeding, result in enhanced BW and muscle growth, ultimately reversing posthatch performance retardation commonly reported in chickens (Uni and Ferket 2004; Kornasio *et al.* 2011).

While yolk only attributes 30% of the total egg it contains a high lipid content (Speake *et al.* 1998) and greater complexity of nutrients compared to albumen (Yadgary *et al.* 2010; Yair and Uni 2011; Yadgary and Uni 2012), hormones (Groothuis and Schwabl 2008; Ho *et al.* 2011; von Engelhardt and Groothuis 2011), immunological factors (carotenoids and antibodies) (Buxton 1952), transcription factors (Knepper *et al.* 1999) and miRNA (Wade *et al.* 2016) which may influence embryonic development. Recent findings by Ho *et al.* (2011) demonstrate yolk source alone can significantly alter phenotypic expression and therefore has the ability to exert strong epigenetic effects during embryonic development. Embryos from two commercial strains (broiler and layers) were transferred from their natural yolks to novel yolk environments using a reciprocal cross model. Surprisingly, embryos presented phenotypes reflecting yolk source rather than embryo genotype, specifically changes in body mass, heartrate and development rate were found. Associated with these physiological changes were significant differences in yolk testosterone (TE) and triiodothyronine ($T_3$) concentrations between the breeds. This implies a complex synergistic effect of breed-specific genotype by yolk exists in avian development, with early embryonic development heavily influenced by yolk composition.

The impact of yolk hormones on developmental programming in avians has been well studied (Groothuis *et al.* 2005; Groothuis and Schwabl 2008; Navara and Mendonça 2008). Schwabl (1993) was the first to identify yolk as a source of maternal endocrine regulation, studying the effect of yolk TE on offspring behaviour. Since then studies of yolk hormone-mediated maternal effects have expanded to include corticosterone (Hayward and Wingfield 2004; Henriksen *et al.* 2011b; Henriksen *et al.* 2013; Ahmed *et al.* 2014a), thyroid hormones (McNabb and Wilson 1997; Wilson and McNabb 1997), and sex steroid (Elizabeth *et al.* 1995). Recently leptin was added to this list, due to its association with offspring post-hatch growth (Rao *et al.* 2009). Yolk hormones attribute to changes in offspring behaviour, growth, morphology, immune function and survival (Groothuis *et al.* 2005). Embryonic and postnatal function of many endocrine glands and hormone axes including; HPA, HPG, somatotrophic axis, adrenomedullary system, renin-angiotensin system, endocrine pancreas and hormones regulating appetite and food intake are affected by yolk hormone concentrations.

The cellular source of yolk factors presumably is maternal as the site of yolk synthesis is primarily the hen's liver. The raw materials of yolk are synthesised in the liver and pass into the blood where they are then deposited in the ovary by steroidogenic cells in the follicular wall during the phase of rapid yolk formation (8-9 day period). Due to the longer deposition period of yolk formation, in comparison to albumen (day of lay), yolk is likely to better reflect maternal status. The extent to which steroid hormones in yolk reflect plasma levels of mother is conflicting, with the suggestion hen may maintain partial control of yolk hormone allocation (von Engelhardt and Groothuis 2011). In addition, hormones and/or nutrients interact therefore manipulating the maternal environment will account of hen regulatory effects. While *in ovo* injection models are excellent for the study of singular compounds on embryonic development, for example CHO or corticosterone, the relevance pertaining to maternal programming and adaptive response might be disputed as *in ovo* injection is unlikely to; be evenly distributed in the yolk, reflect natural timing and concentration of exposure, and therefore not mimic naturally occurring maternal effects.

Therefore to study the impact of maternal environment on embryonic environment it may be more useful to manipulate the maternal environment, therefore accounting for the level of transfer naturally occurring and potential interactions with other associated yolk factors, which may elicit stronger effects on offspring phenotype.

### 2.5.1 Maternal dietary programming in avians

Multiple factors influence the egg and its composition, classified into *intrinsic* factors such as season, age, and hen strain and management factors such as health, housing conditions, and quantity and quality of feed. These factors significantly influence deposition of nutrients, hormones, and other compounds, resulting in a more or less favourable *in ovo* environment for embryonic development (Dixon *et al.* 2015). Of all maternal environmental effects studied to date, manipulation of the maternal diet has the greatest potential to elicit an epigenetic response in offspring. Breeder hen nutrition significantly alters offspring vitality and posthatch growth (Peebles *et al.* 2002; Kidd 2003). However, studies conducted in poultry have generally been designed to test effects of supplementing a basal diet on offspring hatchability and immunological effects (Dixon *et al.* 2015). Nutritional modulation of the hen diet and *in ovo* environment has been identified as a mechanism to improve chick immunity and disease resistance (Kidd 2004). Studies pertaining to maternal diet restriction and mismatched dietary environments are limited in poultry. Consequently, studies pertaining to *in ovo* manipulation, i.e. albumen removal and supplementation, have been included to highlight effects of developmental malnutrition and identify potential aspects of embryonic development which may be influenced via maternal dietary manipulation in the future.

Maternal diet plays a significant role in the antioxidant and immune system during chick development. Increasing basal amounts of vitamin and trace mineral mix fed to broiler breeder hens stimulated the immune system in offspring, reflected in increased leukocyte number in one day old chicks (Rebel *et al.* 2004). Additionally, when offspring were then infected with malabsorption syndrome, intestinal lesions recovery occurred and number of infiltrating pronuclear

leukocytes were higher. By 14 days of age cystic crypts and villus atrophy were less severe and mostly disappeared by day 21 (Rebel *et al.* 2004). Supplemental zinc-methionine in the hen diet also increase cellular immune response in offspring (Kidd *et al.* 1992). However high concentrations of zinc (150µg/g) in laying hens may induce marginal immunosuppression in chicks (Stahl *et al.* 1989).

*In ovo* injection of Vitamin E into the amnion has been shown to enhance antibody and macrophage response of broiler embryos (Gore and Qureshi 1997) and also recognised for its action in protecting against oxidative damage in tissues (Tappel 1962). Vitamin E, and selenium in some circumstances, supplementation in hen diets increases concentrations in egg yolk, and the liver and brain of offspring (Surai 2000; Tsai *et al.* 2008). Maternal vitamin E supplementation improves antibody response in hens when faced with an immune challenge, increasing antibody titre transfer from hen to the chick via the yolk sac (Buxton 1952). This antibody response by the reticuloendothelial system of the hen to vitamin E is suggested to be non-linear. Jackson *et al.* (1978) found passively transferred antibody levels were significantly increased in plasma of two and seven day old chicks when hens were fed 150 and 450 ppm vitamin E prior to immunisation, however when hens were fed 90, 300 and 900ppm chicks experienced no change in antibody titres relative to controls. Reproductive performance is also improved with vitamin E, increasing fertility and hatchability in Taiwan native breeder hens (Tsai *et al.* 2008). Selenium (Se) is suggested to have a sparing effect on vitamin E metabolism (Surai 2000) and preferentially deposited into the yolk compared to albumen (Richards 1997; Pappas *et al.* 2005). Supplementation of hen diets with organo-Se also been associated with enhanced docosahexaenoic acid concentration in the brain of offspring, potentially improving brain function (Pappas *et al.* 2006).

Carotenoid supplementation of the hen diet has also gained considerable interest due to beneficial effects on prevention of infections (Chew and Park 2004) and enhanced antioxidant capacity (Stahl and Sies 2003). Supplementation in broiler breeder hen diets increases carotenoid concentration in yolk (Surai *et al.* 2003; Karadas *et al.* 2005a), yolk sac membrane, liver and plasma

carotenoid concentration in chicks (Surai and Speake 1998; Karadas *et al.* 2005a). Increased maternal carotenoid concentration improves hatching performance (Cucco *et al.* 2007), decreases susceptibility to lipid peroxidation (Surai *et al.* 2003) and improves immune response (Haq *et al.* 1996; Koutsos *et al.* 2006). Approximately three weeks supplementation is required for yolk carotenoid concentration to reach new steady state (Surai and Speake 1998; Karadas *et al.* 2005a). Karadas *et al.* (2005b) found increased carotenoid supplementation in the maternal diet increased concentrations of vitamin A, specifically retinyl oleate and retinyl palmitate, in egg yolk and liver of day old quail chicks. Prenatal exposure effects predominate during the first week post hatch, allowing the embryo to incorporate carotenoids into its liver with much greater avidity compared to chicks fed post-hatch only (Karadas *et al.* 2005a). This implies *in ovo* carotenoid exposure is important for subsequent absorption, metabolism and/or tissue deposition of diet-derived carotenoids. Likewise, chicks hatched from hens fed carotenoid depleted diets (and subsequently carotenoid depleted yolk), failed to achieve the same degree of carotenoid deposition as chicks hatched from eggs containing carotenoids, suggesting a PAR in the hen-chick diet may exist (Koutsos *et al.* 2003). The effect of carotenoids is particularly relevant when considering dietary grain source. Corn contains significantly higher levels of carotenoids than wheat. While both grains are used in poultry diets, wheat is predominately used in Europe, Australia, and Canada, and corn the primary source in United States and Brazil. Maize-based broiler breeder diet exhibit increased antioxidant potential in yolk in comparison to a wheat-based maternal diet and was associated with increased total carotenoids and zeaxanthin concentration in embryonic tissues of offspring (Surai and Sparks 2001). A study by Leandro *et al.* (2011) found a diet by housing density interact effected immune response in broiler breeder hens. Hens fed corn and housed at higher density expressed lower plasma MatAb levels than those fed wheat and/or housed in single cages. Likewise, offspring of the corn-fed/high-density housed hens exhibited smaller spleen white pulp compared to hens housed at lower density, while no difference was detected for wheat-fed hens. This suggests maternal diet composition in combination with other maternal environmental conditions can alter offspring immune response and/or performance.

Vitamins and minerals are involved in numerous cofactor functions and enzymes critical to metabolic functions. Supplementing a commercial broiler breeder hen diet with a vitamin and mineral premix altered expression of 11 genes related to intestinal turnover, proliferation and development, metabolism and absorption at both three and 14 days of age (Rebel *et al.* 2006). Vitamin $D_3$ has been of particular interest initiating morphological changes in bone development and gut integrity in offspring. Vitamin $D_3$ content in egg yolk increases via the hen diet (Mattila *et al.* 1999) and improves leg health by increasing tibia ash content, decreasing incidence of Ca rickets and tibial dyschondroplasia (Atencio *et al.* 2005a; Atencio *et al.* 2005b; Driver *et al.* 2006). Maternal vitamin $D_3$ also effects gut morphology in offspring by decreasing weight and length of the small intestine, increasing villi height and increasing crypt depth during late incubation and early posthatch phase (Ding *et al.* 2011).

Essential fatty acids (FA) contribute to tissue structure and cell membrane synthesis. Long-chain polyunsaturated FA (LCPUFA) are highly concentrated in the brain, retina and other neural tissues. The primary LCPUFA's found in the brain are arachidonic acid (AA) and docosahexaenoic acid (DHA). Biosynthesis from precursors, primarily linoleic acid and α-linolenic acid and/or directly from dietary source influence AA and DHA concentrations. Therefore imbalances in FA and/or specific FA deficiencies can affect neonatal development (Wainwright 2007). In birds the source of PUFA for embryo development is yolk. The level of n-3 yolk content can be increased via maternal dietary alteration, and has been associated with significant increases in levels detected in the brain of embryos and chicks, predominately DHA (Cherian and Sim 1991). Chicks from hens fed diets high in PUFA contained higher concentrations of DHA in brain and liver tissue compared to chicks from hens fed low PUFA diets from hatch to 14 days of age (Pappas *et al.* 2006). Maternal diet with altered Omega-6/Omega-3 ratio alters offspring brain DHA content which can last for up to 6 weeks posthatch (Ajuyah *et al.* 2003). High concentrations of n-3 FA in hen diets have been linked to altered behaviour in partridges (Fronte *et al.* 2008). Additionally, chicks hatched from laying hens fed diets with low ratio linoleic to α-linolenic acid compared to low ratio is affects passive immunity of hatching chicks but not humoral response (Wang *et al.* 2004).

*In ovo* nutrient supplementation, specifically CHO and β-hyroxy-β-methylbutyrate (HMB), alters embryonic development resulting in increased hatch weight, liver glycogen (2-to 5-fold), breast muscle size (Uni *et al.* 2005), yolk sac size (Uni and Ferket 2004) and promotion of myoblast proliferation (Kornasio *et al.* 2011). Additionally, when time of first feed is delayed (36 h) chicks subjected to *in ovo* supplementation maintained higher glycogen reserves, body weight, pectoral muscle weight, and body weight gain post-hatch. Increased glycogen reserves observed post hatch in *in ovo* supplemented chicks presumably reflects improved enteric development (increased villi surface area and enhanced mucin mRNA expression) and digestive capacity of small intestine (Tako *et al.* 2004; Smirnov *et al.* 2006). *In ovo* nutrient enrichment with mineral, vitamins and CHO in amniotic fluid also influences bone structure, composition and mechanical properties pre- and post-hatch (Yair *et al.* 2013). Mineral enrichment results in higher consumption of Fe, Zn, and Mn between ED20 and hatch (Yair and Uni 2011). Such apparent effects of *in ovo* supplementation suggest the embryonic environment is suboptimal for optimal embryonic development in fast growing and/or high performance avian species.

Suboptimal maternal nutrition and/or embryo malnutrition significantly alters offspring phenotype. The high protein concentration measured in egg albumen has attributed to a variety of malnutrition studies focussed on prenatal protein and amino acids in avian embryonic development (Muramatsu *et al.* 1990; Finkler *et al.* 1998; Everaert *et al.* 2013; Willems *et al.* 2013). Albumen removal during embryonic development induces a sparing effects on vital organs, particularly the brain (Miller *et al.* 2002; Ruijtenbeek *et al.* 2003; Rodricks *et al.* 2004), accompanied by raised corticosterone concentration (Rodricks *et al.* 2004). Comparatively, carcass, gut and yolk sac weight decrease from ED14 through to hatch and may persist up to seven days of age (Deeming 1989; Hill 1993; Finkler *et al.* 1998; Miller *et al.* 2002; Everaert *et al.* 2013). Willems *et al.* (2013) found no difference in BW when replacing removed albumen with saline, suggesting the difference observed by others can be attributed to changes water content with albumen removal. Long-term effects of albumen removal appear sex-dependant as changes in growth rate of females were much more

definitive than in males (Hill 1993; Everaert *et al.* 2013). Additionally, disproportionate growth retardation has been associated with albumen removal (Miller *et al.* 2002; Ruijtenbeek *et al.* 2003).

Differences in body weight may be attributed to changes in muscle mass and potential muscle atrophy. Albumen restriction prior to incubation was associated in regulating whole-body protein synthesis (Muramatsu *et al.* 1990). During later development albumen deprived embryos display higher plasma uric acid levels and decreased circulating amino acid concentration at hatch indicating altered protein metabolism (Everaert *et al.* 2013). Increased mRNA expression of muscle ring finger-1 (MuRF1), gene related to proteolysis, and an upregulation of atrogin-1 (males only) was also observed at hatch suggesting increased muscle proteolysis (Everaert *et al.* 2013).

Partial albumen removal during embryonic development is associated with poor reproductive performance in layer-type chickens. During the laying phase (18 to 55 wk of age) hens observed decreased egg weight, lay rate, egg weight and quality, but an increase in the proportion of yolk weight (Willems *et al.* 2013). Restricting Dwarf broiler breeders to 90% ad libitum also decreased egg and chick weight compared to *ad libitum* birds (Triyuwanta and Nys 1992). In comparison, *ad libitum* hens observed reduced fertility rate compared to hens restricted (Triyuwanta and Nys 1990). Reproductive performance, particularly in broiler breeders, is highly regulated by nutritional regimes in commercial systems (Mench 2002; Richards *et al.* 2010). Selection for rapid lean muscle gain in broiler birds, has coincidently resulted in hyperphagia and obesity when fed *ad libitum*. Consequently, severe food restriction (or complete withdrawal) is used in the broiler breeder hen to optimise reproductive performance, hatchability and chick juvenile growth (Tona *et al.* 2002; Mohiti-Asli *et al.* 2012). Unsurprisingly, severe food deprivation in broiler breeder hens results in dramatic metabolic and endocrine changes, including depleted liver glucose (Ralph *et al.* 2015) and plasma triglyceride levels (Kubíková *et al.* 2001; Rajman *et al.* 2006; de Beer *et al.* 2008). Feed restriction has been associated with increased plasma concentrations of growth hormone (GH), IGF-1 (Bruggeman *et al.* 1997; de Beer *et al.* 2008), corticosterone (CORT) (Kitaysky *et al.* 1999; Kubíková *et al.* 2001; De Jong *et al.* 2002; Rajman *et al.* 2006; de Beer *et al.* 2008; Ralph *et al.* 2015), thyroxine

($T_4$) and decreased triiodothyronine ($T_3$) (Bruggeman *et al.* 1997; Kubíková *et al.* 2001; Rajman *et al.* 2006). Maternal CORT concentration in particular is recognised as transferable to the yolk (Cook *et al.* 2009; Okuliarová *et al.* 2010; Almasi *et al.* 2012; Babacanoğlu *et al.* 2013), decreasing egg weight and altering yolk testosterone and progesterone concentrations (Henriksen *et al.* 2011a). Elevated maternal and/or yolk CORT has been associated with smaller offspring (Hayward and Wingfield 2004; Saino *et al.* 2005; Henriksen *et al.* 2013), slower plumage development in offspring (Saino *et al.* 2005), higher activity of the hypothalamo-adrenal axis in response to capture and restraint (Hayward and Wingfield 2004), less competitive and fearfulness, lower immunocompetence and elevated plasma testosterone (Henriksen *et al.* 2013). Laying hens subjected to unpredictable feed restriction schedule resulted in offspring presenting longer tonic immobility durations (indicator of fear) than offspring from *ad libitum* fed hens. No change in yolk CORT concentration was found suggesting other steroid hormones may mediate effects of pre-hatch stress in offspring (Janczak *et al.* 2007). Rao *et al.* (2009) found low-protein (10%) maternal diet in Langshan chickens decreased yolk leptin concentration but caused no change in yolk CORT, $T_3$ or $T_4$ concentrations. Changes in offspring development included decreased hatch weight and elevated $T_3$ plasma concentrations, which is in accordance with the faster post-hatch growth rate observed. Similarly to mammalian and rodent studies, changes gene regulation during embryonic development were also observed including; down-regulation of 20-HSD in the yolk sac membrane, upregulation of hypothalamic 20-HSD mRNA expression, GR, thyrotropin-releasing hormone (TRH) and leptin receptor (LepR) mRNA, and increased IGF 1 and IGF 1 receptor mRNA in pectoralis muscle. This study clearly demonstrates simple maternal diet changes can induce programming effects on offspring in chickens.

## 2.6. Summary

A range of maternal and early life environmental conditions influences development, physiology, and behaviour of offspring. More recently the mismatched maternal-offspring dietary environment has been identified as a key factor in influencing predisposition and expression of metabolic and endocrine dysfunction, a phenomena known as PAR. Changes in maternally-derived hormones, nutrients and epigenetic regulatory factors in yolk have all been associated with changes in offspring phenotype. The extent to which maternal diet dictates and programs offspring performance in poultry is highly underestimated. To date, altering the breeder diet to influence embryonic and posthatch development have primarily focused on simple energy and protein changes (Spratt and Leeson 1987), or combinations of nutrients (Pappas *et al.* 2006) in an attempt to improve antioxidant capacity and immune response in offspring. Little consideration has been given to the potential PAR effects of subsequent offspring diet on adult phenotype. Given the positive effects of matched diets across generations in other species and the considerable metabolic diseases observed in poultry, the impact of PAR phenomenon is worth consideration in poultry production.

Due to the numerous environmental insults imposed on the chick at hatch, providing a stronger chick is valuable, hence a greater emphasis has been placed on pre-hatch mechanisms and the application of *in ovo* supplementation to optimise embryonic growth and development. Studies related to changes in physical development, endocrine regulation and homeostatic control of the offspring have focussed on direct manipulation of the *in ovo* environment rather than maternal derived effects. As the egg is produced solely by the hen, the impact of the hen's environment during egg production is critical to egg composition. Therefore the impact of hen's ability to control deposition of regulatory factors into the egg may significantly impact embryonic development.

Broiler breeder management is heavily focussed on cost effective dietary management and high reproductive performance. While diets are formulated to specific nutrient specifications, specific grain sources and dietary management techniques currently employed may have

underlying transgenerational programming effects resulting in positive or negative phenotypes in offspring. Manipulation of the breeder hen diet, associated epigenetic programming yolk factors and contributing effects of offspring diet may prove provide an opportunity to improve productivity with improved animal welfare benefits for hen and offspring.

# 3. The predominating grain source in the diet of breeder hens induces predictive adaptive programming responses in their offspring during early life.

## 3.1. Abstract

Modulation of the maternal diet, in particular protein and energy level, has been demonstrated to significantly alter the developmental trajectory of offspring. Australian broiler breeder hens are generally sourced from either the UK or US. These regions differ in their predominating grain source, either being corn or wheat. Consequently, breeder stock are fed and genetically selected on either corn or wheat-based diets. In Australia, wheat inclusion in broiler diets predominates over corn, and while attempts are made to formulate to all nutrients, the impact simply of grain type as a 'programming' signal in breeder hens sourced from the US and their progeny is not recognised. In this study the consequences of a mismatched dietary environment on offspring performance and the potential epigenetic role of the yolk as a regulatory interface were investigated. All diets were closely-matched nutritionally and contained at least 50% of either corn or wheat. Using a two-by-two factorial design, broiler breeder hens (HenDiet) and resulting offspring were fed (ProgDiet) either a corn-based (C) - or wheat-based (W) diet. This produced two matched (CC, WW) and two mismatched (WC, CW) maternal-offspring dietary environment combinations. HenDiet influenced offspring hatch weight with chicks from C-fed hens significantly heavier at day of hatch (DOH) than chicks of W-fed hens (45.5g vs. 43.6g respectively, $P < 0.01$). Offspring body weight (BW) was significantly influenced by the interaction of HenDiet X ProgDiet X Day ($P < 0.01$). By D7, offspring fed the same diets as their mothers (CC and WW) did not differ in BW and were heavier than those from mismatched maternal-offspring diets (CW and WC). At both D14 to D21 the birds from the CC diet were significantly heavier than those of all other dietary combinations (WW, CW and WC) demonstrating a significant interaction effect of maternal and progeny diet. Somewhat surprisingly, by D28 maternal grain source continued to influence offspring body weight, as offspring from C-fed hens had increased body weights compared to offspring of W-fed hens. Similarly, the percentage of offspring reaching a target BW value, deemed

the threshold body weight (TH), was significantly higher from C-fed mothers than W-fed mothers ($P < 0.005$). Overall, ADG was significantly increased in CC offspring compared to all other combinations (WW, WC and CW). Mortality rate was not related to dietary treatment. C-fed hens produced significantly heavier eggs than W-fed hens (58.1g vs. 54.6 g, $P < 0.001$) accompanied by significantly heavier albumen content (32.5g vs. 30.5g, $P < 0.001$). Significant differences in the relative expression levels of yolk microRNA of hens on C and W diets was found, indicating yolk as a potential epigenetic mechanism underpinning embryonic reprogramming in chickens. Mir-499, mir-138-2//mir-138-1 and let-7f-2, were all significantly ($P < 0.05$) higher in the yolk of C-fed hens compared to W-fed hens. Mir-205a relative expression was significantly higher in yolk of W-fed hens ($P < 0.0001$). These miRNA have previously been associated with reproductive performance in the hen and embryonic development of the chick and provide preliminary findings of the effect of maternal diet on yolk miRNA expression. The current study is the first to demonstrate a "predictive adaptive response" (PAR) in broiler offspring, particularly during early life, in response to maternal grain source. It also demonstrates the impact of dietary misprogramming on the performance and long-term health of offspring and identifies potential programming mechanisms in the yolk.

## 3.2. Introduction

Globally, the two grains most widely used in poultry production are corn or wheat. In a commercial diet the predominating grain source is generally determined by geographical location. Wheat is commonly used in Europe, Australia and Canada, whereas corn is the predominant energy source in United States and Brazil (Bird 1996). In the Australian broiler industry, maternal genetics are primarily sourced from either the UK, where the predominant grain type is wheat, or from the US wherein corn is the predominant grain source. Consequently, offspring of UK breeder hens in Australia will be subjected to similar wheat-based rations as their mothers, while those sourced from the US will be subjected to a different regime (wheat instead of corn). If there is a significant interaction between mismatched maternal diet and offspring diet, this differential feeding of the breeder hens will have important implications for the performance and health of Australian broiler chickens (Surai and Sparks 2001; Eusebio Balcazar 2010; Leandro *et al.* 2011).

Strong evidence is emerging in humans and other animals that there is indeed such an interaction, whereby the maternal environment 'programs' the developing fetus for the anticipated environment (Bernardo 1996; Wu *et al.* 2004). If the environment then differs from the one anticipated, the offspring are effectively 'misprogrammed' with potentially detrimental outcomes for production and health. The concept of fetal programming is considered to operate at several levels in developmental physiology. Major programming mechanisms appear to be resetting of homeostatic 'set points' in the neural and endocrine systems, development of the gastrointestinal and metabolic systems, and the innate and acquired immune systems (Groothuis and Schwabl 2008; Ho and Burggren 2010; Berghof *et al.* 2013). The extent to which such changes can impact on offspring performance is illustrated well by the experiment of Ho *et al.* (2011) in which the *in-ovo* environment strongly influenced the phenotype of the offspring. More recently, the PAR hypothesis proposes the degree of mismatched interaction between pre-and-postnatal environments is of greatest impact to offspring phenotype. It implies postnatal environmental changes may ameliorate or exaggerate the morphological and functional changes programmed *in utero* (refer to Section

2.4.3). Therefore, the postnatal environment, particularly during periods of developmental plasticity, is also an important factor to be considered when characterising adult phenotype.

In mammalian species, factors within the maternal diet are now being acknowledged to modulate and alter the developmental trajectory of the foetus during development. Changes in the cellular epigenome during development have been attributed to the impact of methyl group donors (e.g. folate, choline) and bioactive compounds (e.g. polyphenols) on gene expression, and are therefore of significance when considering the effects of the maternal diet on offspring performance. Limited knowledge exists however of the particular effects each type of modulating factor and/or combination on fetal development and which appear to permanently alter the adult phenotype (Thornburg *et al.* 2010; Masuyama and Hiramatsu 2012; Chango and Pogribny 2015). The effects of maternal nutrition, in addition to effects of specific nutritional factors, glucocorticoids, and endocrine-disrupting chemicals on epigenetic mechanisms and consequences for adulthood disease are becoming a major focus in human nutrition (Canani *et al.* 2011; Hogg *et al.* 2012; Tammen *et al.* 2013; Jiang *et al.* 2014). In avian species, the egg plays a vital role in developmental programming as it provides the interface between the mother and offspring and components of the egg can significantly differ in composition depending on the maternal diet. Compounds deposited within the egg such as hormones and nutrients have previously been shown to alter developmental trajectory in birds (Surai 2000; Uni *et al.* 2005; Groothuis and Schwabl 2008; Wade *et al.* 2016). Recently, Berghof *et al.* (2013) suggested transgenerational epigenetic mechanisms may influence immune system development and increase the efficiency of intestinal absorption. While the effects of maternally-derived hormones and nutrients on embryonic development have been studied substantially, the detection of epigenetic mechanisms through the influence of microRNAs in the egg is relatively recent (Wade *et al.* 2016). MicroRNAs have previously been shown to be involved in chick embryo development and regulation of growth (Darnell *et al.* 2006; Li *et al.* 2011; Ouyang *et al.* 2015; Bao *et al.* 2016; Garcia-Riart *et al.* 2017). It is likely the egg, and more specifically the yolk, may be a potent vehicle for epigenetic programming of the developing chick embryo dependant on the maternal environment (Wade *et al.* 2016). However,

isolation of egg microRNA and demonstration of their impact on embryonic development is lacking. The extent to which the maternal-offspring dietary interaction and potential epigenetic mechanisms via the yolk environment on embryonic development is unknown in avian species. To further investigate the effect of the maternal diet on developmental programming in chickens, and the potential PAR in offspring, we conducted an experiment in which chickens were raised on diets containing the same or different predominant grain type as their mothers. It was hypothesised the mismatch of predominating grain source between mothers and offspring would result in decreased performances in offspring compared to those fed the same grain-based diet as their mothers. In addition, predominating grain source in the hen diet will alter epigenetic compounds deposited into the yolk. Maternal programming effects were determined via changes in growth rate and associated with potential epigenetic mechanisms of the yolk environment characterised by changes in microRNA expression.

## 3.3. Materials and methods

All animal use was approved by the animal ethics committees of The University of Adelaide (S-2013-105A) and the Department of Primary Industries and Regions, South Australia (PIRSA) (#09/13). In total 200 24-week-old birds from a purebred line of broiler breeders (Cobb 500 USA) were assigned one of two diets; corn-based (C) or wheat-based (W) dietary source obtained from commercial sources. Breeder diets were formulated as predominately corn or wheat-based (Table 3.1) and balanced in all major nutrients (Table 3.2). Breeder hens were group housed (10 birds/pen) in single sire pedigree metal pens (2500 x 1900mm) managed at a commercial breeder facility resulting in 10 pen replicates per hen diet. Birds were fed according to the recommendations of the breeder company (Cobb-Vantress) during the experimental period.

Eggs were collected from the breeder hen flock at two time points (35 and 44 weeks of age) for incubation. Eggs were collected daily and stored for a maximum of seven days. Fertile eggs were incubated at the breeder facility using standard conditions of 37.7°C/84%RH followed by 36.9°C/89.5%RH. At hatch, a total of 240 day-old viable male offspring were individually weighed, tagged and placed (10 chicks/pen) into group-rearing pens (1800 x 1200 mm) at the South Australian Research and Development Institute (SARDI) research facilities, Roseworthy Campus, SA. Offspring from each of the C-fed (*n* = 120) and W-fed hens (*n* = 120) were assigned to either a C-based or W-based diet resulting in a total of four treatments (6 replicates/treatment); Corn Breeder/Corn Offspring (CC) *n* = 60, Corn Breeder/Wheat Offspring (CW) *n* = 60, Wheat Breeder/Wheat Offspring (WW) *n* = 60 and Wheat Breeder/Corn Offspring (WC) *n* = 60. Offspring diets included a Starter (1 to 21 day) and Finisher (22 to 56 d) phase (Table 3.1 and Table 3.2). Standard husbandry procedures were followed with chicks placed in shed at 25°C with heat lamps and given a light cycle of 23 hours light for the first 24 hours. From the second day the light cycle was changed to 16 hours light and the shed temperature adjusted according to bird's needs. Feed and water were provided *ad libitum*. Birds were individually weighed weekly (hatch, D7, D14, D21 and D28) until slaughter and average daily gain (ADG) calculated by dividing weekly weight gain by seven days for each week from hatch to D28. Mortality was recorded daily.

**Table 3.1 Ingredient composition (% of ration) of experimental diets for breeder hens (Early Breeder) and offspring (Starter and Finisher).**

| | Early Breeder | | Starter | | Finisher | |
|---|---|---|---|---|---|---|
| **Ingredient, %** | **Corn** | **Wheat** | **Corn** | **Wheat** | **Corn** | **Wheat** |
| Corn | 50.0 | - | 50.33 | - | 50.36 | - |
| Wheat | - | 59.39 | - | 62.71 | - | 66.74 |
| Barley | 0 | 19.97 | 0 | 0 | 0 | 0 |
| Canola | 8.0 | 7.0 | 11.0 | 7.0 | 10.0 | 5.0 |
| Peas | 27.3 | 0 | 5.0 | 0.0 | 10.0 | 5.0 |
| Soybean meal, 47% CP | 4.0 | 0 | 22.61 | 21.00 | 20.0 | 15.0 |
| Tallow | 0 | 0 | 2.33 | 1.50 | 4.35 | 2.80 |
| Meatmeal, 60% CP | 0 | 5.05 | 6.77 | 5.50 | 3.33 | 3.0 |
| L-Lys | 0.0 | 0.16 | 0.06 | 0.25 | 0.06 | 0.23 |
| Met hydroxyl analog, 80% | 0.22 | 0.16 | 0.34 | 0.33 | 0.26 | 0.28 |
| L- Thr | 0.0 | 0.0 | 0.00 | 0.10 | 0.01 | 0.11 |
| Calcium Carbonate | 7.92 | 6.64 | 0.9 | 0.9 | 0.7 | 0.87 |
| Dicalcium Phosphate | 1.45 | 0.39 | 0 | 0 | 0 | 0 |
| Salt | 0.27 | 0.14 | 0.22 | 0.17 | 0.06 | 0.08 |
| Choline chloride LQ75% | 0.06 | 0.3 | 0.05 | 0.05 | 0.03 | 0.05 |
| Sodium Bicarbonate | 0.06 | 0.18 | 0.0 | 0.1 | 0.2 | 0.2 |
| Potassium Carbonate | 0.05 | 0.10 | 0.0 | 0.0 | 0.0 | 0.0 |
| Salkil | 0.4 | 0.4 | 0 | 0 | 0.4 | 0.4 |
| Vitamin and Mineral Premix[1] | 0.27 | 0.27 | 0.35 | 0.35 | 0.2 | 0.2 |
| Enzyme complex[2] | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 |

[1]Vitamin and Mineral Premix provided per kg of feed: 10,000 IU vitamin A (as *trans*-retinyl acetate); 5,000 IU Vitamin $D_3$ (as cholecalciferol); 3.5 mg Vitamin E (as α-tocopherol acetate); 2 mg Vitamin $K_3$; 3 mg Vitamin $B_1$; 8 mg Vitamin $B_2$; 4 mg Vitamin $B_6$; 20 µg Vitamin $B_{12}$; 40 mg Niacin; 12 mg Pantothenic Acid; 20 µg Biotin; 100 mg Folic Acid; 100 mg Mn; 90 mg Zn; 30 mg Fe; 10 mg Cu; 1 mg I ; 0.4 mg Co; 0.3 mg Se; 125 mg ethoxyquin. [2]Contained 150 mg/t Ronozyme NP and 250mg/t Ronozyme WX·CT (DSM Nutritional Products Ltd, Basel, Switzerland).

**Table 3.2 Calculated metabolisable energy and amino acid content of experimental diets for breeder hens (Early Breeder) and offspring (Starter and Finisher).**

| Calculated analysis | Early Breeder | | Starter | | Finisher | |
|---|---|---|---|---|---|---|
| | Corn | Wheat | Corn | Wheat | Corn | Wheat |
| ME (MJ,kg) | 11.6 | 11.6 | 13.0 | 13.0 | 13.8 | 13.8 |
| CP, g $kg^{-1}$ | 150.0 | 150.0 | 237.0 | 235.0 | 200 | 195.0 |
| Fibre, g $kg^{-1}$ | 40.0 | 46.0 | 41.0 | 40.0 | 40.0 | 36.0 |
| Fat, g $kg^{-1}$ | 40.0 | 36.0 | 70.0 | 43.0 | 100 | 63.0 |
| Lysine, g $kg^{-1}$ | 8.5 | 7.6 | 14.1 | 13.9 | 11.3 | 11.2 |
| Methionine (Met), g $kg^{-1}$ | 3.7 | 3.5 | 6.2 | 6.1 | 5.1 | 5.0 |
| Threonine, g $kg^{-1}$ | 5.6 | 5.0 | 9.3 | 9.3 | 7.7 | 7.7 |
| Tryptophan, g $kg^{-1}$ | 1.6 | 1.6 | 2.7 | 2.7 | 2.2 | 2.2 |
| Isoleucine, g $kg^{-1}$ | 6.1 | 4.9 | 9.0 | 9.0 | 8.3 | 7.6 |
| Leucine, g $kg^{-1}$ | 12.2 | 9.9 | 20.0 | 16.6 | 16.0 | 13.1 |
| Arginine, g $kg^{-1}$ | 10.1 | 8.1 | 15.0 | 14.2 | 13.3 | 11.7 |
| Met + Cys, g $kg^{-1}$ | 6.2 | 6.2 | 9.9 | 9.9 | 8.3 | 8.3 |
| Linoleic Acid, g $kg^{-1}$ | 15.5 | 12.0 | 18.9 | 10.6 | 26.5 | 14.9 |
| Potassium, g $kg^{-1}$ | 6.1 | 6.1 | 8.1 | 8.0 | 7.6 | 6.9 |
| Sodium, g $kg^{-1}$ | 1.6 | 1.6 | 1.6 | 1.6 | 1.3 | 1.3 |
| Calcium, g $kg^{-1}$ | 35.0 | 30.0 | 7.9 | 7.9 | 6.9 | 6.9 |
| Av. Phosphorus, g $kg^{-1}$ | 3.7 | 3.7 | 3.1 | 3.1 | 2.6 | 2.4 |

### 3.3.1 Egg Composition yolk and miRNA expression

Fertile eggs (Appendix; Figure 1) were collected from hens at 35 weeks of age (*n* = 400) and total egg, albumen and yolk weight recorded. Yolk content was collected and stored at -80° for mRNA expression. In brief, the egg shell was removed and yolk separated from albumen using a yolk separator. Albumen weight was recorded. Yolk was placed on gauze to remove residual albumen and then suspended in fresh gauze and the yolk membrane pierced. Yolk was gently massaged from the yolk sac to remove yolk contents and the weight of yolk recorded. Yolk was homogenised for 30 seconds and a 15mL sample of yolk was collected and then stored at -80°C.

Yolk miRNA was extracted and quantified as previously described by Wade *et al.* (2016). In brief, frozen yolk was thawed on ice and 1 mL of yolk was then diluted in lysis solution BF at a 1:1 ratio, vortexed thoroughly and incubated at room temperature for 15 min. 400 µL aliquots of the yolk lysis solution was dispensed into five 1.5 mL microcentrifuge tubes. To each of these aliquots 600 µL of Cleanascite™ (3:1 ratio of Cleanascite™ to original sample volume) was added and vortexed until the sample was homogenous. Solutions were then incubated at 4 °C for 1 h. After incubation samples were centrifuged at 13,000*g* for 5 min. Clear supernatant was transferred to a new 1.5 mL microcentrifuge tube and 40 µL of Protein Precipitation Solution BF was added. The solution was vortexed and incubated at room temperature for 1 min and then centrifugation for 3 min at 11,000*g*. After this the extraction continues as per the Exiqon© miRCURY™ biofluid RNA isolation kit standard instructions from step 4 onwards. The miRNA was quantified using the Qubit® MicroRNA Assay Kit on a Qubit® 3.0 Fluorometer using software APP v1.02 + MCU v0.21. Primers and probes were purchased from Applied Biosystems™ from their TaqMan® MicroRNA assay range. 10 ng of miRNA was used in a 15 µL complimentary DNA (cDNA) synthesis reaction using the Taqman® microRNA reverse transcription kit (Applied Biosystems™) as per manufacturer's instructions in conjunction with the appropriate primers, no more than six miRNAs were amplified in a given cDNA reaction. The RT-qPCR reactions were formulated as per manufacturer's instructions using 1 µL of the above cDNA per 15 µL reaction and using Taqman® universal master

mix II, no UNG (Applied Biosystems™). RT-qPCR was undertaken on a Bio-Rad C1000 thermal cycler with CFX96 real-time system with the following protocol; 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min with fluorescence being read at the end of the 60 °C step. Acquisition and analysis of data was undertaken using the software BioRad CFX manager version 3.0.1215.0601. Ct values were recorded at a relative fluorescent unit value of 20,000. Individual samples with total counts below 12,000 were removed from the analysis and the number of reads mapped to each miRNA sequence were grouped based on the sequence. Expression levels were then normalised relative to total counts for each individual yolk samples and given an arbitrary expression value of 1000.

### 3.3.2 Statistical analysis

Total egg and egg component weights were analysed using a one-way analysis of variance (ANOVA) (SPSS 24.0, SPSS Inc., Chicago, IL) with HenDiet as a fixed effect. Statistical significance of yolk miRNA expression was calculated using one-way ANOVA with HenDiet as a fixed effect. Offspring BW and ADG data were analysed using a linear mixed model with type 3 sums of squares. Data were checked for normality by examining the distribution of residual plots. Individual chick was used as the unit with day of measure (for BW) or period (for ADG) fitted as the repeated measure. Period was defined as the 7 day span over which the birds were measured. Day or Period, HenDiet and ProgDiet were fitted as fixed effects. Although egg collection (replicate) occurred at two different ages, other factors may also have varied between these time points, therefore replicate was fitted as a random effect. Pairwise comparisons were used to determine significant differences between treatments (CC, WW, WC and CW). Data is expressed as least squares means with the standard error of the mean (SEM) and a $P$ value < 0.05 was deemed significant.

Considerations were also made for the analysis of offspring performance as chicks were exposed to an unexpected immune challenge from hatch. This was the unfortunate result of the disease free status of the breeders, contributing to the lower than expected BW on offspring. Therefore, in addition to weekly BW, weekly threshold values (TH) and mortality were analysed. TH

values were established to take into consideration specifications for Cobb500 GGP and male broiler target weights. Dietary management of the offspring was based on commercial broiler specifications and fed *ad-libitum*, however breeders were of GGP stock and therefore offspring unlikely to reach target BW recommended for commercial broilers, therefore a TH value was defined to consider both GGP and commercial broiler specifications. TH were deemed as follows: hatch = 42g, D7 = 180g, D14 = 450g, D21 = 900g and D28 = 1500g. Birds were then scored as; Above TH = 1, and Below TH = 0. Mortality and TH were all analysed using a generalised linear mixed model with binomial distribution and logit-link function with individual chick used as the unit of measure and day as the replicate. Day, HenDiet and ProgDiet were fitted as fixed effects and replicate as random effect. Pairwise comparisons were used to determine statistical significance between dietary combinations. Data are expressed as least squares means with the standard error of the mean (SEM) and a P value $< 0.05$ was deemed significant.

## 3.4. Results

### 3.4.1 Egg composition

HenDiet significantly altered egg weight and composition. Eggs from C-fed hens were heavier than those from W-fed hens (58.1 ± 0.6 g vs 54.6 ± 0.5 g respectively, $P < 0.001$). Albumen weight of C-fed hens was significantly greater than that of W-fed hens (32.5 ± 0.4 g vs. 30.5 ± 0.4 g, $P < 0.001$). No effect of HenDiet on yolk weight was found. Egg albumen and yolk weights were directly related to total egg weight for both W-fed and C-fed diets (Figure 3.1). Regression models for W-fed egg components are as follows: $W_{yolk} = 0.25W_{eW\text{-}fed} + 2.5$; $r^2 = 0.5$ and $W_{albumen} = 0.63M_{eW\text{-}fed} - 4.04$; $r^2 = 0.81$. Regression models for C-fed egg components are as follows: $W_{yolk} = 0.26M_{eC\text{-}fed} + 1.34$; $r^2 = 0.51$; $W_{albumen} = 0.61M_{eC\text{-}fed} - 2.77$; $r^2 = 0.76$.

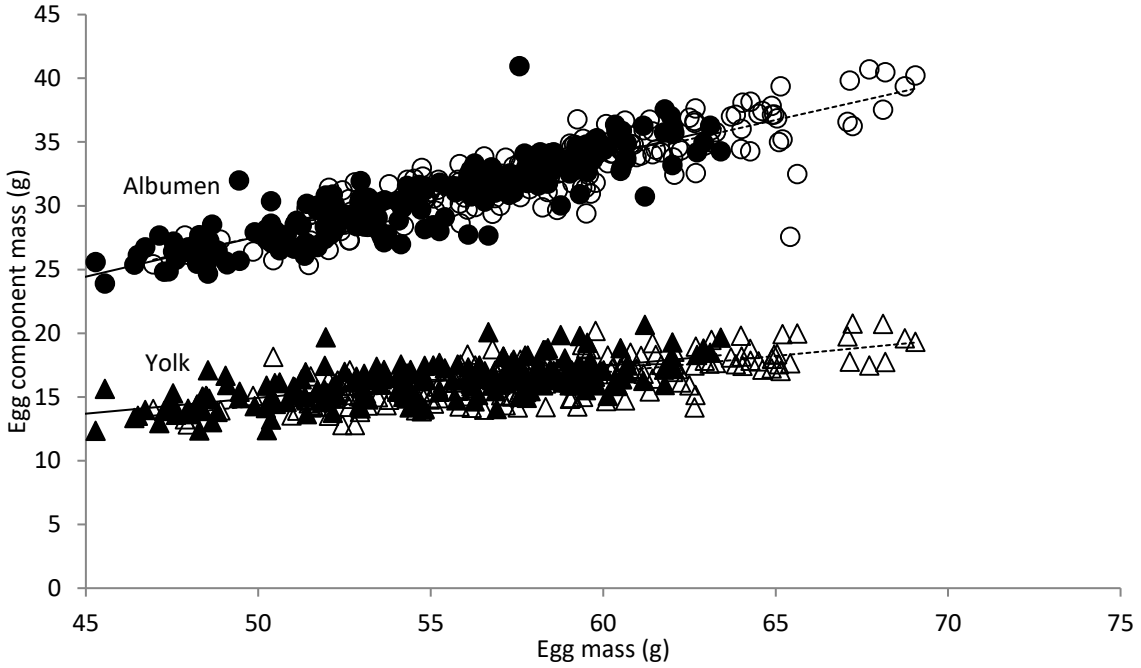


**Figure 3.1 Egg component weight of W-fed and C-fed eggs as a function of egg weight (● = albumen, ▲ = yolk). Filled symbols represent W-fed egg components and open symbols represent C-fed egg components. Broken lines (C-fed) and solid lines (W-fed) through data points indicate best fit regression of the weight of each egg component and egg weight.**

### 3.4.2 Yolk microRNA

The relative expression of yolk mir-205a was highly significantly ($P < 0.0001$) and influenced by HenDiet. The level of expression was higher in yolk of W-fed hens compared to C-fed hens (Figure 3.2). Relative expression levels of miR-499, miR-138-2//miR-138-1 and let-7f were significantly higher in yolk of C-fed hens ($P < 0.05$), as seen in Figure 3.2. There was a trend for increased level of expression in yolk of C-fed hens compared to W-fed hen yolk for let-7c (11.7 ± 2.84 *vs* 3.2 ± 2.2 relative expression value respectively, $P = 0.057$) and miR-4792 (71.3 ± 17.7 *vs* 26.2 ± 13.7 relative expression value respectively, P = 0.091). Comparatively, there was a trend for miR-6497 expression to be higher in yolk of W-fed hens than yolk of C-fed hens (329.0 ± 54.0 *vs* 147.7 ± 69.7 relative expression value respectively, $P = 0.086$). It is worth noting, very few sequences identified as miRNA in the analysis, with most of the sequence data produced classified as 'unknowns', despite being the correct size for miRNA.

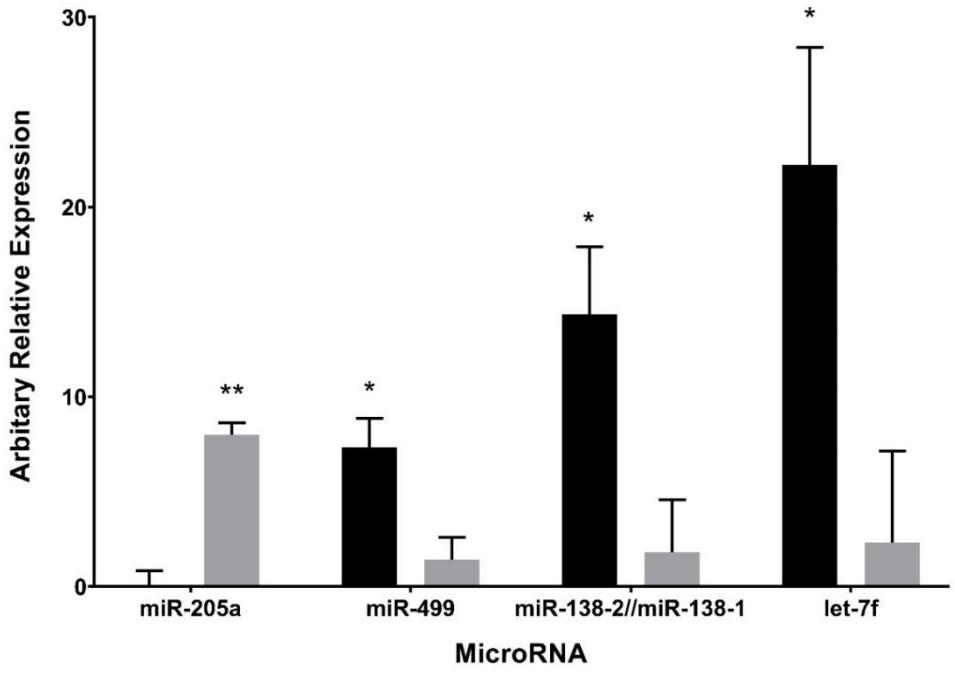


**Figure 3.2 Effect of HenDiet on relative expression levels of yolk miRNAs. HenDiet was either C-fed (black) *n* = 3 corn or W-fed (grey) *n* = 5 wheat, breeder hens. ** denotes significance level of *P* < 0.0001 and * denotes significance level of *P* < 0.05.**

### 3.4.3 Body weight

A significant effect of HenDiet was observed for hatch weight ($P < 0.01$) with chicks from C-fed hens significantly heavier than chicks from W-fed hens (45.5 ± 0.4 g vs. 43.6 ± 0.4 g respectively). Overall, there was a significant main effect of HenDiet ($P < 0.0001$), ProgDiet ($P < 0.05$) and Day ($P < 0.0001$) on offspring BW. A significant ($P < 0.01$) interaction of HenDiet*ProgDiet*Day was also observed as seen in Figure 3.3 (See Appendix; Table 1 for all data). At D7 offspring fed the same diets as their mothers (CC and WW) were not significantly different from each other. The combination of CC was significantly heavier than both mismatched combinations (CW and WC), however WW was not significantly different from either CW or WC combinations. At D14 and D21, offspring of CC maintained significantly higher BWs than all other combinations (WW, WC and CW). At D28 offspring of CC and CW (offspring of C-fed hens) were not significantly different from each other.

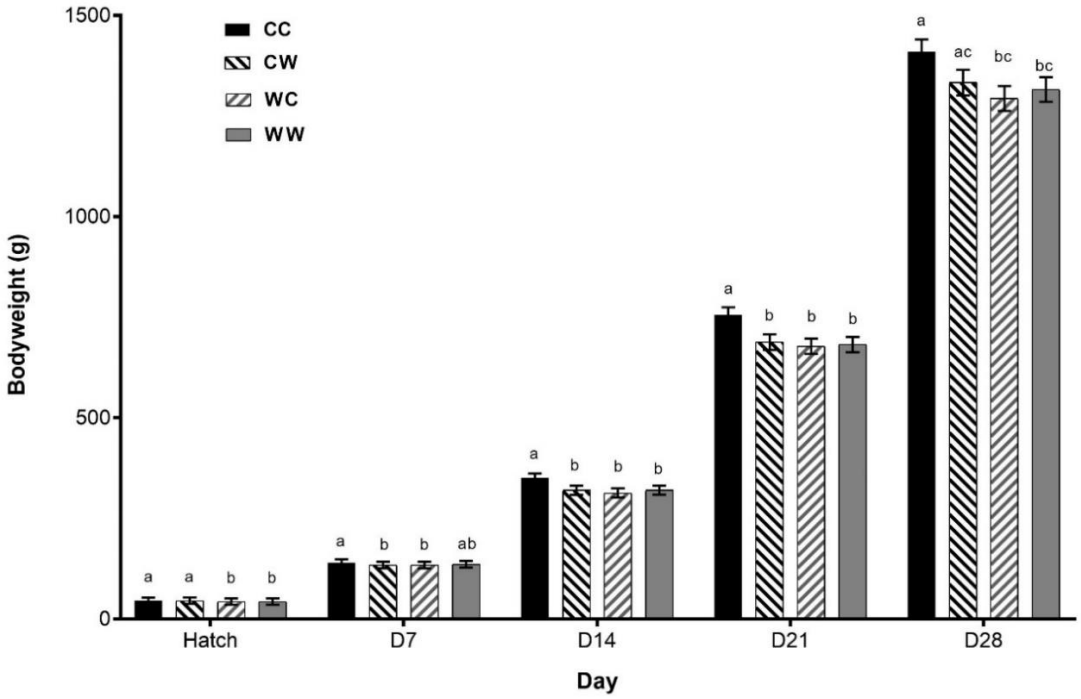


**Figure 3.3 Interaction of HenDiet and ProgDiet on offspring BW (means ± SEM) from hatch to 28 days of age. Letters indicate diet (C = C-fed, W = W-fed). First letter indicates HenDiet and second letter indicates ProgDiet (i.e. CW = C-fed breeder and W-fed offspring). [ab] denotes significance level of *P* < 0.05.**

### 3.4.4 ADG

There was a main effect of HenDiet ($P < 0.05$) and Period ($P < 0.0001$) but not ProgDiet ($P = 0.299$) for offspring ADG. In contrast to BW, there was no significant HenDiet*ProgDiet*Period ($P = 0.231$) effect. A significant HenDiet*ProgDiet ($P < 0.001$) was observed whereby CC maintained a significantly higher ADG than all other combinations (CW, WC and WW) (See Appendix; Table 1 for all data).

### 3.4.5 Threshold

There was a significant main effect of Day ($P < 0.0001$) and HenDiet ($P < 0.005$) on the prevalence of offspring reaching TH value (see Section 3.3.2 Statistical analysis for definition). Overall, offspring of C-fed hens had a significantly higher prevalence of reaching TH than offspring

of W-fed hens (13% vs 7.9% respectively, $P < 0.005$). At hatch 78% of chicks had reached the relative TH value and then decreased significantly ($P < 0.05$) by D7 (2%), D14 (2%) and D21 (4%). No significant difference in TH prevalence was observed between hatch and D28 (19.5%). A trend towards significance was observed for HenDiet*ProgDiet*Day ($P = 0.058$).

### 3.4.6 Mortality

There was no effect of HenDiet ($P = 0.780$) or ProgDiet ($P = 0.423$) on offspring mortality. As expected there was a significant effect of Day ($P < 0.05$). Mortality during the first week of growth was 1.7% and within the expected range for experimental conditions. By D28 mortality was 6.3% and considered slightly higher than expected for normal experimental conditions however as birds were exposed to a mild immune challenged this number was considered reasonable.

## 3.5. Discussion

The results of this study are, to the best of our knowledge, the first to demonstrate developmental programming based on predominating grain source of the maternal diet. We also demonstrated for the first time that a mismatch of grain source, being primarily wheat or corn, between maternal and offspring diets, initiates a strong PAR effect on offspring BW and ADG, particularly during early life.

Maternal dietary grain source significantly influenced egg weight, as C-fed breeders produced heavier eggs than W-fed breeders. This was accompanied by a significant increase in albumen weight of breeders fed a corn-based diet. Research suggests albumen weight increases proportionately more in response to an increase in egg weight and therefore supports the current findings (Romanoff and Romanoff 1949; Nelson *et al.* 2010). The addition of corn and corn by-products in layer diets has previously been associated with increased egg size and Haugh units and is in keeping with the current findings (Deaton and Quisenberry 1964; Kim *et al.* 1976; Lumpkins *et al.* 2005; Skřivan *et al.* 2010). Results pertaining to corn effects on egg composition however are inconsistent with Liebert *et al.* (2005), Lázaro *et al.* (2003) and Safaa *et al.* (2009) all reported no effect on performance or egg quality when corn was substituted by wheat as the main ingredient in poultry diets.

Egg weight is positively correlated with hatch weight, viability and chick 'quality' defined by body conformation (hatch weight and length) and adult body weight (Day 41) (Wyatt *et al.* 1985; Vieira *et al.* 2005; Abiola *et al.* 2008; Nelson *et al.* 2010; Ulmer-Franco *et al.* 2010; Iqbal *et al.* 2016). The significantly heavier weight of offspring at hatch from C-fed hens compared to offspring of W-fed hens was therefore not surprising. Previous studies focussed on the impact of maternal protein restriction on offspring have implied the high protein content of albumen is recognised as the primary protein source for whole-body tissue synthesis during embryonic development (Muramatsu *et al.* 1990; Moran 2007; Willems *et al.* 2014). Therefore the increase in albumen content of C-fed breeder eggs may attribute to greater protein assimilation into tissue during

embryonic development, and therefore embryo weight (Nelson *et al.* 2010). An alternative explanation for the increased hatch weight of C-fed offspring may be the result of developmental programming. The phenomenon of developmental programming, or 'fetal programming' as it is commonly referred to in mammalian research, heavily focusses on the *in utero* environment as a means to permanently alter neonatal phenotype. Ho *et al.* (2011) illustrated a complex synergistic effect of breed-specific 'genotype by yolk' environment exists in avian development, whereby embryo phenotype is the result of breed-specific genetics and the *in ovo* environment. Therefore the interaction of hen genotype, presumably genetically selected on corn-based diets, which are then provided a corn-based diet may provide the optimum *in ovo* environment for embryonic development.

The interaction between the pre-and-postnatal environments, particularly during periods of developmental plasticity, are thought to induce a PAR in offspring dependant on the postnatal environment. Somewhat surprisingly, a strong interactive effect of maternal-offspring dietary environment on offspring growth was found at D7, whereby offspring fed similar grain-based diets as their mothers were comparatively heavier than offspring exposed to mismatched conditions, irrespective of the grain source being corn or wheat. Similarly there was a strong interaction effect of maternal grain source and offspring grain source on overall ADG. This response in offspring BW clearly illustrates the dramatic transgenerational effect grain source can have on offspring performance. As diets were formulated to the same nutrient specifications, the current results strongly suggest developmental programming is operating at a much broader level to specific nutritional cues previously reported (Kidd 2003; van der Waaij *et al.* 2011; Chang *et al.* 2016).

From D14 to D21, birds in the CC treatment were consistently heavier than all other treatments (WW, WC and CW), as seen in Figure 3.3. The significant increase in body weight of CC offspring compared to all other dietary combinations may be due to the fact hens used were directly sourced from the USA, and therefore genetic selection for key performance traits i.e. feed efficiency, likely occurred on corn-based diets. Consequently, progeny may be more efficient on

corn-based diets than wheat-based diets and hence the interaction of C-fed hen and C-fed offspring provides the optimal 'genotype by environment' developmental programming response. Interestingly, at D28, offspring of C-fed hens (CC and CW) were similar in body weight, suggesting the maternal diet can induce long-term effects on health and performance, and likely has a greater effect than is currently acknowledged in production systems. It would be of interest to see if this effect of maternal grain source observed is sustained or increased further if offspring performance was extended past 28 days.

Mechanisms underpinning developmental programming have yet to be elucidated, however the maternal diet is known to significantly influence egg composition and offspring performance (Naber 1979). Recently, the isolation and detection of yolk miRNA in chicken eggs has been reported (Wade *et al.* 2016). Similarly, particular miRNAs have been recognised to play important role in embryonic growth and skeletal muscle development of chickens (Darnell *et al.* 2006; Jebessa *et al.* 2018). In the current study a significant difference in the relative expression levels of four miRNAs in yolk were found between C-fed and W-fed hens. The levels of expression for miR-499, miR-138-2//miR-138-1 and let-7f were all significantly higher in the yolk of C-fed hens compared to the yolk of W-fed hens, while miR-205a was significantly higher in the yolk of W-fed hens. These specific miRNAs have previously been isolated in both the ovary of the hen and the chicken embryo.

Of the four miRNAs significantly higher in the yolk of C-fed hens, miR-499, miR-138-2//miR-138-1 and let-7f have all previously been associated with reproductive processes in the chicken ovary. Let-7 miRNA (in particular Let-7f and let-7c) family are abundantly expressed in the sexually mature chicken ovary (Kang *et al.* 2013; Liu *et al.* 2018), the ovary and oocyte of bovines (Tesfaye *et al.* 2009; Tripurani *et al.* 2010; Huang *et al.* 2011; Miles *et al.* 2012), as well as in murine ovaries and testis (Reid *et al.* 2008). In atrophic ovaries of broody hens, miR-499 was associated with estrogen and gonadotropin-releasing hormone signalling via differential expression of MMP2 gene, and cell cycle pathway via the SKP2 gene (Liu *et al.* 2018). Similarly, Lee *et al.* (2012) reports miR-

499 is closely related to regulatory pathways of oviduct development and differentiation in chickens and also influences pleiotrophin, which was found to be abundant in the glandular epithelial cells of adenocarcinoma of cancerous ovaries of the hen. Research by Jeong et al. (2013) indicates miR-138 is also involved reproductive tract of chickens, specifically during molting process, decreasing expression of p20K gene which has been linked to cellular stress responses and possess abilities to induce cell cycle arrest and apoptosis. The increased expression level of yolk miRNAs associated with reproduction in C-fed hens suggests diet may influence reproductive performance.

Recent studies indicate egg miRNAs may play a role in epigenetic programming during chick development (Wade *et al.* 2016). In the current study, all four yolk miRNAs shown to be significantly different between C-fed and W-fed hen have been associated with embryonic development of the chick. A higher expression of yolk miR-205a was found in W-fed hens, and has previously been identified in endoderm and ectoderm during chick embryo development (Darnell *et al.* 2006). Recent studies indicate miR-205a up-regulation inhibits myoblast proliferation and promotes myoblast differentiation in the chicken embryo (Wang *et al.* 2018) and is down-regulated in the interdigital mesoderm of chick embryonic hind limb during interdigital remodelling (Garcia-Riart *et al.* 2017). In the adult broiler, miR-205a is both abundant and highly differentially expressed in fast and slow-growing breast muscle tissue (Ouyang *et al.* 2015).

In the yolk of C-fed hens, miR-499, miR-138-2//miR-138-1 and let-7c/f were significantly higher than levels in yolk of W-fed hens and have all been associated with cell proliferation, differentiation and apoptosis during embryonic development. In chicken embryo skeletal muscle miR-499 is recognised to potentially target six mRNAs including EGLN1, BNIP3, RFTN2, LDHA, ACD2 and SIK1 (Jebessa *et al.* 2018). While in adult broilers miR-499 is both abundant and highly differentially expressed in fast and slow-growing breast muscle tissue and involved in growth related target genes ADAM17 and FLNB (Ouyang *et al.* 2015). miR-138 has been shown to be a novel regulator of hypothalamic cell migration in the chicken embryo, suggested to inhibit Reln expression and target reelin (Kisliouk and Meiri 2013). The let-7 family act as post-transcriptional

regulators of differentiation in blastodermal cells during early development in the chicken embryo by repressing the expression of the TGFBR1 and LIN28B, which intrinsically controls blastodermal cell differentiation (Lee et al. 2015). Similar to miR-205a, let-7f and let-7c are expressed in interdigital mesoderm of chick embryonic hind limb during interdigital remodelling (Garcia-Riart et al. 2017) and abundantly expressed in adult broiler breast muscle tissue (Wang et al. 2012; Ouyang et al. 2015). Additionally, let-7 family have been recognised in embryonic development neuronal degeneration (Hartl and Grunwald-Kadow 2013). Let-7c has previously been found to be expressed in hindbrain and spinal cord, whereas let-7f transcript was detected in the hindbrain of the central nervous system (Darnell et al. 2006).

Interestingly, an association between miR-499 expression level and disease susceptibility has previously been reported in birds. Down-regulation of miR-499 expression was observed in the intestine of White Leghorn chicken line identified as 'Marek's disease susceptible' phenotype compared to a disease resistant phenotype after inducing necrotic enteritis (Dinh *et al.* 2014). Similarly, miR-499 expression was up-regulated in liver of normal chickens compared to chickens identified with 'Runting and stunting syndrome' (Zhang *et al.* 2015). The significant effect of hen diet on offspring reaching 'threshold' value coupled with the significant difference in miRNAs associated with disease susceptibility is interesting and warrants further investigation.

To our knowledge, this is the first study to demonstrate miRNAs detected during ovary formation in the hen and embryonic development are present in the yolk of fertile eggs prior to embryogenesis and level of expression differs depending on hen diet. While encouraging, the results obtained presented low reads and therefore some caution is required when interpreting. While miRNA analysis is very sensitive, validation with Real Time PCR is warranted. Furthermore, it was noted very few sequences identified as miRNA, and most of the sequence data produced was classified as 'unknowns', despite being the correct size for miRNA. Given that current identification of miRNAs in yolk is minimal, it may be that a number of these sequences may in fact be novel, egg-specific miRNAs. The current results are the first to demonstrate predominating grain source in

breeder diet may significantly alter yolk miRNA expression and attribute to the re-programming of offspring to optimise feeding regime and warrants further investigation.

While the corn and wheat diets were formulated to contain very similar levels of the essential nutrients, some nutrients and factors were not considered in dietary formulation of the contributing grain source. These nutrients are included into the diet as a standard pre-formulated 'premix' to maintain specific concentrations (i.e. vitamins and minerals) or not generally identified as 'essential' nutrients for growth and performance. It is therefore possible these dietary factors may modulate the developmental programming and PARs observed in offspring. In this context, the concentration of carotenoids may be of relevance when comparing corn and wheat grain sources in the current experiment. Surai and Sparks (2001) report corn-based diets contain high levels of carotenoids while wheat-based diets are comparatively low (i.e. 11.8 vs 5.6 mg respectively), a reflection of higher natural carotenoid concentration in corn. While essential micro minerals such as vitamins A and E are formulated in the diets via a pre-formulated vitamin and mineral mix, carotenoids are not of high consideration in the formulation of either the pre-formulated vitamin/mineral mix or the general diet formulation. Therefore increases in carotenoids due to grain source are not a factor in diet formulation, therefore dietary concentrations between the corn and wheat diets in the current experiment may have significantly differed. Carotenoids concentration has gained considerable interest in breeder diets due to its immunomodulatory potential (Chew and Park 2004) and antioxidant capacity (Stahl and Sies 2003) and has been associated with regulation of cell proliferation, differentiation, gene expression, signalling and gap-junction communication (Zhang *et al.* 1992; Livny *et al.* 2002; Bertram 2004; Rühl *et al.* 2004; Sharoni *et al.* 2004; Palozza 2005). It is presumed the high antioxidant capacity of corn affects maternal antibody (MatAb) levels in the breeder and subsequent deposition into the egg. Yolk MatAb levels, and subsequent levels in chicks, have previously been demonstrated to be proportional to level of circulating antibodies in hen (Rahman *et al.* 2002; Hamal *et al.* 2006). Leandro *et al.* (2011) found breeder hens fed corn-based diets expressed higher levels of plasma antibodies compared to breeders on wheat-based diets under low-stress situations. This may be

particularly relevant to the current study as the maternal diet appeared to initiate a strong programming effect on the prevalence of offspring reaching a pre-determined 'threshold' value, observed by a higher percentage of offspring from C-fed hens reaching the target weight than that of W-fed hens. In the event of offspring being immunologically challenged, the greater proportion of offspring from C-fed breeders reaching TH, may reflect an increased ability to perform better due to increased MatAb levels deposited into the yolk and/or antioxidant capacity and immunomodulatory effect of yolk carotenoids. What is particularly interesting is chicks hatched from hens fed carotenoid-depleted diets (and subsequently carotenoid-depleted yolk), fail to achieve the same degree of carotenoid deposition as chicks hatched from eggs containing carotenoids (Koutsos *et al.* 2003). This suggests a PAR in the hen-chick diet may exist whereby carotenoid exposure during embryonic development enhances the chick's subsequent absorption, metabolism and/or tissue deposition of diet-derived carotenoids later in life. Unfortunately egg and offspring carotenoid concentrations was not analysed in our study. In addition calculation of dietary vitamin A and/or carotenoid levels of the diets, hence carotenoid 'activity', was not established therefore it is difficult to ascertain the impact of carotenoids as a programming mechanism.

One factor not considered in the current experiment is the effect of the microbial programming. The maternal diet induces changes in gut microbial composition which has in turn been associated with programming of offspring health and performance (Abecia *et al.* 2014; Paul *et al.* 2016). The human fetus has previously been considered microbiologically sterile prepartum, however research now suggests colonisation and/or contact of the fetus with the maternal GIT microbiota initiates in utero (Thum *et al.* 2012). Ma *et al.* (2014) found high-fat maternal or postnatal diet, persistently alters the intestinal microbiome profile of offspring in Japanese macaque, which in turn affects intestinal maintenance and metabolic health. Furthermore, there is some suggestion nutritional intervention applied to both the mother and her offspring (e.g. maternal diet and offspring diet interaction) significantly alters the microbiome of offspring in ruminants (Abecia *et al.* 2013; Abecia *et al.* 2014). The significant maternal and offspring diet interaction observed during early development in the current study may indicate microbial

programming exists in poultry and may be beneficial for health of the newly hatch chick. Poultry research relating to intestinal microbiota and development is largely constrained to direct effects of diet and environment on an individual. A recent review by Berghof *et al.* (2013) highlights the maternal gut microbiota in broiler breeders can act as a transfer mechanism as well for innate immunity in offspring, however the influence of the maternal diet on offspring microbiome and long-term health is lacking and warrant further investigation.

Mismatched feeding management between generations may contribute to health problems and poor performance based on misdirected transgenerational epigenetic information (Berghof *et al.* 2013). We recognise that the current diets are unlikely to be completely balanced in all nutrients, and these variables may have contributed to the results as nutritional status of the mother can directly impact embryonic growth and development (Barker 1997). Nevertheless, these results indicate the potential implications of ingredient type on birds genetically selected on a specific dietary regime. The current results demonstrate that, when controlling housing, sex and handling conditions, dietary environment can induce a PAR in poultry. A mismatch between breeder environment and broiler environment may contribute to unfavourable effects in performance with more attention given to transgenerational effects initiated by grain source.

# 4. Elevated corticosterone in egg yolk of hens fed a restricted diet to maintain low body weight, alters embryonic and post-hatch development of their progeny

## 4.1. Abstract

Maternal environment has been associated with a wide range of metabolic and physiological changes in offspring phenotype. The severity to which programming effects occur is associated with early neonatal development. Such programming effects have been attributed to prenatal exposure to hormones, particularly the glucocorticoids. Broiler breeder hens are severely feed restricted to maintain egg production, initiating a chronic stress response and increasing circulating corticosteroid (CORT) levels. In this study we tested 1) the effects of broiler breeder dietary management, via body weight, inducing chronic stress indices during the lay period and impact for yolk CORT; 2) resulting effects on embryonic development and; 3) the contributing impact of maternal diet and early neonatal diet on progeny performance. Broiler breeder hens (Cobb 500) were maintained at three body weight groups (HenBW); 3.4 ± 0.04 kg (Low), 3.6 ± 0.04 kg (Medium) and 4.0 ± 0.04 kg (High) from 31 to 35 weeks of age, during which time physiological measures of stress and egg composition were recorded (weeks 31 and 35). Embryos from each HenBW group were examined at two time points during embryonic development (ED14 and ED20) to quantify differences in morphology attributing to HenBW. At hatch, progeny from each hen group were allocated to either *ad libitum* or restricted diets for 21 days, after which all progeny were fed *ab libitum* to D42, at which point tissue and weights were measured.

*Broiler breeder hens.* Serum CORT concentration was significantly higher in Medium hens compared to Low and High hens (9.0 ± 0.4 *vs*. 7.0 ± 0.4 and 7.9 ± 0.5 ng $mL^{-1}$ respectively, $P < 0.0001$). Heterophil to lymphocyte (H/L) ratio was also significantly ($P < 0.0001$) higher in Medium hens compared to Low and High hens. Yolk CORT concentration was significantly higher in eggs from Low and Medium hens compared to High body weight hens (11.8 ± 0.03, 12.4 ± 0.03 *vs*. 9.4 ± 0.03 ng $g^{-1}$ respectively, $P < 0.001$).

*Embryonic Development*. HenBW resulted in sex-dependent changes in tissue and organ weights at ED14 (body weight, fatpad, Proventriculus) and ED20 (duodenum, gizzard and breast muscle).

*Posthatch Development*. Restricting chicks from feed for 5hrs per day changed growth rate during first 21 days indicating the restriction method implemented was effective. At D42 changes in tissue weight were attributed to HenBW with no effect of ProgDiet. Duodenum, jejunum, ileum and pancreas weight were decreased in progeny from Low and Medium HenBW treatments compared to High HenBW treatment ($P < 0.05$). These results indicate changes in broiler breeder hen body weight alter serum CORT and is reflected in yolk CORT concentration. Changes in hen body weight appear to significantly alter embryonic development causing long-term physiological effects, irrespective of early post-hatch dietary management. This suggests maternal dietary environment elicits strong long-term programming effects on offspring development, irrespective of early post-hatch management and potentially attributed to differences in yolk CORT concentration.

## 4.2. Introduction

During critical periods of embryonic development changes in the *in utero* environment can elicit marked programming effects resulting in permanent modifications to offspring development (Barker 1997; Chmurzynska 2010). Suboptimal intrauterine conditions have been associated with low birth weight, resulting in a suite of adult pathologies commonly referred to as the 'metabolic syndrome' including diabetes, insulin resistance, hypertension and renal failure (Fowden *et al.* 2006; Langley-Evans 2006). Intrauterine growth retardation (IUGR) can be induced via a range of insults including maternal stress, hypoxia and dietary manipulation. In particular, maternal dietary restriction (via both total caloric and protein) initiates strong programming effects (Woodall *et al.* 1996; Bertram *et al.* 2001; Whorwood *et al.* 2001; Maloney *et al.* 2003). Maternal undernutrition increases fetal exposure to glucocorticoids, altering enzyme sensitivity and hormone regulation via changes in tissue specific gene expression (Lindsay *et al.* 1996; Nyirenda *et al.* 1998; Bertram *et al.* 2001; Whorwood *et al.* 2001; Langley-Evans 2006). Of the hormones known to regulate embryonic development, glucocorticoids are the most commonly associated with maternally-derived programming responses, identified as potent regulators of embryonic maturation of organs and development (Seckl 2001; Fowden and Forhead 2004). Glucocorticoids can act directly, on genes, and indirectly, by altering the bioavailability of other hormones, therefore influencing growth and development of key tissues and whole organ systems (Fowden and Forhead 2004). Overexposure to glucocorticoids retards fetal growth in rats, sheep, monkeys and humans and has major effects on differentiation of a wide range of vital tissues and organs including lungs, liver, kidneys, muscle, fat and gut (Young *et al.* 1987; Fowden *et al.* 1998; Seckl 2001).

In avian species, *in ovo* exposure to the predominating glucocorticoid, corticosterone (CORT), occurs in the egg (Groothuis *et al.* 2005). Egg CORT concentration is influenced by several factors including breed (Navara and Pinson 2010; Ahmed *et al.* 2013), housing conditions (Lay *et al.* 2011) and physiological status of the hen (Saino *et al.* 2005). Maternal stress in birds modulates the deposition of steroid hormones in the egg and is therefore known to influence offspring phenotype

(Groothuis *et al.* 2005; Groothuis and Schwabl 2008; Henriksen *et al.* 2011b; Ruuskanen 2015). In avian offspring, CORT exposure reduces hatch weight (Janczak *et al.* 2006; Henriksen *et al.* 2013) and alters morphology (Eriksen *et al.* 2003; Chin *et al.* 2009) and immunocompetance (Rubolini *et al.* 2005; Henriksen *et al.* 2013). Long-term consequences of elevated CORT in the egg are primarily on growth traits (Hayward and Wingfield 2004) and behaviour via re-programming of the hypothalamic-pituitary-adrenal (HPA) axis (Ahmed *et al.* 2014a; Ahmed *et al.* 2014b). Administration of CORT to juvenile birds enhances hepatic lipogenesis and redistribution of energy to abdominal stores from peripheral tissues and relative organ weights. Breast muscle development in particular appears highly susceptible to CORT concentration (Davison *et al.* 1983; Lin *et al.* 2006; Cai *et al.* 2009; Shini *et al.* 2009).

The 'fetal programming' phenomenon describes a process whereby the manipulation of maternal dietary conditions resulting in suboptimal *in utero* conditions dictates the response in the fetus leading to enhanced susceptibility to adult disease. However, the early postnatal environment, a period of developmental plasticity, can also 'programme' function, thereby modulating the severity of phenotypic response in offspring (Armitage *et al.* 2005; Godfrey *et al.* 2007). IUGR followed by accelerated postnatal growth is associated with alterations in adipocyte cell size and impaired protein expression of insulin-signalling proteins which are indicative of metabolic disease risk (Berends *et al.* 2013). Research suggests it is the degree of 'mismatch' between maternal and offspring environments, defined as the 'Predictive Adaptive Response' (PAR) hypothesis, which dictates the severity of adverse phenotypic response in offspring (Armitage *et al.* 2005; Lillycrop 2011). Evidence in rat and pig models indicate it is the matching across generations which is important, as evidenced by trials showing maternal over-nutrition during pregnancy combined with high-fat feeding in postnatal life did not induce deleterious effects (Norman and LeVeen 2001; Khan *et al.* 2004).

Perhaps the most extreme industry example of such programming events occurs in the modern broiler breeder hen and her offspring. The modern broiler phenotype and genotype is the result of

intense genetic selection for rapid growth and enhanced muscle mass. Thus, broiler birds commonly present with hyperphagia and poor regulatory feed intake (Richards *et al.* 2010). This satiety effect, if not managed in the broiler breeder hen, not only predisposes birds to obesity but accelerates ovarian follicular maturation such that greater ovulations occur than the oviduct can effectively process (Renema and Robinson 2004). As a result, poor fertility and hatchability, decreased number of settable eggs, and, more erratic oviposition occurs. In order to regulate body weight to optimise egg production and improve flock uniformity, broiler breeder hens are subjected to severe feed restriction during the rearing and breeding phases (Richards *et al.* 2010). As such daily feed allocations can be 25 – 50% of *ad libitum* intake (Yu *et al.* 1992; Savory *et al.* 1993b). Such severe feed restriction is thought to induce a chronic stress response, significantly increasing the primary circulating CORT and H/L ratio in broiler breeders which may persist for up to 10 weeks from onset of restriction (Mench 1991; Hocking and Maxwell 1992; Hocking *et al.* 1996; De Jong *et al.* 2002; Scanes 2016).

Considering the significant effects of yolk CORT concentrations on avian development, and the elevated circulatory CORT levels associated with broiler breeder dietary restrictions, it is likely programming effects will occur, altering embryonic developmental trajectory. A recent study by van der Waaij *et al.* (2011) suggests the same phenomenon occurs in broiler breeder hens whereby restrictive feeding of broiler breeder hens and subsequent *ad libitum* feeding of offspring reduced growth and increased abdominal fat. Furthermore, given the effect of glucocorticoids on tissue differentiation and accretion observed in mammals and the apparent impact of maternally-derived CORT on body weight and growth rate in avians, it is surprising that there are few studies of the effects of exposure of avian embryos to elevated CORT levels *in ovo* on subsequent organ function. The objective of this study was to quantify the impact of severe quantitative feed restriction on chronic stress responses and level of CORT deposition into yolk during the lay period in broiler breeder hens and consequences for offspring. It was hypothesised yolk CORT levels in broiler breeder hens would be elevated in hens with lower body weights (and more severe feed restriction) and would alter morphological development of offspring *in ovo* and therefore post-hatch

performance. Indices recognised to alter in response to severe feed restriction in broiler birds were used to validate the impact of feed restriction and body weight in the broiler breeder hens. To determine if programming effects occur in poultry similar to that of IUGR in mammals, tissues and organ weights, previously recognised to alter in mammalian models, were measured.

## 4.3. Materials and methods

All animal use was approved by the animal ethics committees of The University of Adelaide (S-2014-013) and the Department of Primary Industries and Regions, South Australia (PIRSA) (#1/14). A total of 36 Cobb 500 broiler breeder hens (*Gallus gallus domesticus*) were group housed in treatment groups (*n* = 12/pen) at a commercial breeder facility. From hatch until 21 weeks of age birds were managed and fed under normal industry conditions. At 21 weeks of age birds were selected from the flock and feed intake gradually increased to achieve desired body weight treatments (HenBW) while still allowing for reproductive performance. From 31 to 35 weeks of age hens were maintained using the following feed allocations; 140 g/bird/day, Low; 145 g/bird/day, Medium; and 160 g/bird/day, High. Hens were fed a commercial diet once daily at 8:00h, except on day of blood sampling wherein hens were fed after blood collection. Weekly body weight was recorded prior to feeding to ensure hen body weights were maintained in the three groups. At two time points during the experiment (31 and 35 weeks of age) a 2mL blood was collected via the brachial vein within 3 min of handling using a 23 gauge needle. Blood was left to clot for 24 hr, centrifuged for 10 mins at 4°C (4500 × g), and serum was stored at -20°C for further analysis.

At 31 and 33 weeks of age fertile (Appendix; Figure 1) eggs (*n* = 20 per treatment) were collected and egg, albumen and yolk weight was measured. Yolk was sampled at 31 weeks of age and stored at -20°C for CORT analysis. In brief, the egg shell was removed and yolk separated from albumen using a yolk separator. Albumen weight was recorded. Yolk was placed on gauze to remove residual albumen and then suspended in fresh gauze and the yolk membrane pierced. Yolk was gently massaged from the yolk sac to remove yolk contents and the weight of yolk recorded.

Yolk was homogenised for 30 seconds and a 15mL sample of yolk was collected and then stored at -20°C.

Additional eggs were collected over a three week period from each group of hens. Eggs were randomised and incubated at 38$^{o}$C and 55% humidity from day 0 to 15, then 36.7$^{o}$C and 60% until hatch at day 21 at Roseworthy Campus, The University of Adelaide. Embryos from a subset of eggs were euthanised by cervical dislocation at ED14 (*n* = 12/HenBW) and ED20 (*n* = 18/HenBW) and body weight, residual yolk sac, duodenum, jejunum, ileum, gizzard, breast muscle, abdominal fat pad, liver, heart, proventriculus, Bursa of Fabricius and spleen weight recorded. Remaining eggs were incubated until hatch and chicks were weighed, ID tagged and placed into group-rearing pens based on hen treatment. The progeny experiment was a three-by-two factorial design; HenBW (L, M and H) and two dietary treatments of the progeny (ProgDiet) whereby progeny were allocated to either restricted (R) or *ad libitum* (AL) feed with two pen replicates per interaction. Progeny were either feed restricted for 5 h/day or fed *ad libitum* from D1 to D21 using a standard commercial meat bird starter diet (Ridley Turkey and Meat Chicken Grower). From D21 to D42 all progeny were fed a commercial meat bird finisher diet (Ridley Turkey and Meat Chicken Finisher) *ad libitum*. Standard husbandry procedures were followed with chicks placed in a shed at 25$^{o}$C with a light cycle of 23 h light for the first 24 h. From D2 the light cycle was 16 h light and the shed temperature was adjusted accordingly. All birds were orally vaccinated for infectious bronchitis virus (IBV) at D7. Body weight was recorded weekly. At D42 birds were euthanized and tissues and organs were collected. Duodenum, jejunum, ileum and gizzards were flushed with phosphate buffered saline (PBS) prior weighing. Gross tissue weight was recorded for BW, duodenum, jejunum, ileum, gizzard, breast muscle, liver, heart, proventriculus, pancreas, bursa of fabricius and spleen.

### 4.3.1 White blood cells

One drop of whole blood was smeared onto a glass slide immediately after collection to create a monolayer of cells. Slides were fixed in 70% methanol for 60 secs and allowed to dry and later stained with Wright-Giemsa Stain using a Hematek Stain Pak Automatic Stainer (Bayer,

Mishawaka, U.S.A.). Each slide was counted three times using 40 × magnification (Olympus, Tokyo, Japan) under a blinded analysis (Gross and Siegel 1983). Cells count were heterophils, lymphocytes, monocytes, basophils, and eosinophils until a total of 100 cells were counted (Appendix; Figure 2). The ratio of heterophil to lymphocyte cells was then calculated.

### 4.3.2 Corticosterone analysis

The total concentration of serum CORT was measured using a commercially available competitive enzyme immunoassay kit (DetectX Corticosterone, K014-H5, Arbor Assay, USA). The intra-assay coefficient of variation was < 10%, and the inter-assay coefficient of variation was < 3.4%. Samples were assayed in duplicate. The limit of detection was 0.016 ng $mL^{-1}$.

Corticosterone was extracted from yolk using previously-described methods (Cook *et al.* 2009). Briefly, the egg yolk (~0.1 g) was taken and 0.5 mL of distilled water was added and vortexed until mixed. The mixture was extracted with 3 mL hexane:diether (30:70 ratio) and vortexed and left to settle before snap freezing in an ethanol/dry ice bath. The supernatant was collected, dried and 1 mL of ethanol was added to the samples which were frozen at -80°C overnight. Samples were centrifuged and the supernatant collected and dried. Samples were resuspended in 500 µL of phosphate buffer saline and analysed. Yolk extracts were analysed for CORT using a validated RIA Corticosterone 125I RIA KIT (MP Biomedicals, Orangeburg, NY). The intra-assay coefficient of variation was < 15%, and the inter-assay coefficient of variation was < 8%. Samples were assayed in duplicate. The limit of detection was 2.2 ng $mL^{-1}$.

### 4.3.3 Statistical analysis

To validate HenBW treatment groups were maintained at significantly different body weights throughout the experimental period body weight was analysed using a linear mixed model with type 3 sums of squares (SPSS 24.0, SPSS Inc., Chicago, IL). Individual Hen ID was used as the unit with hen week of age fitted as the repeated measure. Age and hen body weight (HenBW) were fitted as fixed effects. Pairwise comparisons were used to determine significant differences. Serum CORT and white blood cell parameters were analysed by two-way ANOVA with HenBW and Age

fitted as fixed effects. Yolk was analysed using one-way ANOVA with HenBW as fixed effect and egg weight fitted as a co-variate.

Embryonic tissues weights were compared using two-way ANOVA with fixed effects of HenBW, Sex and interactions fitted into the model. Body weight on day of sampling (i.e. ED14 or ED20) was fitted as a co-variate. Progeny body weight and growth rate was analysed using a linear mixed model with type 3 sums of squares. Data were checked for normality by examining the distribution of residual plots. Individual Chick ID was used as the unit with chick day of age (Day) fitted as the repeated measure. Hatch weight was fitted as a covariate. Day, HenBW (Low, Medium and High), ProgBW (Ad libitum or restricted). Male and Female progeny were analysed separately. Progeny tissue weights were analysed using ANOVA with HenBW, ProgDiet and Sex fitted as fixed effects and D42 body weight fitted into the model as a co-variate. Data were checked for normality and homogeneity of variance and no transformations were undertaken. Level of significance was deemed to be $P < 0.05$ and trend deemed to be $P < 0.1$.

## 4.4. Results

### 4.4.1 Broiler breeder hens

#### 4.4.1.1. Body weight

There were no significant interactions between Age and HenBW during the experimental sampling period ($P$ = 0.988). Similarly, there was no significant effect of Week on HenBW (P = 0.690). There was a significant ($P$ < 0.001) between subjects effect of HenBW. Pairwise analysis identified Low, Medium and High treatment groups to be significantly different from each other (3.4 ± 0.04 kg, 3.6 ± 0.04 kg and 4.0 ± 0.04 kg respectively) as seen in Figure 4.1.

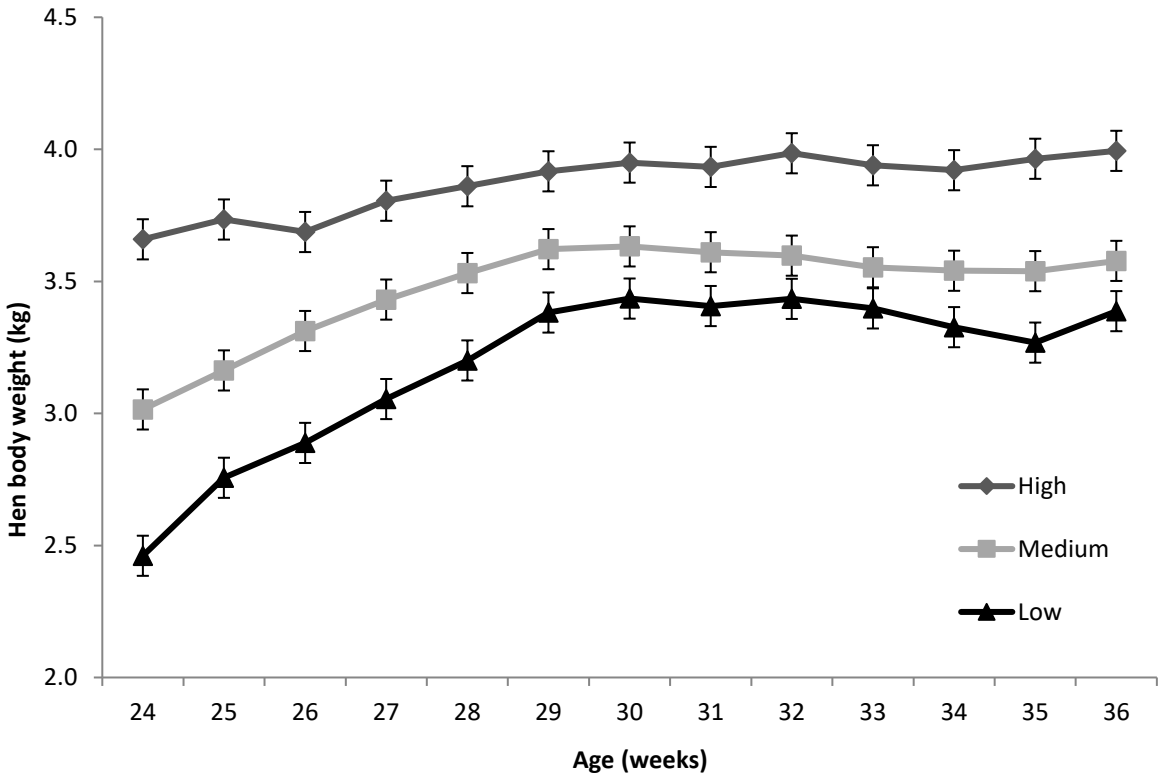


**Figure 4.1 Body weights (kg) of hens maintained at Low, Medium and High body weights maintained throughout sexual maturity (Weeks 30) and during egg laying (Weeks 30-36). Values are means ± SEM**

#### 4.4.1.2. Egg composition

Egg weight significantly ($P < 0.0001$) increased with hen age from 31 weeks of age to 35 weeks age (58.0 ± 0.5 g *vs.* 60.7 ± 0.5 g respectively). There was no effect of HenBW on egg weight ($P = 0.216$). Albumen weight was significantly ($P < 0.05$) heavier in Low treatment hens compared to Medium and High hens (34.2 ± 0.3 g *vs*. 33.3 ± 0.3 g and 33.0 ± 0.3 g) but no significant change with hen age was found. Yolk weight significantly increased with hen age at 31 and 35 weeks (15.5 ± 0.2 g *vs.* 16.5 ± 0.2 g respectively, $P < 0.0001$) and was significantly lower in Low treatment hens compared to High hens (Low 15.6 ± 0.2 g, Medium 16.0 ± 0.2 g and High 16.5 ± 0.2 g, $P < 0.05$).

#### 4.4.1.3. Corticosterone concentration

During the treatment period, there was a significant ($P < 0.05$) effect of Age and HenBW ($P < 0.005$) but not Age*HenBW interaction ($P = 0.454$) on serum CORT concentration. Serum CORT concentrations decreased with an increase in hen age, and was significantly higher at 31 weeks of age compared to 35 weeks of age (8.5 ± 0.3 ng $mL^{-1}$ *vs*. 7.4 ± 0.3 ng $mL^{-1}$ respectively). The serum concentrations of CORT were significantly higher in the Medium body weight hens than in High and Low hens (9.0 ± 0.4 ng $mL^{-1}$ *vs.* 7.0 ± 0.4 ng $mL^{-1}$ and 7.9 ± 0.5 ng $mL^{-1}$ respectively, Figure 4.2 A). A significant ($P < 0.005$) effect HenBW of yolk collected at 35 weeks of age suggests a similar yolk CORT concentration response to that observed in serum. Yolk CORT concentrations were significantly higher in Low and Medium (11.8 ± 0.03 ng $mL^{-1}$ and 12.4 ± 0.03 ng $mL^{-1}$ respectively) treatment group compared to High HenBW group (9.4 ± 0.03 ng $mL^{-1}$) as seen in Figure 4.2 B.

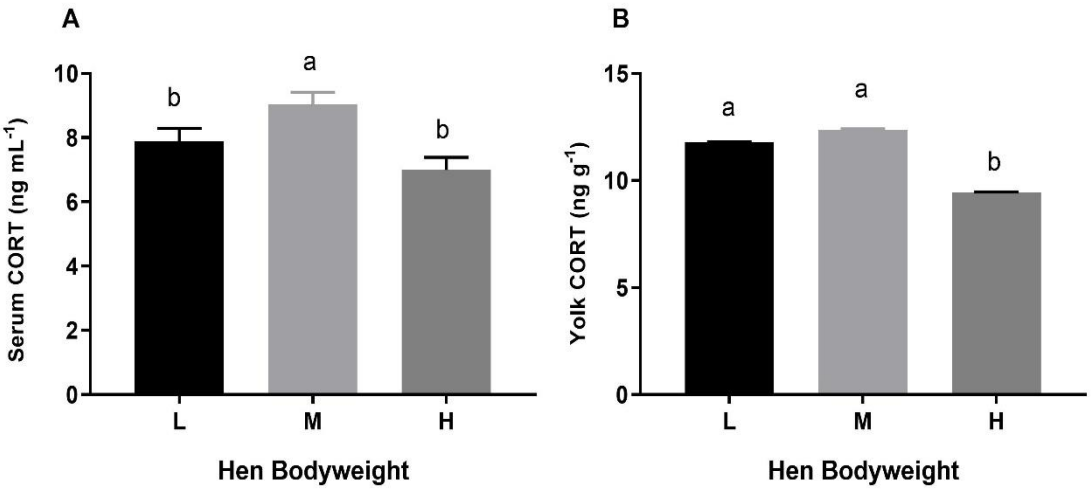


**Figure 4.2 Effect of hen body weight on serum (A) and yolk (B) corticosterone concentrations. Values are mean ± SEM, *n* = 12/treatment for serum CORT and *n* = 20/treatment for yolk CORT. [ab] Different superscripts above the bars indicates significantly different mean values at *P* < 0.05.**

#### 4.4.1.4. White Blood Cells

Overall, the number of white blood cells increased with hen age. The H/L ratio increased from 31 weeks of age to 35 weeks of age (0.6 ± 0.05 *vs*. 1.0 ± 0.05 respectively, $P < 0.0001$). The same trend was observed for basophil number (3.5 ± 0.3 *vs*. 5.5 ± 0.3 respectively, $P < 0.0001$) and the number of monocytes (0.7 ± 0.2 *vs.* 2.2 ± 0.2 respectively, $P < 0.0001$). Eosinophil number decreased between 31 and 35 weeks of age (2.7 ± 0.3 *vs.* 1.5 ± 0.3 respectively, $P < 0.001$). Effects of HenBW were also observed with H/L ratio significantly higher in Medium treatment group compared to Low and High groups (1.1 ± 0.1 *vs.* 0.7 ± 0.1 and 0.6 ± 0.1, respectively, $P < 0.0001$). Monocyte number was significantly lower in the High treatment group compared to Low and Medium groups (0.6 ± 0.6 *vs.* 1.6 ± 0.3 and 2.0 ± 0.3, respectively, $P < 0.001$) and a similar response was found in eosinophil numbers (1.0 ± 0.3 *vs*. 2.4 ± 0.3 and 2.8 ± 0.3, respectively, $P < 0.0001$). There was a significant ($P < 0.0001$) interaction of HenBW*Age for the H/L ratio and basophil number, and trend effect ($P < 0.051$) on monocyte number (Table 4.1).

**Table 4.1 Effect of Hen body weight (Low, Medium or High) and hen age (31 and 35 weeks of age) on white blood cell counts.**

| | Low | | Medium | | High | |
|---|---|---|---|---|---|---|
| | **31** | **35** | **31** | **35** | **31** | **35** |
| *n* | 12 | 11 | 12 | 11 | 12 | 11 |
| H/L ratio | $0.5 \pm 0.1^{a}$ | $0.9 \pm 0.1^{b}$ | $0.7 \pm 0.1^{ab}$ | $1.5 \pm 0.1^{c}$ | $0.6 \pm 0.1^{a}$ | $0.5 \pm 0.1^{a}$ |
| Basophil | $2.7 \pm 0.5^{a}$ | $6.3 \pm 0.5^{b}$ | $3.5 \pm 0.5^{ac}$ | $6.2 \pm 0.5^{b}$ | $4.2 \pm 0.5^{c}$ | $3.9 \pm 0.5^{ac}$ |
| Eosinophil | 3.5 ± 0.4 | 1.3 ± 0.4 | 3.0 ± 0.4 | 2.6 ± 0.4 | 1.6 ± 0.4 | 0.5 ± 0.5 |
| Monocyte | $0.8 \pm 0.4^{x}$ | $2.5 \pm 0.4^{y}$ | $0.9 \pm 0.4^{x}$ | $3.1 \pm 0.4^{y}$ | $0.4 \pm 0.4^{x}$ | $0.9 \pm 0.4^{x}$ |

$^{ab}$ Different superscripts within a row indicate $P < 0.05$, $^{xy}$ Different superscripts within a row indicate $P < 0.1$

### 4.4.2 Embryonic development

At ED14 there were no main effects of HenBW or sex on residual yolk sac, duodenum, jejunum, ileum, breast muscle, Bursa of Fabricius, proventriculus, heart and spleen weight. A sex-dependent effect of HenBW was observed for proventriculus ($P < 0.05$) weight in embryos and a trend effect of HenBW on embryonic body weight and fat pad weight ($P < 0.1$) as seen in Figure 4.3 (See Appendix; Table 2 for all significant interactions and data). By ED20, female body weight tended to be heavier than males (43.1 ± 0.7 g *vs.* 41.1 ± 0.8 g respectively, $P = 0.071$). There was no significant effect of HenBW on body weight, residual yolk sac, jejunum, ileum, proventriculus, liver, heart, bursa of fabricius and spleen gross weight. There was a sex-dependent effect of HenBW on duodenum ($P < 0.05$) and breast muscle ($P < 0.05$) as seen in Figure 4.4 (See Appendix; Table 3 for all significant interactions and data). Overall there was a trend effect of HenBW on gizzard weight ($P < 0.1$) irrespective of embryo sex with progeny from Medium body weight hens significantly lighter than progeny from Low and Medium body weight hens (Low 1,566.5 ± 55.2 g, Medium 1,383.7 ± 56.8 g and High 1,530.4 ± 55.6 g, $P = 0.061$).

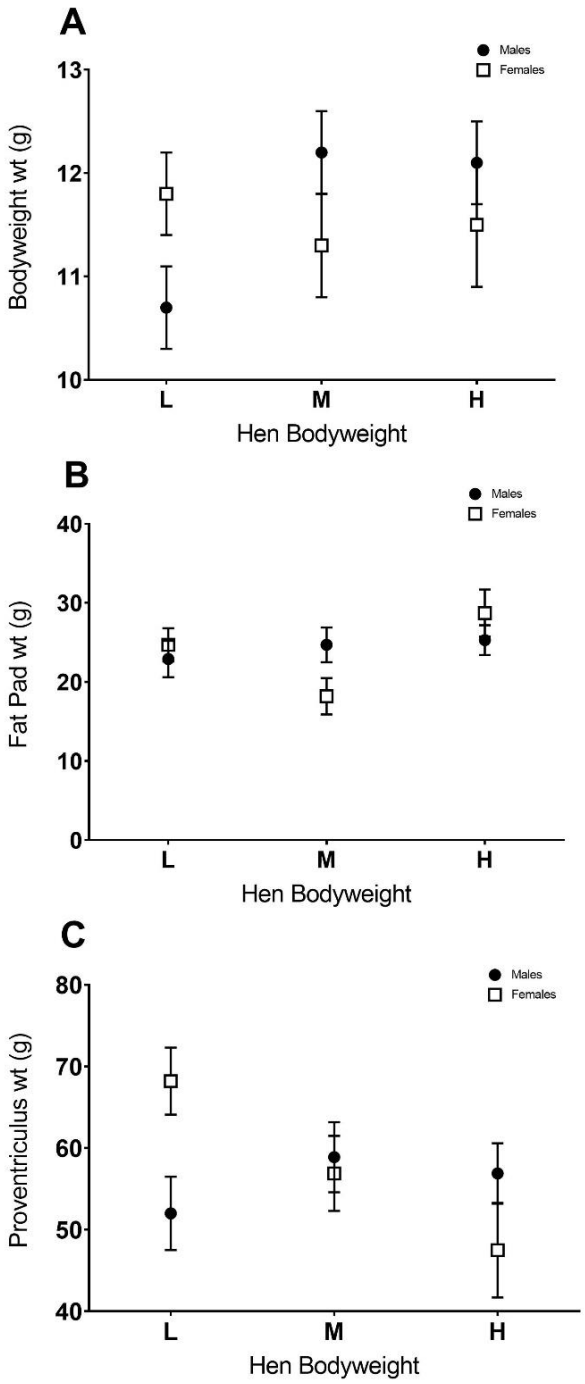


**Figure 4.3 Effect of HenBW and sex on tissue and body weight of progeny at ED14. Individual graphs of tissues with significant or trending differences; body weight (A), fat pad (B) and proventriculus (C). Circles indicate males and squares indicate females. Values are means ± SEM. $^{ab}$ Differing superscripts above the bars indicate $P < 0.05$, $^{xy}$ Differing superscripts above the bars indicate $P < 0.1$.**

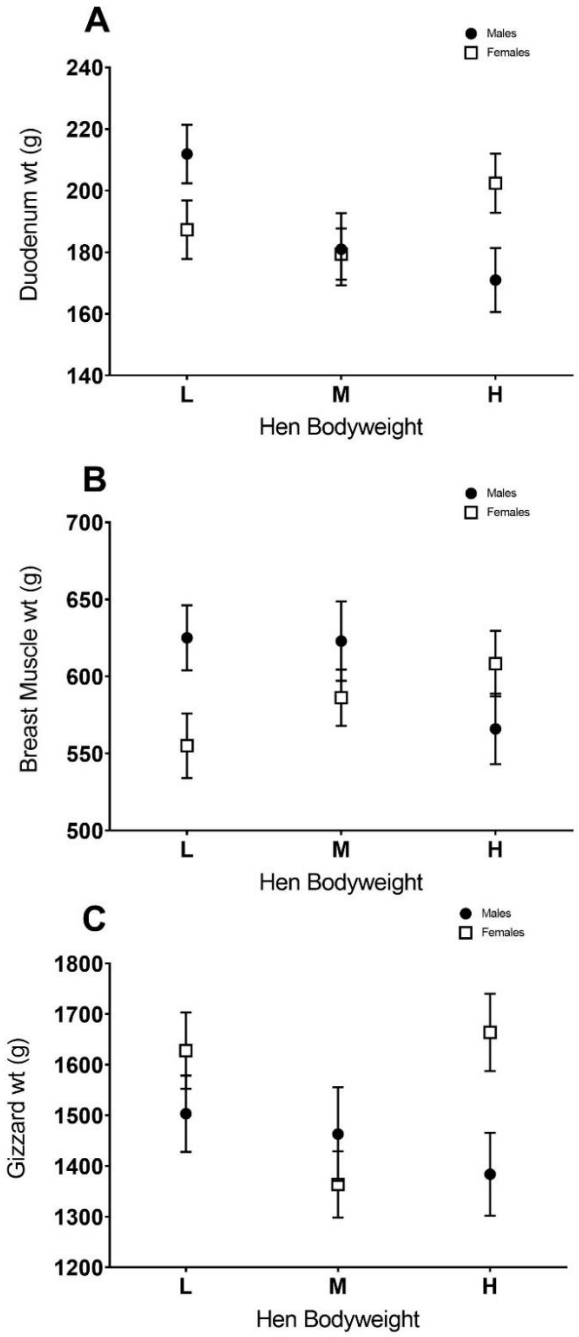


**Figure 4.4 Effect of HenBW and sex on tissue weight of progeny at ED20. Individual graphs of tissues with significant or trending differences; duodenum (A), breast muscle (B) and gizzard (C). Circles indicate males and squares indicate females. Values are means ± SEM. $^{ab}$ Differing superscripts above the bars indicate P < 0.05, $^{xy}$ Differing superscripts above the bars indicate *P* < 0.1.**

### 4.4.3 Posthatch development

#### 4.4.3.1. Weekly body weight and growth rate

There was no effect of HenBW ($P = 0.432$) or sex ($P = 0.489$) on hatch weight of progeny. In regards to progeny growth, there were significant main effects of Day ($P < 0.001$) and Sex ($P < 0.001$). No significant interaction effect of Day*HenBW*ProgDiet ($P < 0.05$) was found in either male or female progeny body weight or growth rate. In female progeny there was no significant of HenBW*ProgDiet for either body weight ($P = 0.756$) or growth rate ($P = 0.946$). There was however a significant Day*ProgDiet effect on body weight ($P < 0.05$) and growth rate ($P < 0.05$) as shown in Table 4.2. In male progeny there was a significant effect of HenBW*ProgDiet for both body weight ($P < 0.005$) and growth rate ($P < 0.05$), as seen in Table 4.3, but no Day*ProgDiet effect on body weight ($P = 0.694$) and growth rate ($P = 0.776$).

**Table 4.2 Effect of ProgDiet on body weight (g) and growth (g/week) in female progeny**

| | **Females** | | |
|---|---|---|---|
| Day/Week | **Ad libitum** | **Restricted** | ***P* - Value** |
| *Body weight (g)* | | | |
| D7 | 114.0 ± 2.4 | 111.5 ± 2.4 | 0.467 |
| D14 | 317.5 ± 5.9 | 299.6 ± 5.7 | *< 0.05* |
| D21 | 694.4 ± 10.7 | 653.4 ± 10.4 | *< 0.01* |
| D28 | 1165.0 ± 17.3 | 1110.5 ± 16.8 | *< 0.05* |
| D35 | 1724.1 ± 24.7 | 1698.5 ± 23.9 | 0.458 |
| D42 | 2292.0 ± 30.5 | 2282.9 ± 29.6 | 0.831 |
| *Growth rate (g/week)* | | | |
| 1 | 70.7 ± 2.4 | 68.2 ± 2.3 | 0.431 |
| 2 | 203.6 ± 3.9 | 188.1 ± 3.8 | *< 0.01* |
| 3 | 376.8 ± 5.5 | 353.8 ± 5.3 | *< 0.005* |
| 4 | 470.6 ± 7.8 | 457.1 ± 7.6 | 0.218 |
| 5 | 559.1 ± 11.9 | 588.0 ± 11.6 | 0.087 |
| 6 | 567.9 ± 13.8 | 584.4 ± 13.4 | 0.392 |

**Table 4.3 Effect of HenBW and ProgDiet on body weight (g) and growth (g/week) in males**

| | HenBW | | |
|---|---|---|---|
| **ProgDiet** | **Low** | **Medium** | **High** |
| *Body weight (g)* | | | |
| Ad libitum | 1206.1 ± 33.8 [ab] | 1234.8 ± 23.9 [a] | 1166.9 ± 22.1 [b] |
| Restricted | 1162.3 ± 30.7 [b] | 1154.4 ± 22.1 [b] | 1236.2 ± 18.5 [a] |
| *Growth rate (g/week)* | | | |
| Ad libitum | 456.8 ± 21.7 | 462.7 ± 15.3 | 436.5 ± 14.2 |
| Restricted | 445.6 ± 13.3 [ab] | 430.0 ± 14.2 [a] | 473.5 ± 11.9 [b] |

[ab] Different superscripts within a row indicate $P < 0.05$

#### 4.4.3.2. Day 42 body weight and tissue weights

At D42 male progeny were significantly heavier than female progeny (2.8 ± 0.03 kg vs. 2.3 ± 0.03 kg respectively, $P < 0.0001$). A sex effect was observed for duodenum weight, being significantly heavier in males compared to females (15.3 ± 0.4 g vs. 13.7 ± 0.3 g respectively, $P < 0.005$) while breast muscle weight was significantly heavier in female progeny compared to male progeny (458.1 ± 5.7 g vs. 410.8 ± 8.2 g respectively, $P < 0.0001$). Overall, there was a significant effect of HenBW on progeny duodenum ($P < 0.0001$), jejunum ($P < 0.0001$), ileum ($P < 0.05$), pancreas ($P < 0.001$) and breast muscle ($P < 0.05$) (Figure 4.5). An interaction effect of HenBW and ProgDiet was observed for ileum in progeny ($P < 0.05$). An interaction effect of HenBW and ProgDiet was also observed in jejunum of male progeny only ($P < 0.05$) (Figure 4.6).

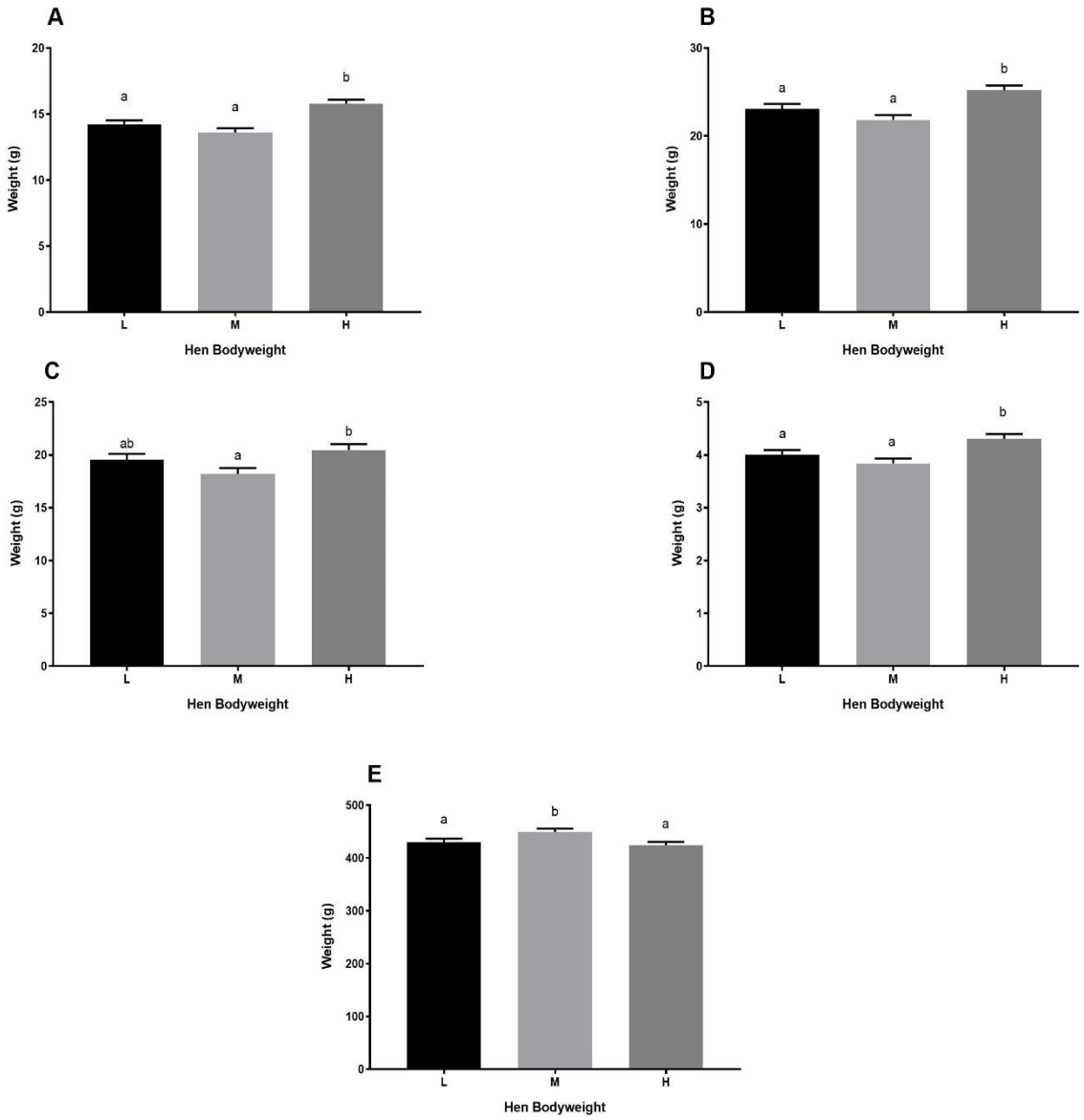


**Figure 4.5 Effect of HenBW on tissue weight of progeny at D42. Individual graphs of tissues with significant differences; duodenum (A), jejunum (B), ileum (C), pancreas (D) and breast muscle (E) weight D42. Values are means ± SEM. ab Differing superscripts above the bars indicate *P* < 0.05.**

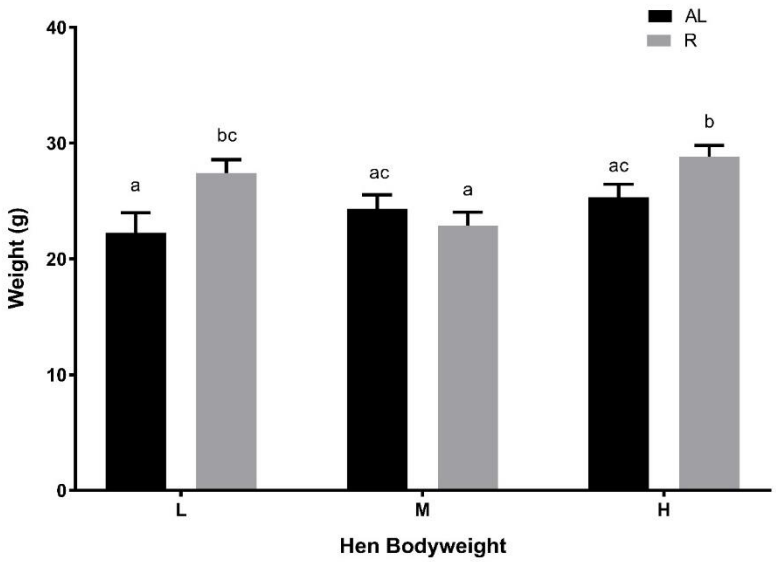


**Figure 4.6 Effect of HenBW (L, M, H) and progeny diet (AL or R) on jejunum weight of male progeny at D42. Values are means ± SEM. [ab] Differing superscripts above the bars indicate *P* < 0.05.**

## 4.5. Discussion

The impact of maternal diet on embryonic and post hatch performance has been recognised in various species (Armitage *et al.* 2004; Armitage *et al.* 2005; Ciacciariello and Tyler 2013). In addition the early posthatch environment can also induce significant effects on offspring performance. Initial differences in body weight were generated by differences in feed intake due to limited feeder space. This varied allocation to feeder space between individual birds in this experiment was random and therefore the differences in initial body weight being due to other factors (i.e. genetics and non-nutritional challenges) is unlikely. Throughout this discussion care is taken to impute the treatment effects to "maintenance of differential body weights already established in a commercial breeder unit" rather than feed intake per se, as this is a small but potential confounding effect in the study design. The current study highlights the dramatic effect physiological status of the broiler breeder hen has on embryonic development and the dramatic long-term implications this has for offspring physiology, irrespective of early posthatch environment.

Chronic stress induced by feed restriction in broiler breeder hens is associated with elevated CORT concentration (Hocking *et al.* 1996). Concentrations observed in the current study are similar to values previously reported, with slight differences in concentration between studies is likely due to various strain, age and environmental differences (Kubíková *et al.* 2001; Love *et al.* 2005; de Beer *et al.* 2008; Ralph *et al.* 2015). It was expected CORT concentration would be highest in hens of low body weight, however hens maintained at medium body weight had significantly higher serum CORT concentration than high body weight hens (Figure 4.2A). This differential response may be attributed to a combination effect of chronic stress, metabolic regulation and reproductive status. CORT concentration in birds fluctuates in response to various factors including reproductive status (Wilson and Cunningham 1981) and intermediate metabolic responses. Ovulation is dependent on body weight, therefore the lay period in medium hens occurred earlier than low body weight hens and may therefore alter serum CORT level. Therefore serum CORT concentration under feed-restricted circumstances may not only represent a stress response but also ovulatory cycle and feed

consumption in broiler breeders. Irrespective of cause, serum concentrations were highest in low and medium body weight hens and goes some way to support previous studies demonstrating various levels of feed restriction in broiler breeders alters circulating CORT concentrations (Kubíková *et al.* 2001; De Jong *et al.* 2002; Love *et al.* 2005; Rajman *et al.* 2006; de Beer *et al.* 2008).

Heterophil to lymphocyte ratio is often used as an indicator of chronic stress in birds, and reportedly increases in response to severe feed restriction (Maxwell *et al.* 1990; Hocking and Maxwell 1992; Maxwell *et al.* 1992; Zuidhof *et al.* 1995). However, glucocorticoids can influence heterophil:lymphocyte ratio (H:L), depressing the immune system and elevating H:L greatly. Administration of CORT or dexamethasone is followed by dramatic increases in H:L ratio (Gross *et al.* 1980; Gross and Siegel 1983; Vicuna *et al.* 2015). It is therefore not surprising hen serum CORT levels were accompanied by significantly higher H:L ratio of Medium hens compared to values observed in Low body weight.

By 35 weeks of age, H:L ratio was significantly higher in Low and Medium hens, with values similar to previously reported (Zuidhof *et al.* 1995; Jones *et al.* 2004). A similar effect was observed for basophil number. As basophilia is reportedly indicative of more long-term restriction, this supports the current findings and may be a more accurate indicator of chronic stress response in poultry (Maxwell *et al.* 1990; Maxwell *et al.* 1992; Hocking *et al.* 1993; Maxwell 1993; Savory *et al.* 1993a; Savory *et al.* 1996). The general increase in white blood cell response observed in Low and Medium hens reflects the response in serum CORT, demonstrating that differences of as little as 400g in body weight can elicit a significant physiological response in broiler breeder hens.

In many circumstances yolk CORT concentration reflects circulating CORT levels in the hen (Hayward and Wingfield 2004; Henriksen *et al.* 2011a; Almasi *et al.* 2012). Yolk CORT concentrations between treatment groups reflected a similar trend to that found in serum values with CORT significantly higher in the Low and Medium body weight hens compared to High hens. The increase in yolk CORT for both Low and Medium body weight groups suggest yolk CORT may be a more useful indicator of physiological response when used in reference to programming effect as yolk

deposition takes place over a 10 day period, in comparison to serum taken at a singular time point. Yolk values observed in the current study are similar to previously reported and imply low body weight is associated with elevated serum and yolk CORT concentrations (Love *et al.* 2008; Cook *et al.* 2009; Almasi *et al.* 2012).

No significant effect of the high hen body weight treatment on total egg weight was found, however, yolk weight was significantly decreased in low body weight hens compared to high body weight hens. This is not surprising as sexual maturity is related to hen body weight and composition therefore heavier birds typically come into lay earlier and with greater lipid content (Summers and Leeson 1983).

Prenatal stress and glucocorticoid manipulation leads to sex-dependent modification of behaviour, brain and organ morphology in many species (Kapoor *et al.* 2006). In poultry, posthatch exposure to glucocorticoids exert numerous physiological effects including decreased growth (Donker and Beuving 1989; Post *et al.* 2003; Song *et al.* 2011), increased liver, intestinal and adipose tissue weight (Bartov *et al.* 1980; Gross *et al.* 1980; Hamano 2006) and depressed gastrointestinal functioning (Scanes 2016). Comparatively, little information exists concerning the effects of CORT exposure *in ovo* on embryonic development.

The current study highlights the profound effect maternal dietary environment and associated changes in yolk CORT concentration have on embryonic and post hatch development. Differences in hen body weight caused significant sex-dependent effects on specific tissues associated with growth and nutrient absorption on both day 14 and 20 of embryonic development.

At ED14, male embryos from Low body weight hens tended to be lighter than males from Medium and High body weight hens. As mentioned earlier, yolk CORT was significantly increased in Low body weight hens. These results support previous findings in which elevated maternal CORT is associated with decreased growth in offspring (Eriksen *et al.* 2003; Janczak *et al.* 2006; Shini *et al.* 2009; Ahmed *et al.* 2014a). In particular, body weight of males may be more sensitive to changes

in CORT during embryonic development (Rubolini *et al.* 2005; Hayward *et al.* 2006; Uller *et al.* 2009) and may explain the sex-dependent effect observed. In comparison, proventriculus weight relative to body weight was increased in female embryos from Low body weight hens at ED14, significantly heavier than embryos from High body weight hens and male embryos of Low body weight hens. Malheiros *et al.* (2003) also observed an increase in the proportional weight of the proventriculus after dietary supplementation of CORT, albeit in juvenile broilers. This increase was attributed to CORT-induced growth retardation rather than a direct effect of CORT exposure. In the avian, gut maturation occurs early in embryonic development as reflected in low (<1) allometric coefficient, thus as body weight gain is reduced by CORT, organs maintain a higher weight respective to body weight. As a decrease in body weight was observed in embryos at ED14, the same event may be occurring *in ovo*. The significant difference in proventriculus weight between male and female embryos of low body weight hens indicates sexes may actually have opposite and/or differential responses to CORT during development as previously identified by Uller *et al.* (2009).

By ED20 hen body weight had a significant effect on duodenum weight, again only in male embryos. Similar to results of proventriculus weight observed at ED14 in females, duodenum weight was heavier in embryos of low body weight hens (Figure 4.4). While a significant difference in body weight was present at ED14, by ED20 no difference was found, indicating yolk CORT may have a direct effect on gut development. In mammals, prenatal glucocorticoids exert effects throughout the gastrointestinal tract influencing morphological, cytological, and functional differentiation (Trahair and Sangild 1997). During the final stages of incubation in the embryonic chick the intestine undergoes morphological, cellular, and molecular changes including accelerated proliferation and differentiation of enterocytes, increased absorptive capacity and nutrient uptake, and elevated expression of genes involved in digestion and absorption processes in the epithelial enterocytes (Uni *et al.* 2000; Geyra *et al.* 2001). It may be during period of proliferation and differentiation CORT exposure may induce early maturation of intestinal tissue. Similarly, breast muscle weight was also significantly higher in male embryos from low body weight hens. Chin *et al.* (2009) also reports juvenile European starlings exposed to elevated embryonic CORT had heavier

and more functionally mature flight muscles compared with control fledglings. Likewise, Lin *et al.* (2006) suggests the breast muscle of broiler birds is highly sensitive to stress mimicked by CORT administration.

Increased exposure to CORT concentration enhances fatty acid synthesis in the liver and central fat deposition resulting in increased abdominal fat. Consequently, CORT administration increases liver weight, indicating hypertrophy of the liver (Davison *et al.* 1985; Malheiros *et al.* 2003; Lin *et al.* 2004). However, no significant effect of hen body weight was found for either abdominal fat pad or liver weight in the current study. Additionally, studies indicate elevation of maternal CORT has also previously been associated with decreased hatch weight (De La Cruz *et al.* 1987; Hayward and Wingfield 2004; Love *et al.* 2005). Conversely we found no such effect of hen body weight on hatch weight of offspring. It may be the level and or timing of CORT exposure may not have been sufficient to cause a response in offspring. The current results indicate fluctuations in yolk CORT concentration may be associated with sex-specific effects on embryonic development, although further research is required to disseminate if the effects observed in embryonic development are the direct result of yolk CORT.

The first few days posthatch are critical in chick development as the major nutrient source transitions from yolk to exogenous feed (Noble and Ogunyemi 1989; Noy and Sklan 1998; Sklan 2001). During this transition, rapid physical and functional development of the gastrointestinal tract occurs (Uni *et al.* 1999). Recent studies demonstrate early feed restriction of broiler chicks induce prolonged metabolic programming in chicks leading to long-term health effects (Zhan *et al.* 2007; Onbaşılar *et al.* 2009; Yang *et al.* 2010). It was therefore predicted that early posthatch dietary restriction would induce programming effects attributed to the PAR effects between generations. Female offspring fed an *ad libitum* diet during the first three weeks posthatch maintained significantly heavier body weight from D14 to D28 and decreased growth rate, demonstrating 5hrs feed restriction daily significantly affected early feed intake, however this was not observed in males. Interestingly, there was a significant interaction of hen body weight and progeny diet on

progeny body weight in males only. *Ad libitum* fed progeny from the Medium BW hens were significantly heavier than progeny from High BW hens as is in keeping with the predicted programming response, whereby offspring from feed restricted mother display rapid growth and increased feed utilisation when in an adequate dietary environment. However at D42 there was no significant difference in body weight due to hen body weight or offspring diet. A similar study by van der Waaij *et al.* (2011) found *ad libitum* fed offspring, particularly females, of restricted mothers were heavier and had greater abdominal fat at six weeks of age than daughters of ad libitum fed mothers. As hens in the High BW group were still maintained at a level of restriction, be it slightly less than the Low BW hens, the differences between hen treatments were likely not extreme enough to elicit a strong programming effect on offspring body weight when placed under *ad libitum* or restrict fed management. Unfortunately, due to logistics on dissection day, abdominal fat pad weight was not measured at D42, which may have identified differences due to mismatch dietary environments.

Although no difference in body weight was found at D42, there was a profound effect of hen body weight on offspring tissue weight, irrespective of early posthatch diet. Offspring from Low and Medium BW hens had significantly lighter duodenum, jejunum, ileum and pancreas weights (Figure 4.5 and Figure 4.6). This demonstrates programming events *in ovo* have dramatic consequences for life-long physiology and negate early posthatch effects. While maternal diet can influence intestinal gene expression in avian offspring (Rebel *et al.* 2006), information on the cellular and molecular changes occurring the avian embryo during early development is sparse and limited to late embryonic development as emergence of villi begins only during the second half of incubation (Uni *et al.* 2003; Karcher and Applegate 2008; Bohórquez *et al.* 2011). Even less is known of the effects of CORT exposure *in ovo* on intestinal morphology, with majority of studies focussed on gross physiology and behavioural effects (Henriksen *et al.* 2011b). In sheep, fetal cortisol surge is responsible for villi proliferation and the induction of digestive enzymes in the gut. Such maturation changes can be prematurely induced by infusion of cortisol into immature foetuses (Silver 1990; Silver 1992). In the neonatal pig, cortisol secretion stimulates highly idiosyncratic expression of

particular brush-border enzymes and intestinal regions (Sangild *et al.* 1995). Mechanisms by which glucocorticoids stimulate brush-border hydrolase activities is unknown. It is possible such glucocorticoid-induced changes in rodents and sheep result from increased enterocyte turnover and replacement of the villus cell population (Trahair *et al.* 1987; Yeh *et al.* 1987; Leeper and Henning 1990).

As previously mentioned, gut maturation occurs earlier than lung, liver and heart in the developing chick, presumably due to it being the primary organ for energy and nutrient supply for growth (Govaerts *et al.* 2000). Additionally, glucocorticoids induce changes in tissue accretion and differentiation resulting in changes in cell size, proliferation rate, terminal differentiation and maturation (Trahair and Sangild 1997; Quaroni *et al.* 1999; Fowden and Forhead 2004). These two factors may account for the significant differences observed in embryonic intestinal weights of offspring dependant on hen body weight. Yolk CORT concentration exposure may have influenced total cell size and maturation during embryonic development. Consequently, early gastrointestinal maturation *in ovo* likely alters posthatch intestinal structure and function, limiting the plasticity and morphological capacity of the intestine. These effects may only become apparent during rapid growth when a high reliance for nutrient absorption occurs. In juvenile broilers, administration of the synthetic glucocorticoid analogue, dexamethasone, significantly alters jejunum mucosal morphology, increasing crypt depth and decreasing villi height, and therefore decreasing absorptive area and capacity (Li *et al.* 2009; Chang *et al.* 2015). Early maturation of intestinal segments *in ovo* resulting in limited plasticity and rapid posthatch growth rate may explain the lower intestinal weights of Low and Medium hen body weight offspring at D42.

Although we observed a change in yolk CORT concentration associated with hen body weight, it is highly likely dietary factors may attribute to the differences in offspring development. Wang *et al.* (2008) suggest cellular signalling defects, redox imbalance, reduced protein synthesis, and enhanced proteolysis may attribute to reduced growth and impaired development of the small intestine, liver, and muscle in IUGR piglets. While rats born to protein-restricted dams observe

higher cell proliferation in all intestinal segments, up-regulation in transporter gene expression and gene expression of intestinal enzymes in offspring (Pinheiro *et al.* 2013a; Pinheiro *et al.* 2013b). Likewise, feed restriction and stress significantly alter other hormones not measured in the current study (Navara *et al.* 2006; Richards *et al.* 2010) and may also influence embryonic and posthatch development (Fowden 1995; Fowden *et al.* 2005; Groothuis *et al.* 2005; Groothuis and Schwabl 2008; Rao *et al.* 2009).

In summary, the results of this study demonstrate relatively small changes in the body weight of the hen not only alters yolk CORT concentration but have life-long detrimental effects on offspring physiology and may therefore influence offspring performance and health. The current results highlight a greater understanding of hen physiology and maternal diet effects in broiler breeders is required and provides an excellent opportunity to improve offspring health and performance in the future. Further studies of the impact of feed restriction in breeder hens commencing from day 1 posthatch should be conducted to remove the confounding effect of starting with hens already differing in body weight at age 24 weeks of age.

# 5. Differences in the nutrient and hormone yolk profile between two genetically diverse commercial poultry strains

## 5.1. Abstract

The role of developmental programming and the impact of the maternal and embryonic environment on subsequent health and performance of progeny is of significant interest in avian models (Hynd *et al.* 2016). Numerous studies demonstrate compounds (i.e. nutrients and hormones) deposited into the avian egg significantly alter offspring development however studies of such mediators generally focus on singular compounds. Therefore the objective of this experiment was to quantify the natural variation in yolk nutrient and hormone deposition from hens differing widely in phenotype which might contribute to large developmental responses. Variables quantified in eggs from broiler (Cobb500) and layer (Isa Brown) birds included: egg weight, albumen weight, yolk weight, concentrations of lipids, protein, glucose, testosterone (TE), corticosterone (CORT), insulin, total triiodothyronine ($T_3$) and total tetraiodothyronine ($T_4$). Both egg weight (60.19 ± 0.29 *vs* 57.58 ± 0.29 g, $P < 0.001$) and yolk weight (16.61 ± 0.12.96 *vs* 12.97 ± 0.07 g, $P < 0.001$) of broilers was greater than that of layers. Yolk glucose concentration was significantly higher in layers (1.93 ± 0.07 vs 1.64 ± 0.07 mg $g^{-1}$, $P = 0.009$) and protein concentration displayed a similar trend ($P = 0.16$). The higher yolk glucose concentration was accompanied by a higher insulin concentration in layer yolk compared to broiler yolk (1.28 ± 0.03 *vs* 1.03 ± 0.031 pg $mg^{-1}$ respectively, $P < 0.001$). Broiler yolk contained higher concentrations of TE (0.64 ± 0.03 *vs* 0.55 ± 0.03 pg $mg^{-1}$ respectively, $P < 0.05$) and a near two-fold higher level of $T_4$ (4.52 ± 0.12 *vs* 2.36 ± 0.13 pg $mg^{-1}$ respectively, $P < 0.001$). CORT and $T_3$ concentrations tended to be higher in broiler yolk. Results highlight the yolk profile is heavily dependent on bird strain. The extent to which differences identified are genetic or environmental in origin is unclear due to confounding diet and genotype effects. Dissecting the individual genetic and maternal environmental effects on yolk deposition may provide a greater understanding of the role of yolk composition in embryonic reprogramming and underlying mechanisms.

## 5.2. Introduction

Selection within the poultry industry has focussed largely on post-hatch phenotype characterised by rapid growth rate and body weight in broilers, and prolific egg production in layers. It has generally been considered that these outcomes largely reflect the impact of the genetics of the progeny on physiology and production. However it is becoming increasingly recognised that there are significant effects of the *in ovo* environment on embryological development, e.g. see Ho *et al.* (2011). Indeed some of the maternal genetic effects on progeny performance may reflect differences in the environment that is established *in ovo* (i.e. the yolk and albumen characteristics). Physiological differences between the two breeds have been identified as early as 60-84h post-conception (Ho *et al.* 2011). Divergence in the physiological and metabolic processes during embryonic development, particularly during the rapid growth phase, include changes in protein accumulation, lipid metabolism, heat production and $O_2$ consumption and respiration (Ohta *et al.* 2004; Sato *et al.* 2006; Everaert *et al.* 2008; Druyan 2010). Such differences are associated with changes in gene regulation of myogenic differentiation in muscle establishment and energy homeostasis, setting the stage for breed-specific phenotypes post-hatch (Yuan *et al.* 2009; Al-Musawi *et al.* 2011).

Until recently the divergence in embryonic development between breeds was thought to reflect genetically-driven differences in the absorption and metabolism of yolk lipids (Sato *et al.* 2006). However, Ho *et al.* (2011) recently identified egg yolk (irrespective of egg and albumen) expresses breed-specific programming effects, with physiological traits (i.e. heart rate, body weight and development rate) reflecting yolk source rather than embryo genotype. The yolk environment between breeds was associated with differences in yolk testosterone (TE) and triiodothyronine ($T_3$) concentration analysed. It appears yolk exclusively can reprogram developmental trajectories of physiological systems and potentially alter post-hatch performance and health.

Maternally-derived nutritional and endocrine factors have been intensively studied for their developmental programming effect on offspring morphology, physiology and behaviour (Miller *et*

*al.* 2002; Uni and Ferket 2004; Groothuis *et al.* 2005; Groothuis and Schwabl 2008; Everaert *et al.* 2013). Differences in yolk hormone concentrations are well known to impact phenotypic expression in chickens (Groothuis *et al.* 2005). Moreover, hormone concentrations are generally much higher in yolk than in albumen (Ahmed *et al.* 2013). Increased yolk corticosterone (CORT) results in slower-growing chicks (Hayward and Wingfield 2004), whereas TE and insulin appear to have stimulatory effects on embryonic growth and differentiation (De Pablo *et al.* 1991; Schwabl 1996b). High thyroxine ($T_4$) content in quail eggs is associated with accelerated differentiation and growth of embryonic pelvic cartilage (McNabb and Wilson 1997). Similarly, nutrient concentrations can directly impact embryonic development, with lipid, glucose and amino acid utilisation critical to growth and muscle protein accumulation (Uni *et al.* 2005; Sato *et al.* 2006; Yair *et al.* 2013). Previous studies indicate yolk compounds associated with embryonic development vary significantly between breeds, however direct comparisons have generally been limited to one or two compounds. In addition, a critical period between 48 and 60 h is suggested in embryonic chick in which the yolk environment has a large impact on the rate of development, implying early exposure to yolk factors may alter phenotypic expression (Ho *et al.* 2011). In this study we aimed to identify key differences in the yolk profile prior to on-set which may in part contribute to the divergence in embryonic development between breeds. Therefore a range of hormones and nutrients associated with embryonic development and developmental programming were measured in yolk of broiler breeder and layer hens. It was hypothesised the hormone and nutritional profile of yolk will differ dramatically between broiler and layer breeds.

## 5.3. Materials and methods

### 5.3.1 Egg collection

Fertile (Appendix; Figure 1) eggs (*n* = 400) were collected from Isa Brown Layer and Cobb 500 Broiler Breeder hens at 35 weeks of age from commercial breeder companies Hi-Chick Breeding Company Pty Ltd (Bethel, SA, Australia), and Baiada Poultry Pty Ltd (Gawler, SA, Australia), as such hens were housed and fed under normal commercial conditions. All eggs were stored at 4°C until individually weighed and the yolk and albumen separated and weighed separately within 24 hrs. In brief, the egg shell was removed and yolk separated from albumen using a yolk separator. Albumen weight was recorded. Yolk was placed on gauze to remove residual albumen and then suspended in fresh gauze and the yolk membrane pierced. Yolk was gently massaged from the yolk sac to remove yolk contents and the weight of yolk recorded. Yolk was homogenised for 30 seconds and a 15mL sample of yolk was collected and then stored at -20°C. Individual yolk (5 mL) samples were pooled based on stratified egg weights to represent uniform egg weight distribution and then re-homogenised. Pooled samples were stored at -20°C to be used for nutrient and hormone analysis (*n* = 20 per breed).

### 5.3.2 Nutrient analysis

#### 5.3.2.1. Dry matter

Yolk samples (0.2 g) were transferred to glass tubes (pre-weighed) and dried in a 105°C oven for DM analysis. Tubes with the remaining DM were placed in a dessicator overnight and then weighed to calculate the percentage of water.

#### 5.3.2.2. Lipid

Total lipids were extracted using a modified chloroform: methanol extraction method (Siebert *et al.* 2006). Two mL of distilled water was added to 0.5 g yolk with two glass beads and vortexed until homogenised. Nine mL of chloroform-methanol (2:1 vol/vol) was added and shaken vigorously. Samples were allowed to stand for separation and then centrifuged at 2,000 rpm for 5 min. The upper layer was removed by aspiration and the remaining layer transferred to glass tubes

and dried under $N_2$ in a 40°C water bath. Lipid content was represented as weight of lipids per g of yolk.

#### 5.3.2.3. Protein

Yolk samples (0.1 g yolk) were weighed into separate microcentrifuge tubes with 1mL distilled water and two glass beads and vortexed for 2 min. Sample homogenates were diluted to 1:50 using distilled water containing 0.01 % Tween 20 (P1379, Sigma, St Louis, MO). A 5 µL aliquot of the diluted homogenate was transferred to a 96 well ELISA plate and dye reagents added (Bio - Rad protein assay, Hercules, CA). A standard curve was generated using dilutions of BSA standard solution. The absorbance was measured at 595 nm and the weight of protein per g of yolk calculated. Intra-assay coefficient of variation for yolk protein was 15%. Duplicate samples were all analysed in a single assay.

#### 5.3.2.4. Glucose

Yolk samples (0.1 g yolk) were weighed into separate microcentrifuge tubes with 1mL distilled water and two glass beads and vortexed for 2 min. Sample homogenates were diluted to 1:20 using distilled water containing 0.01 % Tween 20 and vortexed for 2 min. A 50 µL aliquot of the diluted sample was transferred to a 96 well ELISA plate and 200 µL enzymatic glucose reagent added (Sigma, St Louis, MO), incubated for 30 min at 37°C and the absorbance measured at 595 nm. A standard curve was generated and the weight of glucose per g of yolk calculated accordingly. Intra-assay coefficient of variation for yolk glucose was 10%. Duplicate samples were all analysed in a single assay.

### 5.3.3 Hormone analysis

#### 5.3.3.1. Testosterone and corticosterone

A modified extraction procedure of Cook *et al.* (2009) was used to prepare both TE and CORT egg yolk samples. In brief, 0.1 g of yolk was placed in a 13 mL glass conical tube with 0.5 mL of distilled water, and vortexed with two glass beads to ensure a homogenous suspension. Five mL of petroleum ether: diethyl ether (30:70 vol/vol) was added and vortexed for 1 min. The aqueous

phase was snap-frozen and the ether phase decanted into fresh tubes. The ether extraction was dried down under a stream of $N_2$ then resuspended in 90 % ethanol and incubated at -80°C overnight. Thawed samples were centrifuged at 2500 rpm for 5 min and decanted into fresh tubes. Hexane (2 mL) was added and gently vortexed for 1 min and then centrifuged for 5 min at 2500 rpm. The hexane layer was discarded and ethanol phase dried down under a stream of $N_2$. Sample precipitate was resuspended in 500 µL of PBS (supplemented with 0.1% Gelatin). Extraction efficiency was calculated at 70%. Yolk TE and CORT concentrations were measured with a RIA using corticosterone (27840-100mg, Sigma Aldrich Pty Ltd, Sydney, AUS) and testosterone (108T-077, Sigma Aldrich Pty Ltd, Sydney, AUS) as standards. The assay utilized [$^3$H]-corticosterone and [$^3$H]-testosterone (Amersham Pharmacia Biotech, UK, Buckinghamshire HP, England) as tracers. Limit of detection was 0.01ng $mL^{-1}$ and the intra-assay coefficient of variation below 8% for TE. Limit of detection for CORT was 0.02ng $mL^{-1}$ and the intra-assay coefficient of variation below 7%. Duplicate samples were all analysed in a single assay.

#### 5.3.3.2. Insulin

Insulin was extracted from the pooled yolk samples using a modified procedure by De Pablo *et al.* (1982) to increase extraction efficiency. Samples were diluted by adding 0.1 g of yolk in 50 µL distilled water and vortexed. One mL of ice cold 0.2 M HCL: 75% ethanol was added and shaken (150 oscillations per min on a shaker) at 4°C overnight. Samples were then centrifuged at 3000 rpm for 20 min at 4°C and the supernatant decanted into fresh tubes and concentrated to 1/20$^{th}$ of the starting volume by air evaporation at room temperature. Sample precipitate was resuspended in 500 µL of 0.05 M ammonium carbonate and neutralised using sodium hydroxide and centrifuged at 3000 rpm at 4°C for 20 min. The supernatant was decanted into fresh tubes and lyophilised by freeze drying. Samples were resuspended in deionised water and insulin concentration measured using the protocol described by McMurtry *et al.* (1983). The limit of detection was 0.1ng $mL^{-1}$ and the intra-assay coefficient of variation below 8%. Duplicate samples were all analysed in single assay. Extraction efficiency was 66%. Yolk insulin concentrations were measured with RIA using

insulin (donated by JP Murtry US Department of Agriculture, Co. USA) as a standard. The assay utilized [$^3$H]-insulin (Amersham Pharmacia Biotech, UK, Buckinghamshire HP, England) as a tracer.

#### 5.3.3.3. Triiodothyronine and tetraiodothyronine

The same extraction procedure of Ho *et al.* (2011) was used prior to RIA detection. Samples were analysed using a competitive binding Total $T_3$ RIA Kit (IM3287) and a Total $T_4$ RIA Kit (IM3286) purchased from BeckmenCoulter (Immunotech, Prague, Czech Republic). The manufacturer's instructions provided with the kit were followed exactly. The limit of detection for $T_3$ was 0.5nm $L^{-1}$ and the intra-assay coefficient of variation below 8%. The limit of detection for $T_4$ was 6.5nm $mL^{-1}$ and the intra-assay coefficient of variation below 7.5%. Duplicate samples were all analysed in a single assay.

### 5.3.4 Statistical analyses

Regression analysis was used to quantify the relationships between total egg weight and egg component weight within breeds. Total egg and egg component weights between breeds was analysed using a General Linear Model with egg weight fitted as a covariate for both yolk and albumen variables (SPSS 18.0, SPSS Inc, Chicago, IL). Differences in yolk nutrient and hormone concentrations between breeds were analysed using a General Linear Model. As pooled yolk samples were used to measure yolk nutrient and hormone concentrations, egg weight was not fitted as a covariate in the model therefore only concentrations are reported and total egg levels not calculated. Statistical significance was accepted at $P < 0.05$.

## 5.4. Results

### 5.4.1 Egg components

Egg albumen and yolk weights were directly related to total egg weight for both broiler and layer breeds (Figure 5.1). Total egg weight was significantly higher in broilers than layers (60.19 ± 0.29 g and 57.58 ± 0.29 g respectively, $P < 0.0001$). Similarly, yolk weight of broiler eggs was significantly higher than that of layer eggs (16.61 ± 0.07 *vs* 12.96 ± 0.07 g respectively, $P < 0.0001$). The opposite was observed for albumen weight with layer eggs significantly higher albumen weight than broiler eggs (35.48 ± 0.1 vs 33.46 ± 0.1 g respectively, $P < 0.0001$). Regression models for layer egg components are as follows: $W_{yolk} = 0.23W_{el} - 0.53$; $r^2 = 0.48$ and $W_{albumen} = 0.67M_{el} - 3.92$; $r^2 = 0.82$. Regression models for broiler egg components are as follows: $W_{yolk} = 0.24M_{eb} + 2.75$; $r^2 = 0.47$; $W_{albumen} = 0.60M_{eb} - 1.77$; $r^2 = 0.73$.

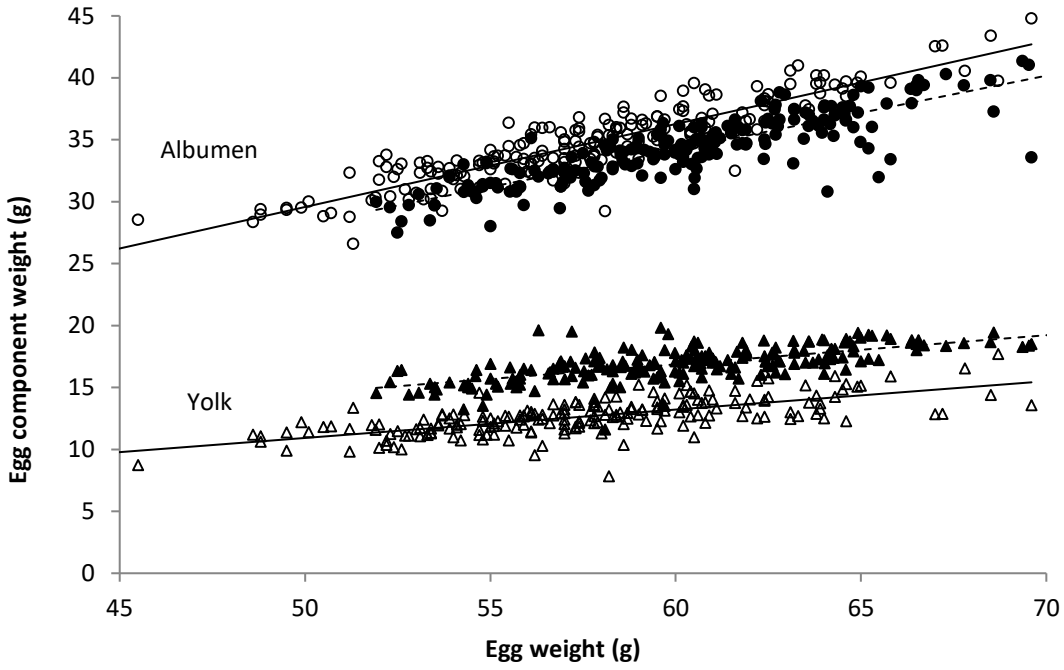


**Figure 5.1 Egg component weight of broiler and layer eggs as a function of egg weight (● = albumen, ▲ = yolk). Filled symbols represent broiler egg components and open symbols represent layer egg components. Broken lines (broiler) and solid lines (layer) through data points indicate best fit regression of the weight of each egg component and egg weight.**

### 5.4.2 Yolk nutrients

Results of the yolk nutrient analysis of pre-incubated pooled broiler and layer chicken eggs are shown in Table 5.1. Yolk glucose concentration was significantly higher in layer eggs than broiler eggs (1.93 ± 0.07 *vs* 1.64 ± 0.07 mg $g^{-1}$ respectively, $P < 0.05$). Yolk protein concentration displayed a similar trend as glucose, with layer eggs exhibiting higher concentrations than broiler eggs however was not significant. Yolk lipid and water content were not significantly different between breeds.

**Table 5.1 Nutrient and hormone concentration in the yolk of pre-incubated eggs from broiler breeder and layer hens. Data are expressed as means ± SEM.**

| | Broiler | Layer | *P*-Value |
|---|---|---|---|
| DM, % | 49.74 ± 0.16 | 49.33 ± 0.20 | 0.150 |
| Lipid, mg/g | 308.15 ± 0.43 | 306.40 ± 0.46 | 0.782 |
| Protein, mg/g | 156.66 ± 6.01 | 168.95 ± 6.17 | 0.162 |
| Glucose, mg/g | 1.64 ± 0.07 | 1.93 ± 0.07 | 0.009 |
| Insulin, pg/mg | 1.03 ± 0.03 | 1.28 ± 0.03 | 0.000 |
| $T_3$, pg/mg | 0.28 ± 0.01 | 0.271 ± 0.01 | 0.566 |
| $T_4$, pg/mg | 4.52 ± 0.12 | 2.36 ± 0.13 | 0.000 |
| TE, pg/mg | 0.64 ± 0.03 | 0.55 ± 0.03 | 0.016 |
| CORT, pg/mg | 17.43 ± 0.53 | 16.76 ± 0.53 | 0.371 |

### 5.4.3 Yolk hormones

A statistical interaction between breed and yolk hormone was detected for specific yolk hormones. Yolk $T_4$ concentration of broiler eggs was significantly higher than that of layer egg yolk by approximately two-fold (4.52 ± 0.12 *vs* 2.36 ± 0.13 pg $mg^{-1}$ respectively, $P < 0.0001$; Table 5.1). No significant difference in yolk $T_3$ concentration was detected between breeds. Yolk TE concentration was significantly higher in broiler eggs at 0.64 ± 0.03 pg $mg^{-1}$ than layer egg yolk at 0.55 ± 0.03 pg

$mg^{-1}$ ($P < 0.05$). In contrast, yolk insulin was significantly higher in layer eggs than observed in broiler eggs (1.28 ± 0.03 *vs* 1.03 ± 0.03 pg $mg^{-1}$ respectively, $P < 0.001$; Table 5.1). Although yolk CORT showed a similar trend to $T_4$ and TE, no significant difference was detected between breeds.

## 5.5. Discussion

The objective of this study was to quantify differences in the content of compounds likely to influence embryonic development in birds differing widely in phenotype. No attempt was made to separate genetic from environmental (e.g. hen nutrition) effects on egg composition as it is the combination of the two that is likely to influence the embryonic environment effect demonstrated by Ho et al. (2011). We have compared broiler and layer eggs to establish a more-detailed analysis of the egg and yolk profile focusing on compounds likely to influence embryonic development.

Initial egg weight is the primary determinant of chick weight, with incubation egg weight loss, incubation time and conditions, shell and residue weight, breeder age, strain and sex all secondary determinants (Wilson 1991; Dzialowski and Sotherland 2004). In accordance with previous studies, we found broiler hen egg weight was significantly greater than that of layers and this may contribute to hatch weight differences between breeds previously observed (Everaert *et al.* 2008; Druyan 2010). Ho *et al.* (2011) and Ohta *et al.* (2004) both reported no significant difference in egg weight between breeds, however neither studies stated hen age at time of collection therefore discrepancies in egg weight between studies are likely confounded by environmental conditions and hen age (Yadgary *et al.* 2010).

In keeping with previous studies, broiler eggs contained significantly greater yolk and less albumen than layer eggs (Sato *et al.* 2006; Ho *et al.* 2011; Nangsuay *et al.* 2015). Factors attributing to yolk weight differences are likely numerous, however, hen genotype is known to significantly influence yolk deposition. Miyoshi and Mitsumoto (1980) and Hartmann *et al.* (2000) both demonstrate a selection response for yolk proportion, whereby yolk-to-albumen ratio and yolk as a percentage of total egg weight was altered by selection. Additionally, genotypic differences in yolk metabolism are established during embryonic development and may contribute to a greater capacity for yolk deposition in the adult broiler during ovulation. By ED18 broilers maintain greater yolk consumption and absorption than layers, reflected in heavier heart, liver and pectoral muscle weights (Ohta *et al.* 2004; Sato *et al.* 2006; Everaert *et al.* 2008; Al-Musawi *et al.* 2011; Nangsuay *et*

*al.* 2015). Additionally, significant changes in hypothalamic gene expression relating to energy homeostasis and obesity differences can also be observed (Pal *et al.* 2002; Sato *et al.* 2006; Everaert *et al.* 2008; Yuan *et al.* 2009; Druyan 2010). The apparent accelerated fat utilisation and energy conversion efficiency of broilers may be conducive to increased yolk weight observed.

Yolk weight *per se* appears to have little impact on embryonic development, but yolk composition can significantly influence neonatal quality (Finkler *et al.* 1998; Ho *et al.* 2011). Yolk is the primary source of maternally-derived macromolecules including; hormones, nutrients, carotenoids and antibodies, hence critical to physiological development (Schwabl 1993; McNabb and Wilson 1997; Bellairs 2005; von Engelhardt and Groothuis 2011; Yadgary and Uni 2012). Originating from the maternal diet, these resources can exert large effects on embryo viability with levels highly influenced by genetic variation in absorption, metabolism and deposition (Lillie *et al.* 1951). As expected we found significant differences in both the hormone and nutrient yolk profiles between breeds.

Egg nutrients severely impact late-term embryonic and hatchling development as studies using *in ovo* supplementation highlight nutrients, such as carbohydrates and proteins, initiate strong physiological and morphological effects on embryonic development (Uni and Ferket 2004; Smirnov *et al.* 2006; Kornasio *et al.* 2011). In the current study glucose concentration was significantly higher in layer yolk compared to that of broilers, and while not significant, protein concentration displayed a similar trend. In avians, insufficient glycogen levels force mobilisation of muscle protein for gluconeogenesis, thereby reducing early embryonic growth and development (Kornasio *et al.* 2011). *In ovo* supplementation of carbohydrates and β-hydroxy-β-methylbutyrate in broiler breeder chicks has been shown to improve liver glycogen by 2- to 5-fold, elevate relative breast muscle size by 6 to 8%, and, increase hatching weights by 5 to 6%, (Uni *et al.* 2005). It is thought elevating glycogen levels *in ovo* reduces the reliance on glucose from gluconeogenesis, minimising muscle protein utilisation and increasing pectoral muscle weight percentage.

Breed differences in yolk glucose and protein concentrations are influenced by hen genotype and the environmental conditions prevailing during egg laying. Braun and Sweazea (2008) observed a negative relationship between plasma glucose concentration and body weight of birds, hence the lower body weight of layers may be associated with higher circulatory glucose levels, which, in-turn, may be reflected in elevated concentrations of glucose in the yolk. The greater rate of muscle deposition in broiler birds may also increase glucose and protein requirements, thereby lowering plasma concentrations in broiler breeder hens. While genetically-driven differences in yolk nutrient concentrations are therefore likely, they are confounded by the hen environment and particularly her nutritional status. Prolonged feed deprivation in broiler breeders can decrease plasma protein and albumen concentrations (Rajman *et al.* 2006). Interestingly, it does not appear to affect plasma glucose, due presumably to glucose homeostatic mechanisms. This suggests yolk protein concentration is heavily influenced by maternal diet, while yolk glucose concentrations may be predominantly genetically driven. Verification of this speculation will require an experimental design that allows the effect of genotype and nutritional environment to be separated using a crossover design in which the two strains are offered the two dietary regimes typically used for each strain.

Levels of maternal hormones in egg components during avian embryological development are important because the embryo has limited capacity to produce its own endogenous hormones (De Pablo et al 1982). Yolk hormones concentrations and their effects on development have been extensively studied (Schwabl 1993; Groothuis *et al.* 2005; Gil 2008b; Müller *et al.* 2012). Of the hormones known to influence early development, we found $T_4$, TE and insulin concentrations significantly differed between broiler and layer yolks.

The significantly higher TE level in broiler yolk than layer is in accordance with previous findings and may contribute to phenotypic differences observed during offspring development (Ho *et al.* 2011). Yolk TE levels were lower than reported in previous studies however numerous factors contribute to deposition including; strain, breeds, age and other hormones alter yolk TE

concentrations (Schwabl 1993; Henriksen *et al.* 2011a). Studies indicate increased yolk TE is associated with increased overall body size, muscle mass and metabolic rate at hatch, characteristics typical of the broiler phenotype (Schwabl 1996b; Lipar and Ketterson 2000; Eising *et al.* 2001; Groothuis *et al.* 2005). Additionally, a 2-fold difference in yolk $T_4$ levels was also detected. Yolk $T_4$ concentration was significantly higher in broilers and in accordance with levels previously reported (Hilfer and Searls 1980; Sechman and Bobek 1988; Prati 1992). Elevated yolk $T_4$ concentration has been associated with accelerated differentiation and growth of embryonic pelvic development in Japanese quail (McNabb and Wilson 1997). The higher levels in yolk TE and $T_4$ of broilers is likely to contribute to the divergence in growth and development, both pre-and-post hatch, of broiler and layer breeds.

In contrast, insulin levels were significantly higher in layer yolk than in broiler yolk. This may reflect breed differences in the nutritional status and metabolic regulation of the hen. As insulin regulates the abundance of circulating glucose in the hen's circulatory system, it is therefore unsurprising that, in keeping with yolk glucose differences, insulin levels were also significantly greater in layer yolk.

Our results demonstrate that layer and broiler embryos are subjected to markedly different nutritional and hormonal yolk environments during the critical phases of embryological development. Such differences reflect a combination of maternal environmental effects (Schwabl 1996a; Gil *et al.* 1999; Verboven *et al.* 2003; Tschirren *et al.* 2004) and maternal genetic effects (Okuliarova *et al.* 2011) and have been shown to be important determinants of the phenotype of the progeny (Ho *et al.* 2011). There is significant scope for manipulating the *in ovo* environment by altering the genotype and environment of the hen (Hynd *et al.* 2016). The differences in nutrient and hormonal profiles of the eggs of layers and broilers presented in the present chapter provide a baseline for future epigenetic and developmental studies in avians.

# 6. General discussion

The experiments described in this thesis underpin a novel and potentially-economically valuable phenomenon for the poultry industries. Evidence is presented that the genotype and management of hens can influence the circulating levels of hormones and the composition of their eggs and that these changes coincide with marked physiological changes in their offspring post-hatch. The experiments within this thesis identify developmental programming potential in avian species in a context relevant to current broiler breeder hen management. The overarching hypothesise of this thesis was firstly; manipulation of the breeder hen diet signals changes to the developing embryo via yolk composition and initiates developmental programming effects, and secondly; phenotype of the offspring is heavily influenced by the combined effect of both the maternal diet and offspring diet, defined as 'Predictive Adaptive Response' (PAR). The individual projects of this thesis demonstrate dietary grain source and physiological state (via hen body weight) can induce programming in a 'mismatched' environment, potentially via yolk signalling compounds such as microRNAs, hormones and nutrients. Such fluctuations in yolk composition are associated with developmental programming effects *in ovo* and can alter adult offspring growth long-term. These results signify epigenetic mechanisms have significant potential to improved productivity and welfare in the poultry industry.

The maternal diet elicits strong programming effects during both embryonic and posthatch development. Recently, it has been proposed that the degree to which these programming effects are expressed during adulthood vary depending on the insult and the extent to which the post-hatch environment is correctly (or incorrectly) anticipated. This phenomenon, in which maternal diet dictates and programs offspring performance, has significant implications for the chicken meat industry given the marked mismatch between the diet of the hen and that of their offspring. In the case of the broiler chicken, the post-hatch nutritional environment rarely reflects that anticipated by the broiler breeder hen. This thesis highlights the profound effect dietary management practices currently imposed on the broiler breeder hen have on programming the developing embryo and subsequent effects on lifetime performance under the PAR model. It also identifies that dietary

changes of the broiler breeder greatly influences the physiological status of the hen and associated with fluctuations in various biological compounds in within the yolk.

## 6.1. Maternal diet alters physiological state of hen and egg composition

Maternal dietary insults, in particular effects of undernutrition or overnutrition, initiate strong programming responses in the developing neonate (Barker 1997; Tzanetakou *et al.* 2011; Chango and Pogribny 2015). Fluctuations in maternal diet result in altered metabolic, endocrine and health status of the mother, therefore potentially altering nutrient and hormone exposure to the developing embryo via the egg. In Chapter 4 it was found changes in hen body weight associated with feed allocation alters circulating corticosterone concentration and reflected in corticosterone concentrations of the yolk, as has previously been reported (Henriksen *et al.* 2011a). Surprisingly, reducing feed allocation by as little as 15g per day (or altering average body weight by 400g) increased serum corticosterone concentrations, identified between the low body weight hens compared to those of the heavier body weight group and on slightly greater feed allocation. This implies even slight changes in dietary intake can illicit significant physiological changes in the hen, yolk composition and potentially the programming of offspring.

While it was observed low maternal body weight resulted in increased serum and yolk corticosterone concentrations and influence developmental programming, other hormones have also previously been attributed to changes in offspring behaviour and physiology which may be associated with maternal diet (Groothuis and Schwabl 2008; Henriksen *et al.* 2011b). Profiling of yolk hormones and nutrients in Chapter 5 identified differences between the two most divergent commercial poultry strains, expanding on potential epigenetic mechanisms outlined previously by Ho *et al.* (2011). Interestingly, broiler breeder eggs profiled in Chapter 5 displayed similar differences in yolk testosterone, corticosterone and thyroid hormones to those previously observed in the blood of feed-restricted broiler breeder hens (Kubíková *et al.* 2001; Rajman *et al.* 2006). As yolk hormones of commercial breeder hens (Chapter 5) paralleled similar trends to circulating levels previously reported in feed-restricted breeder hens, the yolk hormone profile of commercial

breeder hen, presumably exposed to feed restriction conditions, likely reflect the impact of both strain and management on circulating levels. These hormones are known to influence embryonic development and therefore are prime candidates as potential programming factors within the yolk which are not currently considered in commercial broiler production.

Feed restriction in hens has also been found to alter other hormones and nutrients including $T_3$, $T_4$, triacylglycerol and cholesterol (Rajman et al 2006, Kubikova et al 2001). In this context, it is interesting to note the significant difference in glucose and insulin concentration of broiler and layer yolks identified in Chapter 5, and implies potential differences in maternal glucose regulation effects between strains, or dietary management. Maternal glucose regulation is typically influenced by physiological changes during gestation and maternal nutrition in mammals (Tzanetakou *et al.* 2011). Likewise, changes in carbohydrate type and fibre content influence glycaemia attributing to abnormal hyperglycaemia and attribute to impaired glucose tolerance in offspring. These maternally-derived factors may results in altered metabolic profile of the yolk and reprogramming of glucose regulation in offspring. Evidence suggests repeated maternal insulin resistance and glucose intolerance is 'predominant' enough to be transmitted transgenerational (Reusens et al 2001). In Chapter 4, in which maternal body weight was altered via feed restriction, unfortunately only yolk corticosterone was measured. Measurement of other hormones, particularly metabolic hormones would have gone some way to identifying the effects of maternal body weight and feed allocation on glucose regulation in the offspring and may be highly relative to broiler production considering the mismatch between feed restricted breeder hens and *ad libitum* fed progeny.

When considering endocrine mechanisms contributing to developmental programming, it is worth considering the disparities in yolk hormone concentrations consistently found in the literature. In this thesis, three key factors were highlighted which may attribute to differences in yolk hormone levels and potential reasons for disparities. Firstly, understanding of the mechanisms, pathways and processes by which hormone deposition into the egg occurs and the regulatory capacity of the hen to manipulate deposition and interactions with other compounds is required

and has been a point of discussion in the literature (Groothuis and Schwabl 2008). Studies of placental regulation of hormone and nutrient uptake into the mammalian fetus have been invaluable in understanding links between maternal regulation, fetal development and offspring health (Jansson and Powell 2007). Secondly, as identified in Chapter 4, changes in concentrations may occur over time, suggesting an acclimatisation to the maternal diet/stress imposed, or a change in physiology due to other parameters such as sexual maturity. It is therefore worth consideration when identifying fluctuations in physiological state, the potential 'acclimatisation' response in hens as this may impact on the level of exposure to the progeny. Finally, methods of hormone analysis in yolk are varied and appear to significantly influence results due to the extraction efficiency and technique used and must be a consideration for future analysis (Henriksen *et al.* 2011b).

Chapters 3 to 5 highlight the extent to which maternal diet and strain effect total egg and associated yolk and albumen weights. While egg component weight is acknowledged to correlated to hatch weight and post-hatch performance, permanent embryonic programming events are more likely attributed to compounds deposited into the egg rather than egg, yolk and albumen weight *per se*. Residual yolk is generally present in the chick at hatch suggesting in general, the quantity of yolk is not a limiting factor during embryonic development, and therefore unlikely to initiate significant programming effects during embryonic development (Romanoff 1960). Additionally, changes in embryo phenotype have been demonstrated to reflect yolk type, irrespective of genotype (Ho *et al.* 2011). The epigenetic modulators in yolk have yet to be conclusively identified but will almost certainly include a suite of hormonal, nutritional and microRNA components working together to alter embryonic gene expression, in turn altering physiological 'set points' in the offspring. Recently, microRNAs have been detected in yolk and a prime candidate of epigenetic signalling in the embryo (Wade *et al.* 2016). Results in Chapter 3 expand on these findings; signifying differences in yolk microRNA expression can be attributed to changes in the maternal diet. While preliminary, these results are significant as they reveal the maternal diet can significantly influence epigenetic regulatory factors present in the yolk. As discussed in Chapter 3, changes in microRNA

expression level can occur in the yolk by simply altering dietary grain source. While results are preliminary, the microRNAs detected have previously been identified in the chicken embryo development and various reproductive processes of the hen. Combined with yolk data from Chapters 4 and 5, these results highlight the maternal diet can significantly alter epigenetic regulatory factors present in the yolk and are particularly important when identifying mechanisms of maternal dietary developmental programming.

## 6.2. Impact of maternal diet on embryonic development initiated *in ovo*

Fluctuations in the fetal/*in ovo* environment have previously been attributed to a range of endocrine, metabolic, physiological and functional adaptations in the developing embryo and fetus. Evidence suggests maternal undernutrition alters fetal hypothalamic-pituitary-adrenal (HPA) axis development and function via increased exposure to glucocorticoids (Oliver *et al.* 2007). In Chapter 4, a link between maternal body weight, yolk corticosterone and changes in gross body and organ weight of the developing embryo was observed in offspring. Differences in organ weight could be identified as early as embryonic day (ED) 14 (proventriculus, abdominal fat pad and body weight), and also during later stages of development (ED20) affecting the duodenum, breast muscle and gizzard weight. Interestingly, these responses in organ and tissue weight appear to be sex-dependant. Research relating to corticosterone exposure on embryonic phenotype is limited, with most studies related to posthatch development (Henriksen *et al.* 2011b). Findings in Chapter 4 are the first to our knowledge, to identify corticosterone related programming effects on embryonic organ and tissue development. Research indicates limiting dietary protein in hen alters 20-hydroxysteroid dehydrogenase (20-HSD) mRNA in the yolk sac membrane and in addition to 20-HSD glucocorticoid receptors, thyrotropin-releasing hormone and leptin receptor mRNA at ED14 (Rao et al. 2009). Similarly, yolk and albumen corticosterone concentration alters hepatic mRNA expression of 11β-HSD1 and 11βHSD-2 in the developing embryo (Ahmed *et al.* 2013). These findings suggest corticosterone sensitivity *in ovo* may result in phenotypic changes during embryonic development with consequences for post-hatch phenotype.

Differences in the duodenum, jejunum and ileum weight relative to body weight in adult progeny (D42) was, somewhat surprisingly, attributed to hen body weight. In addition, changes in jejunum weight and body weight at D42 were attributed to an interaction effect of hen body weight and offspring diet in male adult offspring. This effect on small intestinal weight observed in adult progeny is a significant finding, indicating alteration of enteric development *in ovo* causes permanent long-term effects on the gastrointestinal tract in progeny. Recent studies highlight the great potential *in ovo* feeding technologies have on enteric development as a means to improve chick vitality and performance. Supplementation via the amnion during *in ovo* development is acknowledged to increase relative weight of the small intestine (Bhanja *et al.* 2008), jejunal villi height, surface area, goblet cell density, mucin gene expression and improved disaccharide digestion (Tako *et al.* 2004; Uni and Ferket 2004; Smirnov *et al.* 2006). Furthermore, maturation of the gastrointestinal tract occurs earlier than lung, liver and heart in the developing chick embryo, presumably due to it being the primary organ for energy and nutrient supply for growth, and suggests enteric development may be particularly sensitive to fluctuations in the *in ovo* environment (Govaerts et al. 2000). Results of Chapter 4 expand on the current literature to suggest altered enteric development *in ovo* may not only influence early post-hatch performance but causes long-term effects, and may arise due to maternal effects. Duodenum weight was significantly influenced by maternal body weight during both embryonic development (ED20) and adulthood (D42). Furthermore, the increase in ED20 duodenum weight in embryos of low and medium body weight hens coincided with increased yolk corticosterone concentration. Glucocorticoids are known to influence tissue accretion and differentiation, initiating changes in cell size, proliferation rate, terminal differentiation and maturation and may therefore result in hyperplasia and/or hypertrophy (Trahair and Sangild 1997; Quaroni et al. 1999; Fowden and Forhead 2004). In Chapter 4 only gross weight was measured, therefore to fully quantify potential maternally-derived and/or long-term effects, ascertaining morphological and functional changes of the small intestine both during embryonic and at D42 is warranted.

Differences in embryonic body weight were also attributed to hen body weight, with male embryos from low body hens tending to be lower in body weight at ED14 observed in Chapter 4. This effect has also been reported in mammals with maternal undernutrition decreasing fetal weight during early gestation (Vonnahme *et al.* 2003). In mammals, this occurs as a result of a 'glucose sparing effect' due to the developing fetus requiring large quantities of glucose, primarily as an energy source. As a result the fetus prioritises glucose allocation to the early-maturing organs deemed vital during embryonic development. In the embryonic chick, carbohydrate (CHO) concentration is critical prior to emergence from the shell, with very little CHO remaining in the egg before hatch (Christensen *et al.* 1993). As the amount of CHO in the egg is very low, gluconeogenesis from protein is the source of glucose for accumulation of glycogen that eventually fuels the hatching activities (Klasing 1998). Glucose supplementation at later stages of embryonic development not only improved the broiler chick weight but also help in the development of digestive tract (Bhanja *et al.* 2008; Bhanja *et al.* 2015). Interestingly, lower glucose and insulin levels were observed in broiler breeder yolk compared to layer yolk in Chapter 5. It could be speculated broiler breeder hen dietary management and breed may attribute to decreased glucose concentration in the yolk, resulting in phenotypic difference during embryonic development and the hatch weight of broiler and layer offspring.

The yolk environment can significantly alter embryonic development, attributing to differences in phenotypic expression of the broiler and layer embryo (Ho *et al.* 2011). Fluctuations in yolk hormone concentrations have been linked to the maternal environment and recognised to alter embryonic development and offspring phenotype (Groothuis *et al.* 2005; Groothuis and Schwabl 2008; Müller *et al.* 2012). Furthermore, supplementation of CHO and amino acids *in ovo* result in significant effects on embryonic growth and development, implying nutrients in the broiler egg environment may be lacking at sufficient levels to optimise embryonic development (Uni and Ferket 2004; Uni *et al.* 2005; Yair *et al.* 2013). The differences in nutrient and hormone profile of layer and broiler breeder hen yolk in Chapter 5, and the change in yolk corticosterone concentration dependant on maternal body weight in Chapter 4 indicate the dietary management of the broiler

breeder hen and genotype significantly influence yolk composition and may attribute to divergence in embryo phenotype. Similar to the 'Developmental Origins of Health and Disease' theory previously described in mammals (Martyn and Barker 1994; Barker 1997), the results of this thesis highlight the significant effect the maternal environment has on yolk composition and developmental programming consequences in the avian. While long-term effects of developmental programming on adult health and disease in mammalian species have been established, results in poultry are lacking. Considering the negative effects of rapid embryonic growth on skeletal development and metabolic disorders post-hatch in broilers (Buzała *et al.* 2015), studies pertaining to the long-term consequences of altered embryonic development via the maternal dietary environment in avians may prove advantageous in regards to health and welfare outcomes of progeny.

## 6.3. PAR in poultry

The PAR hypothesis implies the degree in which developmental programming effects during embryonic and fetal development are expressed in adulthood are dictated by the response to environmental cues received in early life. Developmental programming events are thought to occur in an attempt by the mother to enhance survival in the environment 'anticipated' by the mother. However, when the environmental conditions of the progeny differ to that of the mother (mismatched), it can result in adverse phenotypic effects (Bateson *et al.* 2014). While Chapter 5 identifies significant changes in embryonic development, results in this thesis also highlight progeny performance is significantly influenced by the posthatch environment. In both Chapters 3 and 4 a PAR effect was observed in progeny, demonstrating this phenomenon can present under a range of dietary environments. Surprisingly, these effects can be found during either early post-hatch development and long-term.

In Chapter 3, changing the predominant grain in the ration of progeny compared with the ration of the hen appeared to influence the growth response during the first week of growth. Irrespective of the grain source, progeny exposed to the same grain as their mother displayed higher body

weights than those of mismatched dietary environments. This response in offspring suggests PAR and potential transgenerational effects of grain source on offspring performance. While the mechanisms behind this change are unclear, various factors may attribute to this early posthatch programming response. As previously discussed, gastrointestinal weight altered in response to maternal diet in Chapter 4. Additionally, Chapter 3 body weight results of adult progeny (D28) cautiously imply an effect of maternal diet, which may have long-term implications for offspring performance. As the hen diet alters biological factors deposited within the egg/yolk (such as miRNA, hormones and nutrients) it is likely to invoke changes during embryonic development to suit the predicted environment. The concept of nutrigenomics may play a significant role in establishing cellular responses in the developing embryo (Afman and Müller 2006). The difference in gastrointestinal weight observed in Chapter 4 was suggested to be a result of morphological modulation in response to cell maturation effects. These developmental changes *in ovo* may be an adaptive response to enhance survival post-hatch when offspring are exposed to similar environmental conditions to that experienced by the broiler breeder hen.

Emerging evidence suggests the maternal microbiome plays a significant role in maternal dietary programming and provides an interesting concept in the phenomenon of maternal dietary effects and PAR in offspring. Until recently the human fetus was considered sterile, however research now suggests an efflux of commensal bacteria can arise through the placental barrier (Thum *et al.* 2012). Maternal stress during pregnancy is also associated with alterations in proteins related to vaginal immunity and metabolic reprogramming of the gut and brain in mice offspring (Jašarević *et al.* 2015). The maternal diet is now recognised to alter the microbiota in the gastrointestinal tract of neonate by the time of birth (Ma *et al.* 2014; Chu *et al.* 2016). In avians, the embryo develops separate from the mother, however the egg shell is recognised to host bacteria and there is now indications bacteria can be isolated in the gastrointestinal tract of the chicken embryo and new hatched chick (Kizerwetter-Świda and Binek 2008). Experiments within this thesis focus on compositional changes of compounds deposited into the yolk during egg formation (i.e. hormones, nutrients and microRNA), in response to maternal dietary effects. However the

transmission of maternal hen-derived microbiota to the embryo during egg formation in the oviduct has also been identified (Ding *et al.* 2017; Lee *et al.* 2019). Considering the significant influence of the maternal diet on fetal microbiota and metabolic reprogramming in mammals, it is worth considering the hen diet may initiate reprogramming effects via changes in microbiota, altering phenotype during embryonic and early posthatch development. The dietary source to which the chick is then exposed, particularly during early post-hatch growth, in combination with maternally-derived microbiota may influence phenotypic response in offspring (i.e. feed efficiency, metabolic programming and immune function). Consequences of the hen diet on microbiota of the hen and during egg formation must therefore also be recognised as is likely to be of significant interest when studying egg composition and potential developmental programming and PAR responses in avians.

The phenomenon of PAR has been the subject of intense studies in mammalian species, however the level of research within this area has been comparatively lacking in poultry. To date, altering the breeder diet to influence embryonic and posthatch development has primarily focused on simple energy and protein changes (Spratt and Leeson 1987), or combinations of nutrients (Pappas et al. 2006) in an attempt to improve antioxidant capacity and immune response in offspring. Little consideration has been given to the potential PAR effects between maternal diet and subsequent offspring diet on long-term health and performance. In mammals, maternal undernutrition in combination with adequate progeny diet in recognised to accelerate early postnatal growth. In Chapter 4 male progeny of lower body weight hens were significantly heavier post-hatch when fed *ad libitum*. A similar study by van der Waaij et al. (2011) found *ad libitum* fed offspring, particularly females, of restricted mothers were heavier and had greater abdominal fat at six weeks of age than daughters of *ad libitum* fed mothers. The increased rate of gain was attributed to body fat rather than muscle tissue, with suggestions this is triggered by catch-up growth on insulin resistance. Prenatal stress is also associated with a number of physiological pathways linked to offspring obesity, a result of glucocorticoid reprogramming (Drake *et al.* 2007; Bouret 2009; Gluckman and Hanson 2009). In Chapter 4 the programming effects observed were associated with differences in yolk corticosterone level and appeared sex-specific. Yolk

corticosterone exposure has previously been associated with changes in the HPA axis response sensitivity, reflected in changes in body weight of male offspring (Rubolini *et al.* 2005; Hayward *et al.* 2006; Uller *et al.* 2009). Changes in behaviour, corticosterone release and gene regulatory expression level of male offspring have all been associated with maternal stress (Goerlich *et al.* 2012). While an increase in breast muscle at D42 was observed in progeny of lower body weight hens, abdominal fat was not measured at D42. It would have been interesting to see if differences in females or males were apparent and the proportions of abdominal fat and breast muscle. In addition, hens in the high body weight group were still maintained at a level of restriction, albeit slightly less than other treatment groups, therefore differences between hen treatments may not have been extreme enough to elicit a strong programming effect on offspring body weight when placed under *ad libitum* or restrict-fed management.

The impact of maternal dietary effects may also be of significant consideration in the context of offspring health and disease challenge. In Chapter 3, changes in the predominating grain source of the hen appeared to influence growth response of offspring exposed to an immune challenge. Offspring exposed to a differing grain type to that of the hen reduced the proportion of progeny reaching expected target weights. This suggests specific nutrients in the maternal diet can influence progeny health. Rebel *et al.* (2006) for example, demonstrated the offspring of hens fed an improved diet altered gene expression in intestinal development and immune function. The mismatch between broiler breeder and offspring dietary environment is also suggested to potentially result in negative innate immunity effects in the broiler, see review by Berghof *et al.* (2013). Exposure to specific nutrients *in ovo* may influence the efficiency of uptake posthatch resulting in better health and performance of offspring. Compounds such as carotenoids have immunomodulatory potential and therefore of significant interest in commercial nutrition as a health-promoting agent (Bendich 1989). In chickens, maternal carotenoid status has been identified as important in the incorporation of tissue carotenoids in offspring (Koutsos *et al.* 2003; Karadas *et al.* 2005a). Therefore incorporation of specific compounds into the maternal diet and offspring diet may provide early posthatch and long-term health benefits to offspring (Kidd 2003, 2004; Hocking

2007). Due to the numerous environmental insults imposed on the chick at hatch, providing a stronger chick is valuable, hence a greater emphasis has been placed on pre-hatch mechanisms and the application of *in ovo* supplementation to optimise embryonic growth and development. With increasing restrictions on the use of antibiotics and hormones as growth promoters in food-producing livestock, the opportunity to utilise the maternal diet to improve progeny performance is worth consideration in poultry nutrition.

## 6.4. Conclusions and future directions

To conclude, manipulation of the yolk environment via the broiler breeder hen diet provides a mechanism to alter embryonic development and induce long-term effects in the offspring. Furthermore, the mismatch in dietary environment between generations and concept of PAR has significant consequences for progeny health and performance. The series of experiments conducted within this thesis demonstrate the maternal diet alters biologically-active factors (e.g. hormones, nutrients and miRNA) associated with epigenetic modulation, further expanding on results of Ho *et al.* (2011) and Wade *et al.* (2016). It also demonstrates maternal physiology and diet mediate physiological changes, specifically related to organ and tissue weight during embryonic development in a sex-dependant manner.

While tremendous gains have been made in the broiler industry through genetic selection, nutrition and management, considerations of the *in ovo* environment and long-term developmental programming effects have been underestimated. Furthermore, the mechanisms underpinning fetal and embryonic programming are yet to be elucidated in avian species. The findings of this research will underpin what could be the new frontier for the poultry industries of the future, resulting in healthier, more productive meat chickens which rely less on antibiotics, are less susceptible to diseases and less susceptible to metabolic and physiological pathologies. Greater consideration of the management of the breeder hen will also contribute to better hen welfare.

# Appendix

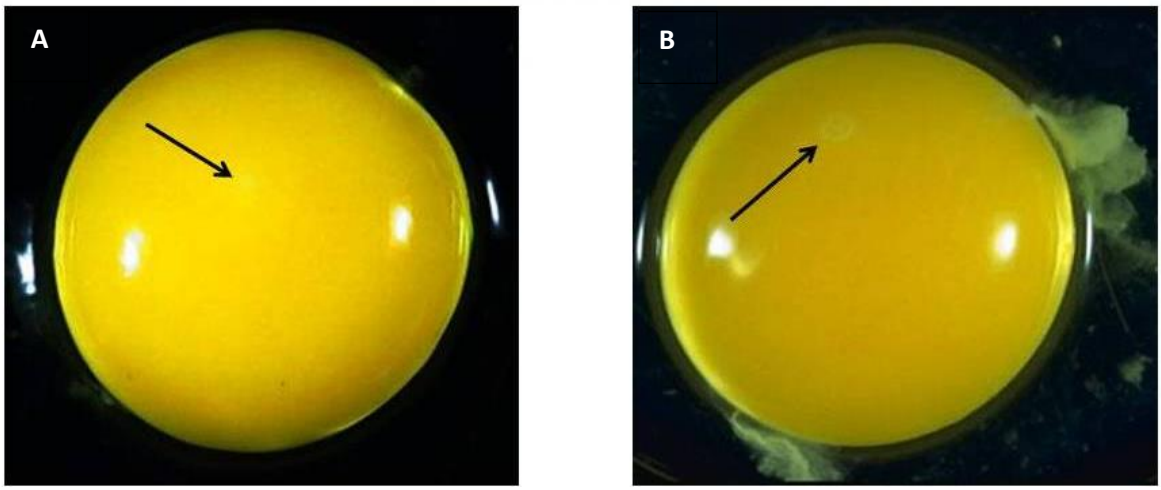


**Figure 1. Identification of fertile egg (A) and infertile egg (B)**

**Table 1. Effect of HenDiet (C-fed or W-fed) and ProgDiet (C-fed or W-fed) on average daily gain from hatch to 28 days of age (mean ± SEM).**

| Hen Diet | **Corn** | | **Wheat** | |
|---|---|---|---|---|
| Offspring Diet | **Corn** | **Wheat** | **Corn** | **Wheat** |
| **Body weight, g** | | | | |
| Hatch | 45.4 ± 7.9[a] | 45.7 ± 7.9[a] | 43.5 ± 7.9[b] | 43.3 ± 7.9[b] |
| D7 | 140.3 ± 8.2[a] | 134.3 ± 8.2[b] | 134.2 ± 8.2[b] | 136.5 ± 8.2[ab] |
| D14 | 350.5 ± 11.2[a] | 320.1 ± 11.3[b] | 313.1 ± 11.3[b] | 320.1 ± 11.1[b] |
| D21 | 755.8 ± 19.0[a] | 688.3 ± 19.5[b] | 677.7 ± 19.3[b] | 682.0 ± 19.0[b] |
| D28 | 1410.2 ± 3.3[a] | 1333.1 ± 31.6[ac] | 1293.5 ± 30.9[bc] | 1315.7 ± 30.4[bc] |
| **ADG, g** | | | | |
| P1 (Hatch – D7) | 13.6 ± 1.6 | 12.7 ± 1.6 | 13.0 ± 1.6 | 13.3 ± 1.6 |
| P2 (D7- D14) | 30.0 ± 1.8 | 26.5 ± 1.8 | 25.5 ± 1.8 | 26.2 ± 1.8 |
| P3 (D14 – D21) | 57.9 ± 2.2 | 51.8 ± 2.2 | 51.9 ± 2.2 | 51.5 ± 2.2 |
| P4 (21- D28) | 92.9 ± 3.3 | 93.0 ± 3.4 | 88.5 ± 3.4 | 91.0 ± 3.3 |
| Average (P1- P4) | 48.2 ± 1.7[a] | 46.5 ± 1.7[b] | 45.2 ± 1.7[b] | 45.1 ± 1.7[b] |

[abc] Different superscripts within a row indicate $P < 0.05$

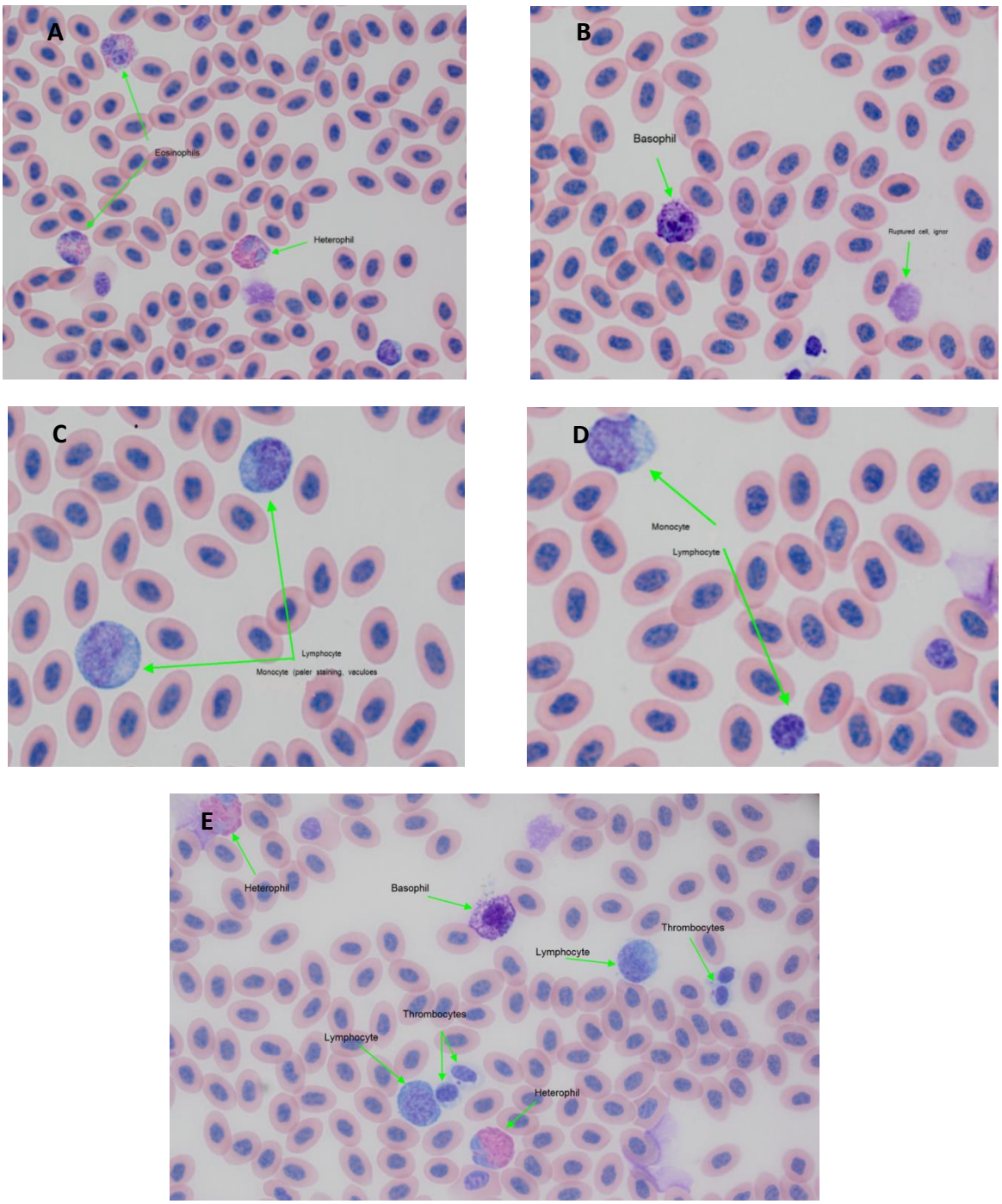


**Figure 2. Avian blood cells (A, B, C, D and E) under x100magnification. Heterophil (rod-like, pale pink), Eosinophils (rounded, light blue cytoplasm, dark pink), Basophil (dark purple, grain-like, no cytoplasm), Lymphocyte (darker purple/blue, less cytoplasm, nucleus always round, Monocyte (paler staining, cytoplasmic vacuoles, nucleus more variable in shape; sigmoid, bean shaped)**

**Table 2. Effect of Hen body weight (Low, Medium or High) and embryo sex on Embryonic Day 14 body weight, residual yolk sac weight and various tissue weights.**

| | Low | | Medium | | High | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| *n* | 6 | 6 | 7 | 5 | 9 | 3 |
| Body weight, g | $10.7 \pm 0.4^{x}$ | $11.8 \pm 0.4^{xy}$ | $12.2 \pm 0.4^{y}$ | $11.3 \pm 0.5^{xy}$ | $12.1 \pm 0.4^{y}$ | $11.5 \pm 0.6^{xy}$ |
| Residual Yolk Sac, g | 11.2 ± 0.8 | 11.1 ± 0.7 | 11.9 ± 0.7 | 11.7 ± 0.8 | 12.3 ± 0.6 | 12.8 ± 1.0 |
| **Tissue weight, mg** | | | | | | |
| Duodenum | 36.0 ± 3.4 | 36.9 ± 3.1 | 39.8 ± 3.3 | 39.1 ± 3.5 | 39.3 ± 2.8 | 38.5 ± 4.4 |
| Jejunum | 44.2 ± 4.9 | 49.0 ± 4.5 | 41.7 ± 4.7 | 39.7 ± 5.0 | 39.7 ± 4.0 | 52.5 ± 6.3 |
| Ileum | 33.7 ± 2.8 | 33.0 ± 2.6 | 32.3 ± 2.7 | 30.3 ± 2.9 | 31.0 ± 2.3 | 35.5 ± 3.7 |
| Gizzard | 244.7 ± 15.0 | 259.0 ± 13.7 | 240.9 ± 14.4 | 215.5 ± 15.3 | 246.5 ± 12.3 | 261.4 ± 19.4 |
| Liver | 211.3 ± 8.8 | 227.8 ± 8.0 | 234.3 ± 8.4 | 213.0 ± 8.9 | 205.2 ± 7.2 | 219.8 ± 11.4 |
| Heart | 96.9 ± 6.2 | 97.8 ± 5.7 | 93.3 ± 5.9 | 87.7 ± 6.3 | 93.2 ± 5.1 | 83.7 ± 8.0 |
| Fat Pad | $22.9 \pm 2.3^{xy}$ | $24.7 \pm 2.1^{x}$ | $24.7 \pm 2.2^{x}$ | $18.2 \pm 2.3^{y}$ | $25.3 \pm 1.9^{x}$ | $28.7 \pm 3.0^{x}$ |
| Proventriculus | $52.0 \pm 4.5^{a}$ | $68.2 \pm 4.1^{b}$ | $58.9 \pm 4.3^{ab}$ | $56.9 \pm 4.6^{ab}$ | $56.9 \pm 3.7^{a}$ | $47.5 \pm 5.8^{a}$ |
| Spleen | 7.3 ± 0.7 | 7.3 ± 0.7 | 8.3 ± 0.7 | 6.5 ± 0.8 | 7.5 ± 0.6 | 7.4 ± 1.0 |
| Bursa of Fabricius | 11.3 ± 1.2 | 10.3 ± 1.1 | 12.5 ± 1.1 | 11.9 ± 1.2 | 10.8 ± 1.0 | 13.3 ± 1.5 |
| Breast Muscle | 501.1 ± 21.2 | 487.0 ± 19.4 | 502.5 ± 20.4 | 440.5 ± 21.6 | 478.4 ± 17.4 | 491.6 ± 27.5 |

$^{ab}$ Different superscripts within a row indicate $P < 0.05$, $^{xy}$ Different superscripts within a row indicate $P < 0.1$

**Table 3. Effect of Hen body weight (Low, Medium or High) and embryo sex on Embryonic Day 20 body weight, residual yolk sac weight and various tissue weights.**

| | Low | | Medium | | High | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| *n* | 9 | 9 | 6 | 12 | 8 | 10 |
| Body weight, g | 41.9 ± 1.2 | 42.2 ± 1.2 | 41.4 ± 1.5 | 41.7 ± 1.1 | 40.2 ± 1.3 | 45.3 ± 1.2 |
| Residual Yolk Sac, g | 7.4 ± 0.4 | 8.0 ± 0.4 | 7.8 ± 0.5 | 8.6 ± 0.3 | 8.2 ± 0.4 | 8.4 ± 0.4 |
| **Tissue weight, mg** | | | | | | |
| Duodenum | 211.9 ± 9.5$^{a}$ | 187.3 ± 9.5$^{abc}$ | 181.0 ± 11.7$^{c}$ | 179.4 ± 8.3$^{bc}$ | 171.0 ± 10.4$^{b}$ | 202.4 ± 9.6$^{ac}$ |
| Jejunum | 268.4 ± 12.8 | 258.3 ± 12.8 | 248.0 ± 15.8 | 246.4 ± 11.1 | 224.7 ± 14.0 | 268.0 ± 13.0 |
| Ileum | 265.7 ± 17.1 | 241.7 ± 17.1 | 255.4 ± 21.0 | 236.7 ± 14.8 | 228.6 ± 18.6 | 277.3 ± 17.3 |
| Gizzard | 1503.7 ± 75.4$^{xy}$ | 1628.0 ± 75.3$^{y}$ | 1463.4 ± 92.5$^{xy}$ | 1363.8 ± 65.4$^{x}$ | 1384.1 ± 82.0$^{x}$ | 1664.1 ± 76.3$^{y}$ |
| Liver | 756.4 ± 23.3 | 763.1 ± 23.3 | 730.4 ± 28.6 | 755.0 ± 20.2 | 705.5 ± 25.3 | 776.8 ± 23.6 |
| Heart | 275.4 ± 8.8 | 274.0 ± 8.8 | 272.7 ± 10.8 | 257.3 ± 7.6 | 267.7 ± 9.6 | 283.5 ± 8.9 |
| Fat Pad | 474.6 ± 24.3 | 453.5 ± 24.2 | 439.5 ± 29.8 | 448.8 ± 21.0 | 422.6 ± 26.4 | 491.3 ± 24.6 |
| Proventriculus | 234.4 ± 13.1 | 229.9 ± 13.1 | 212.0 ± 16.1 | 229.5 ± 11.4 | 214.3 ± 14.2 | 228.0 ± 13.3 |
| Spleen | 17.9 ± 2.1 | 19.3 ± 2.1 | 23.6 ± 2.5 | 22.1 ± 1.8 | 21.5 ± 2.2 | 22.5 ± 2.1 |
| Bursa of Fabricius | 44.2 ± 3.6 | 37.3 ± 3.6 | 38.5 ± 4.5 | 37.9 ± 3.2 | 34.7 ± 4.0 | 39.6 ± 3.7 |
| Breast Muscle | 625.1 ± 21.1$^{a}$ | 555.1 ± 21.0$^{b}$ | 623.0 ± 25.8$^{a}$ | 586.3 ± 18.3$^{ab}$ | 566.0 ± 22.9$^{ab}$ | 608.4 ± 21.3$^{ab}$ |

$^{ab}$ Different superscripts within a row indicate $P < 0.05$, $^{xy}$ Different superscripts within a row indicate $P < 0.1$

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