The sex dependent impacts of maternal feed restriction stress and elevated cortiocosterone *in ovo* on meat bird growth, development and hypothalamic gene expression

By

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Abstract

Developmental programming is a recognised phenomenon across species. Programming of offspring during embryonic development to adapt to their environment can be advantageous, such as improved nutrient efficiency during times of famine. Incorrect programming, however, can result in lifelong problems, with evidence in humans of diseases such as diabetes, occurring due to misprogramming.

Maternal factors, such as nutrition and stress, during pregnancy and egg development can play a major role in offspring development. Restricted nutrition and increased stress in mothers can affect lifelong growth, metabolism, behaviour and immunity of their offspring. There is also evidence of differing effects of developmental programming between sexes, including response to stress and metabolism.

Commercial meat birds are intensely selected for growth and thus may be affected by changes in maternal nutrition and stress. This thesis aimed to identify maternal factors that may influence, progeny growth, metabolism, development, immunity and behaviour in commercial meat birds with an emphasis on differences between male and female progeny.

In the initial experimental trial broiler breeder hens were feed restricted to three bodyweight groups (low, medium and high). Eggs were collected over a 2-week period from each group and their progeny grown to 42 days of age. Progeny growth and immune response to a LPS challenge was measured. Breeder hens at the lowest bodyweight showed increased stress behaviours and heterophil: lymphocyte (H:L) ratio. Male progeny bodyweight from low bodyweight hens was reduced from

35 to 42 days old and females from heavy hens responded to the immune challenge with a reduced bodyweight and increased H:L ratio at 23 days of age.

The increased stress behaviour and H:L ratio count of hens maintained at the lowest bodyweight and highest feed restriction, impacted on the growth and immunity of their offspring. A possible reason behind this was through changes in stress hormones of the hen, such as corticosterone which may have then impacted on her offspring. To investigate this theory, commercial meat bird eggs were exposed to increased corticosterone and the birds grown out to determine the impact of elevated corticosterone exposure *in ovo* on meat bird growth and development.

Eggs were injected with corticosterone into the chorioallontoic membrane (CAM) at embryonic day eleven. Birds were grown to 42 days old, with growth, organ weights, H:L ratio and behaviour measured. Bodyweights at day 42 were reduced in birds exposed to increased corticosterone *in ovo*. This result was similar to the result in the first experiment where bodyweight was reduced in males from low bodyweight hens. Although, there were no difference between sexes, it does suggest corticosterone elevations during embryonic development can impact on growth late in life with similar results across studies.

There were, however, sex differences in organ weights at day 42. Male birds exposed to increased corticosterone had increased spleen weights compared to control males, while in corticosterone exposed females they were reduced. Males given corticosterone also showed a reduced stress response during the tonic immobility behavioural test.

The different effects seen between sexes in both the hen restriction trial and the corticosterone *in ovo* trial demonstrated that embryonic exposure to cortiocosterone

is impacting on meat birds throughout their life but can differ between sexes. This difference between sexes was then incorporated into the final study, which aimed to detail how corticosterone may be affecting lifelong bird development, growth and immunity and behaviour through programming of the hypothalamic-pituitary-adrenal (HPA) axis during development.

To investigate this, gene expression within the hypothalamus of birds given corticosterone *in ovo* was measured. Genes measured were the glucocorticoid receptor (GR), corticotropic releasing hormone (CRH), arginine vasotocin (AVT), 11ßhydroxysteroid dehydrogenase Type 1 (11ß-HSD1) and 20-hydroxysteroid dehydrogenase (20-HSD). At embryonic day 14, the expression of GR, CRH, AVT and 20-HSD was reduced in birds injected with corticosterone at embryonic day 11. Expression of 11ß-HSD1 was increased in females compared to males.

The results of the final trial show that corticosterone exposure *in ovo* can alter gene expression of important genes involved in the HPA axis. This could result in a lifelong programming of the HPA axis and account for the changes in growth, organ development, immunity and behaviour observed in the previous trials.

It is clear that the impact of maternal stress in broiler breeder hens can impact on her offspring, likely via increases in stress hormones and alterations of the HPA axis pathway. It is likely that there are other hormones and pathways impacted and more research is needed to understand the full effects of hen feed restriction on her offspring. The differences between sexes further complicates this work and also warrants further research.

If more can be understood about the impacts of feed restriction on male and female meat birds, the industry can better understand how management of hens can affect

the future production, health and welfare of their offspring. With further research in this area, breeder hen nutrition can be managed to reduce stress and the developmental programming effects on the subsequent generation to improve growth, health and welfare of meat birds. Understanding the differences between how male and female meat birds may be affected by hen feed restriction stress, may also lead to improved management of birds throughout their lives

Declaration of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Thesis Format

This thesis contains the findings of three experimental studies investigating the impact on meat birds of increased maternal stress, through developmental programming. The studies within this thesis begin with broiler breeder hen feed restriction stress and the effects on the offspring, through to the effects of the stress hormone, corticosterone on the developing avian brain.

Chapter 1 provides an introduction and extensive review on the concept of developmental programming with a focus on maternal stress effects across species. Possible impacts of maternal stress on the developing Hypothalamic-Pituitary-Adrenal (HPA) axis in offspring from stressed mothers is also discussed, with emphasis on feed restriction induced stress and the reprogramming of the HPA axis in developing meat birds. Finally, differences in progeny development between males and females are discussed and the potential impacts on the chicken meat industry is considered.

Chapter 2, the first experiment, entitled, ‘Effect of Restricted Feed Intake in Broiler Breeder Hens on Their Stress Levels and the Growth and Immunology of Their Offspring’ focuses on feed restriction and stress in broiler breeder hens, with three groups of hens maintained at different bodyweights during lay. The flow on effects of the hen’s bodyweight on the growth and development of their progeny were then investigated. The progeny were also given an immunological challenge using a Lipopolysaccharide (LPS) to measure differences in immune response in birds from hens maintained at differing bodyweights.

Chapter 3, the second experiment entitled, ‘Injection of Corticosterone *In Ovo* Leads to Reduced Growth and Sex-Dependent Effects on Organ Weights in Meat Birds’

utilised *in ovo* corticosterone injections into eggs and its effects on growth and organ weights from hatch to day 42 were measured. This experiment was a continuum from experiment one and was conducted to further understand the effects of corticosterone on male and female meat bird growth and development. Differences between sexes was also a focus of this experiment due to the significant sex differences in growth and immune response discussed in Chapter 2.

Chapter 4 is entitled 'Effects of Corticosterone Injection at Embryonic Day Eleven on Broiler Growth and Tonic Immobility'. This chapter continued to explore the impacts of corticosterone elevations *in ovo* on growth and behaviour, with behavioural differences found between sexes.

Chapter 5 is entitled 'Increased Corticosterone *In Ovo* Reduces Embryonic Meat Bird Hypothalamic Gene Expression with Differing Effects in Males and Females'. Real-time quantitative PCR (qPCR) techniques were employed to further investigate potential pathways that may be responsible for the differences found in male and female meat birds exposed to *in ovo* corticosterone injections in experiment 2 (Chapter 3). Expression of candidate genes involved in the HPA axis pathway were measured from embryonic day 14 to day 21 in both male and female chicks.

Chapter 6, the final chapter, discusses the major findings of each chapter. Final conclusions are made and impacts to the poultry industry and future research are discussed. Recommendations for the management of broiler breeder hens and their offspring are also made, with the aim of reducing the impacts of feed restriction maternal stress shown throughout this thesis.

Project Aims

The objective of this thesis was to investigate the effects of chronic feed restriction in broiler breeder hens, and how subsequent stress can impact both male and female progeny growth and development through programming mechanisms.

The hypothesis was that increased maternal stress in broiler breeder hens will impact on the growth, development, behaviour and immunity of their offspring.

The individual projects of this thesis aimed to:

1. Determine the effects of feed restriction and reduced bodyweights on stress response in broiler breeder hens.
2. Investigate the effects of reduced hen bodyweight on growth and immune response of their offspring.
3. Assess the effects of elevated corticosterone on both male and female meat bird growth and organ development using *in ovo* corticosterone injections
4. Assess the effect of elevated *in ovo* corticosterone on male and female meat bird behaviour and stress response
5. Determine the effects of elevated corticosterone on Hypothalamic-Pituitary-Adrenal (HPA) axis development and gene expression in male and female meat birds.

Chapter 1: Literature Review

Introduction

The concept of ‘developmental programming’ was first proposed in humans, with the establishment of the “thrifty phenotype” hypothesis (Hales *et al.* 1991). The basis of this hypothesis is that the environment of the mother can significantly influence the health of her offspring, through ‘programming’ of the offspring’s physiological systems to better cope with the prevailing environmental conditions. The evolutionary value of this reprogramming to suit the environment is clear, but in situations in which the environment changes between generations, potential ‘misprogramming’ may result, consequently reducing viability and health of the progeny.

Developmental programming and misprogramming effects were demonstrated on a large scale during the Dutch Famine in the Second World War. During this famine, pregnant women gave birth to smaller offspring, who later in life experienced rapid compensatory weight gain, cardiovascular disease, increased insulin levels and elevated glucose levels (Hales 2001). Programming of these children to be “thrifty” by their pregnant mothers during famine had altered their metabolism. These children were programmed to have improved metabolism during times of food shortage, but in times of ample food this same programming led to metabolic disorders, known collectively as ‘metabolic syndrome’. The importance of maternal nutrition during pregnancy was therefore recognised and it has since been realised that other factors during pregnancy, such as maternal stress can also influence offspring development (Wadhwa *et al.* 1993 Couret *et al.* 2009; Davis *et al.* 2011).

The effects of maternal stress on developmental programming can be wide-ranging and have been shown across mammalian species. In humans, reduced birthweight (Wadhwa *et al.* 1993) and elevated plasma cortisol (Davis *et al.* 2011) levels have

been reported in babies from stressed mothers. In pigs, maternal stress has resulted in decreased immune cell numbers (Couret *et al.* 2009) and in rats, prenatal stress led to increased basal and stress induced circulating corticosterone in the offspring (Vallée *et al.* 1999). The across species effects of prenatal stress on their offspring, particularly increases in circulating corticosterone, are suggestive of an altered sensitivity of the brain and the hypothalamic-pituitary-adrenal (HPA) axis to stress. This highlights the implications of maternal stress during embryonic development and is suggestive of the sensitivity of the brain and HPA axis to stress.

The HPA axis has a vital role in maintaining the homeostatic balance of stress hormones. Cortisol maintenance allows for the benefits of short-term cortisol elevation, for energy mobilisation (Lynn *et al.* 2003), whilst preventing long-term elevation and negative effects such as, decreased growth and immune suppression (Lin *et al.* 2006). The glucocorticoid receptor (GR) within the paraventricular nucleus (PVN) of the hypothalamus is vital to the homeostatic regulation of the HPA axis through negative feedback (Talge *et al.* 2007). The GR responds to elevated circulating plasma glucocorticoids, such as cortisol, and as such, the sensitivity of the GR influences circulating cortisol levels. This is via the negative feedback mechanism of elevated cortisol cellular uptake and activation of the GR, resulting in reduced cortisol through the HPA axis pathway (Stephens & Wang 2012).

Increased maternal cortisol levels influence the sensitivity and expression of GRs. In mothers with high circulating cortisol, the offspring's circulating cortisol may be elevated by reduced sensitivity of the GR resulting in reduced negative feedback (Seckl 2007). The expression of the GR gene is also thought to be reduced through increased methylation of the promoter region of the glucocorticoid receptor,

decreasing expression and the number of GR receptors available (Talge *et al.* 2007).

Conversely, plasma cortisol concentrations may be reduced in offspring of chronically stressed mothers suffering post-traumatic-stress disorder (PTSD), who have low circulating plasma cortisol (Yehuda 2002). The low maternal cortisol concentrations increase GR sensitivity and expression in the offspring, which reduces circulating plasma cortisol through increased negative feedback sensitivity (Grossman 2003). The differing responses to maternal stress and resulting changes to HPA axis development, mean that understanding the impact of maternal stress is difficult to predict.

Maternal stress and the effects on offspring are not limited to just mammalian species. In avian species, maternal stress has been shown to increase corticosterone deposition within the egg in European starlings (Love *et al*. 2008), resulting in chicks with reduced growth and HPA axis response (Love *et al.* 2008). Domestic, commercial meat birds have been found to be influenced by increases in corticosterone with changes to the HPA axis leading to lifelong alterations in behaviour (Ahmed *et al.* 2014), bodyweight (Janczak *et al.* 2006; Lin *et al.* 2006), blood glucose regulation (Jiao *et al*. 2008) and plasma corticosterone levels (Lin *et al.* 2007). Such dramatic effects of maternal stress on the neuro-endocrine systems of the offspring are particularly relevant to the chicken meat industry as breeder hens are subjected to nutritional restriction as a means of maximising reproductive performance, leading to potential hunger stress.

In Australia, chicken meat production has continued to increase, with 664million birds produced in 2017/18 (Australian Chicken Meat Federation 2019), with this

growth likely to continue, as chicken meat is a cheap source of animal protein. This is due to the rapid growth of meat birds and efficient feed conversion (feed/gain), with an average weight of 2.9kg and a feed conversion ratio (FCR) averaging 1.6 at 42 days old in mixed-sex Cobb 500 birds (Cobb-Vantress 2019). To produce birds with this level of efficiency and growth, meat bird production relies on intense genetic selection at many levels, including the breeder hen flock.

Broiler breeder hens are vital to the cost-effective production of meat birds, with one hen producing up to 100 meat birds annually (Berghof *et al.* 2013). The reproductive performance of the hen (high rate of production of fertile eggs) is therefore crucial to continual efficient production of meat chickens. To ensure this, hens are maintained at an optimal weight, using feed restriction, thus retaining a suitable rate of lay and fertility, as well as preventing metabolic, cardiovascular and skeletal disease associated with increased weight gain (D'Eath *et al.* 2009). However, a side-effect of feed restriction in hens is chronic hunger, with hens feed restricted up to 45% of their *ad libitum* feed intake (Buckley *et al.* 2012). This can lead to a state of stress in hens (de Jong *et al.* 2002), which may then impact on their offspring through developmental programming.

The literature relating to developmental programming and the effects of maternal stress on the physiology, behaviour, immunity, health and growth of offspring is explored. Literature on multi-species effects are reported and the programming effects to the HPA axis and GR receptor examined. The effects of maternal stress in breeder hens on the multi-billion-dollar poultry industry are reviewed.

Developmental Programming

Nutrition and Developmental Programming

Developmental programming is now a well-recognised area of research across species. Environmental influences on the oocyte, embryo or foetus during development elicit significant lifelong impacts on metabolism, physiology and behaviour (Chmurzynska 2010). Developmental programming can act as a survival advantage as the offspring is literally "programmed" by their mother for the same environment she herself is experiencing (Hales 1992).

The "thrifty phenotype hypothesis" was first described in 1991 by Hales and Barker (Hales *et al.* 1991) when they found reduced early life and foetal weight resulted in non-insulin dependent diabetes. The link between foetal, early life and metabolic disorders later in life was then established and demonstrated to hold true across several well-known cases, including the Dutch Famine. In this famine, reduced nutrition in mothers resulted in smaller offspring, that in later life developed higher fasting glucose levels, insulin resistance, cardiovascular disease and increased weight gain (Hales 2001). The role of maternal nutrition and restricted feed during pregnancy was then recognised as a driver of foetal development and lifelong health.

As well as being demonstrated in humans, the role of maternal nutrition on developmental programming in other species has been shown. Extensive research into the effects of reduced foetal nutrition using intrauterine growth restriction (IUGR) in sheep research models, has shown reduced lamb birthweights, insulin-like growth factor (IGF-1) plasma concentrations (Jones *et al.* 1988) and arterial blood pressure in IUGR sheep (Louey *et al.* 2003). In rat models, similar results

have been seen with reduced birth weight in offspring of feed restricted mothers (Vickers *et al.* 2005) and elevated fasting insulin and leptin levels in offspring of mothers restricted to 30% *ad libitum* feed intake (Vickers *et al.* 2001). Rats fed a diet of reduced protein while pregnant, also resulted in offspring with reduced pancreatic β-cell proliferations and islet size (Snoeck *et al.* 1990).

Maternal Stress and Developmental Programming

As well as nutritional influences, maternal stress during pregnancy can also impact on lifelong health, through developmental programming. In humans, maternal stress influences on offspring development have been increasingly documented. Mothers questioned during pregnancy and identified as stressed, were associated with smaller children at birth (Wadhwa *et al.* 1993) and had babies with a reduced head circumference (Lou *et al.* 1994). As well as influencing growth, maternal stress during pregnancy in humans has also been associated with behavioural changes. Children from stressed mothers have increased cortisol to a heel-prick as babies (Davis *et al.* 2011), lower mental and motor development scores at 8 months of age (Buitelaar *et al.* 2003) and increased cortisol when first starting school (Gutteling *et al.* 2005).

Other species have also shown the effects of maternal stress on offspring. In pigs, social housing stress of gilts during late pregnancy resulted in reduced adrenal weight and decreased total white blood cells in piglets from stressed mothers (Couret *et al.* 2009). In rats, maternal stress, can lead to increased dysfunction of the HPA axis, with increased corticosterone levels earlier in life (Vallée *et al.* 1999) and a 49% increase in corticotropic-releasing hormone (CRH) levels as adults (Cratty *et al.* 1995). The effect of maternal stress is wide-reaching and results in increased activation of the mother's HPA axis, resulting in elevated maternal and

foetal glucocorticoids (Knackstedt *et al.* 2005), influencing the developing foetal HPA axis.

Reprogramming of the Hypothalamic-Pituitary-Adrenal (HPA) Axis

The role of the HPA axis in the stress response is well known. During a stressful event, neurons in the paraventricular nucleus (PVN) of the hypothalamus are stimulated to release corticotropic releasing hormone (CRH), which results in an increase in adrenocorticotropic hormone (ACTH) from the anterior pituitary, which acts on the adrenal cortex, elevating corticosterone (Herman *et al.* 2012). Elevation of corticosterone can have many positive short-term benefits, including mobilisation of stored energy (Lynn *et al.* 2003) increased circulating glucose (Lin *et al. 2007)* and increased insulin levels (Jiang *et al.* 2008). However, long-term elevation of corticosterone can be detrimental, leading to reduced growth (Lin *et al.* 2006) and immune changes, including reduced weight of the Bursa of Fabricus and spleen in chickens (Shini *et al.* 2008).

Corticosterone, levels are maintained at a homeostatic balance via a negative feedback loop. When corticosterone levels are elevated, they act on the glucocorticoid receptors (GR) within the hypothalamus, reducing CRH release and therefore, corticosterone (Herman *et al.* 2012). When this feedback mechanism is unable to maintain this balance, corticosterone levels remain elevated. An overexposure to cortisol during pregnancy, can affect this feedback loop, by reducing the sensitivity of the GR and elevating cortisol (Seckl 2007). When this occurs in avian species, it can result in increased corticosterone deposition within the egg and as a consequence of this, increased corticosterone exposure to the embryo (Royo 2008).

The mechanisms behind differences in negative feedback 'set points' and how they may be affected by maternal stress continues to be researched. One area believed to be influenced by these changes is the glucocorticoid receptor (GR), expressed predominately in the paraventricular (PVN) nucleus of the hypothalamus, and higher glucocorticoid affinity receptors, mineralocorticoid receptors (MR), expressed throughout the brain (Talge *et al.* 2007). Both receptors are highly expressed in the brain during development (Seckl 2007). It is thought that as the levels of corticosterone increase, the sensitivity of these receptors is decreased, reducing negative feedback and increasing circulating corticosterone levels (Seckl 2007).

Alternatively, expression of the GR gene may be altered due to methylation of the promotor region of the gene (Talge *et al.* 2007). In rodents, increased nurturing behaviour by the dam, resulted in decreased methylation of the GR gene of the offspring. As methylation blocks transcription, this would result in increased GR expression (Weaver *et al.* 2004). It may therefore also be possible that as the gene promoter to GR is susceptible to methylation and if methylation is increased by maternal stress, GR expression would decrease (Talge *et al.* 2007), see Figure 1 below. Decreased GR gene expression could lead to reduced negative feedback to glucocorticoids, and increased circulating corticosterone.

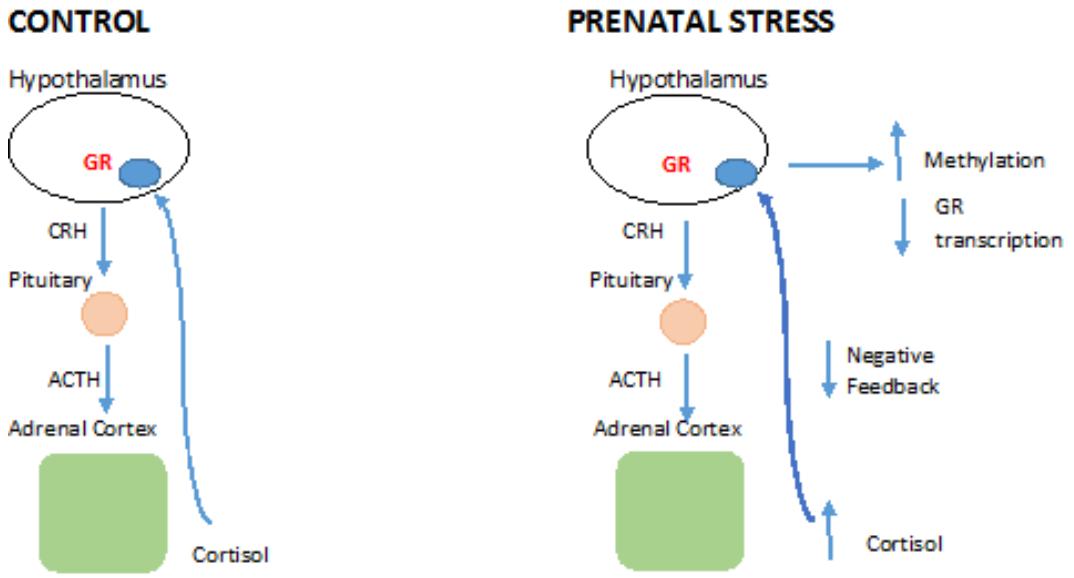

Figure 1: Possible influence of maternal stress leading to increased DNA methylation and reduced transcription of the glucocorticoid receptor (GR) gene within the brain of the offspring. Reduced GR transcription may lead to reduced negative feedback and increased circulating corticosterone levels Adapted from Talge *et al.* 2007.

Exposure to a stressful event in humans leading to PTSD, can show an opposite effect (Yehuda 2002). Patients with PTSD have an increase in negative feedback and reduced cortisol levels (Yehuda 2002). The dexamethasone suppression test (DST) has also shown significantly higher cortisol suppression in PTSD patients, indicating increased glucocorticoid receptor sensitivity in these patients (Grossman 2003). These differences in response to cortisol, shown in Figure 2, may be applicable to poultry and mean that there are possibly differing receptor changes, and response to stress may be relative to time and the intensity of stress exposure.

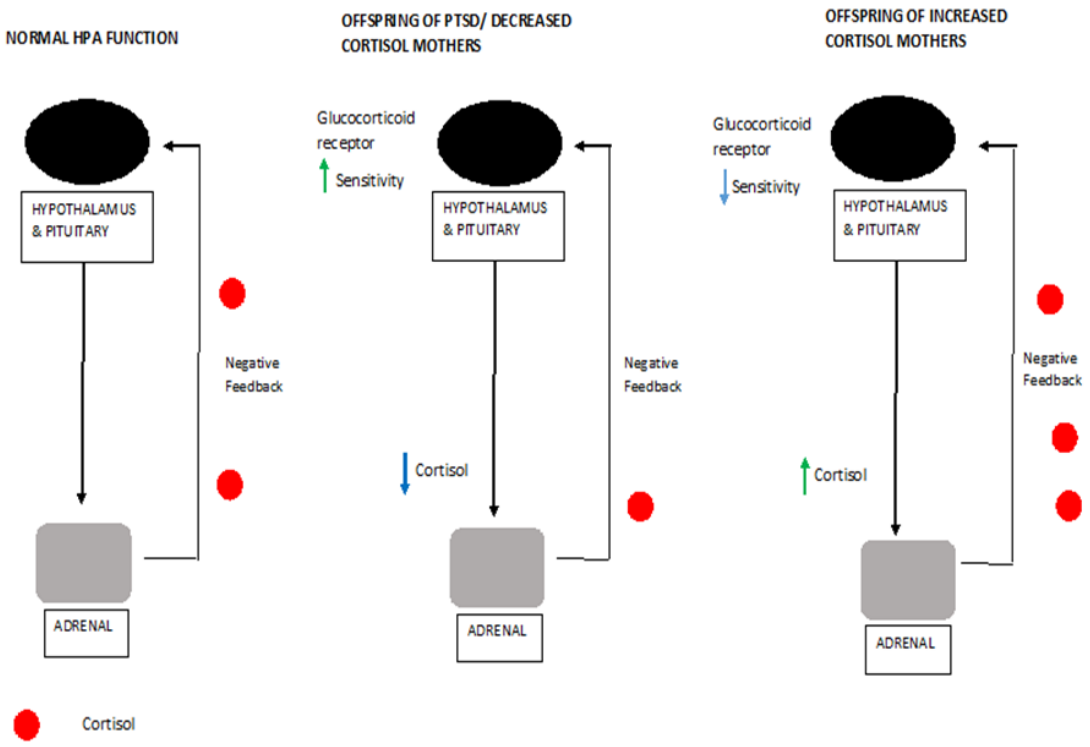

Figure 2**:** Hypothalamic-pituitary-adrenal axis function in normal offspring, offspring of mothers with post-traumatic stress disorder (PTSD) and low cortisol levels and offspring from stressed mothers with increased cortisol. In the normal HPA axis, cortisol levels are maintained at a steady level. In PTSD offspring, glucocorticoid receptor sensitivity is increased, increasing negative feedback sensitivity and reducing cortisol. In offspring of high cortisol mother glucocorticoid receptor sensitivity is reduced, reducing negative feedback efficiency and increasing cortisol.

Developmental Programming and Meat Birds

Meat Bird Production

Meat bird production has grown exponentially, with global chicken meat consumption expected to reach 51.5kg per person by 2022 (Australian Chicken Meat Federation, 2019). Chicken meat is likely to continue to be an important protein source as global population continues to grow and is currently the cheapest animal protein compared to beef, lamb or pork (Australian Chicken Meat Federation, 2019). The rapid increase in consumption of chicken meat can be attributed to the intense genetic section on meat birds over the last 100 years, improving weight gain and feed efficiency to make these gains (Siegel 2014). Genetic selection of meat birds

has been so effective that a bird that in 1985 a meat bird was 1.4kg at 35 days old, eating 3.2 kg of feed, while today they are 2.4kg using 3.7kg of feed at the same age (Siegel 2014). Such impressive improvements within a short period of time using selection on production traits has been highly successful. Selection for growth and fed conversion traits has significantly influenced health and welfare of meat birds. As a rapidly growing bird, there are many health problems due to this growth, such as leg deformities, metabolic disorders and heart failure (Zuidhof *et al.* 2014). Decreased immunity has also been linked to increased growth in meat birds (Yunis *et al*. 2000) due to decreased adaptive immunity and there is also a higher mortality seen in rapidly growing birds (Cheema 2003).

Variations in growth and health have been driven by nutrition and genetic selection at many differing genetic levels, from a pedigree bird, to great-grandparent lines, grandparent lines and through to the parents of meat birds (Siegel 2014; Figure 3). The heavy and careful selection of particular traits at these different genetic levels, as mentioned previously, can lead to massive improvements in growth and feed efficiency as well as other phenotypic changes to health. As seen in the figure below (Eenennaam *et al.* 2014), one pure-line female can equate to over three million meat birds and therefore 5.7 million kg of chicken meat at 1.86kg chicken meat per bird (Australian Chicken Meat Federation, 2019). The importance of any changes within these levels has the potential to significantly impact on meat birds. To further understand what the potential is for such changes, the next level from meat birds, breeder hens, have the potential to be investigated and the impacts to their offspring measured.

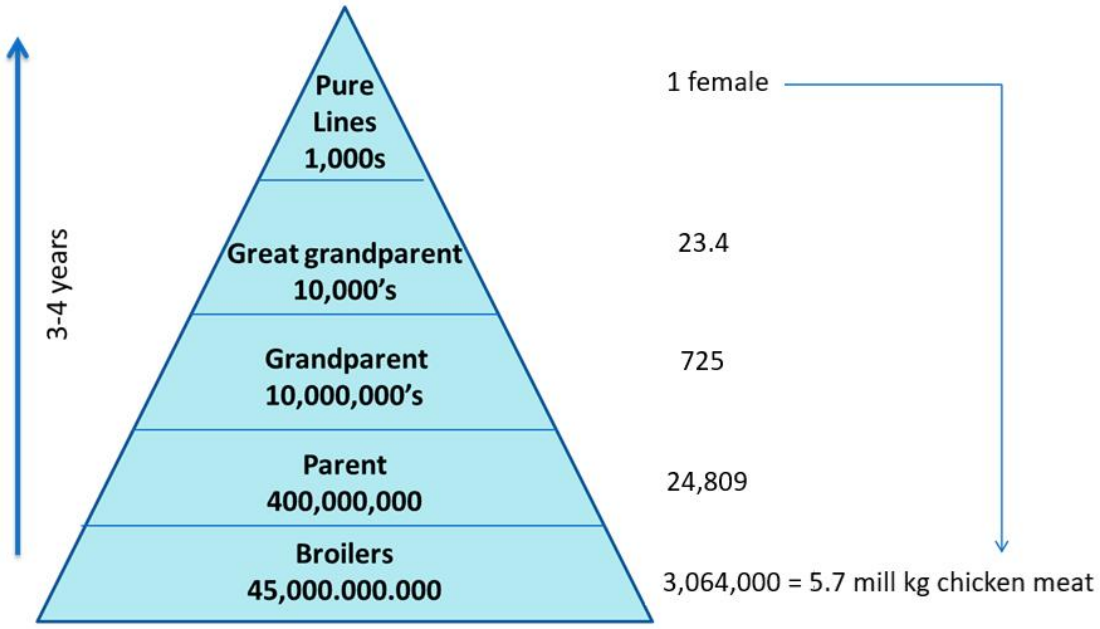

Figure 3: Generations of meat birds used for genetic selection from pure lines, through to parent/breeder flocks and the final broiler meat bird. Also shows the significant impacts of each generation on subsequent generations, through to broilers. Adapted from (Eenennaam *et al.* 2014).

Breeder Hens and Feed Restriction

Breeders or parent birds are the fourth and final generation of birds before meat birds in poultry genetic selection and are parents to meat birds (Australian Chicken Meat Federation 2019) (see Figure 3). Breeder hens make up an important part of the production of chicken meat, with 460 million breeder hens used globally to produce 60 billion meat birds (Berghof *et al.* 2013). With the same genetic potential as their meat bird offspring, breeder hens are prone to rapid weight gain which can result in reduced fertility and lay (Decuypere *et al.* 2010). To counteract this issue hens are feed restricted to prevent excess weight gain and ensure continual production of eggs (Buckley *et al*. 2012).

The level of feed restriction in breeder hens can vary from 25 to 45% of *ad libitum* feed intake (Buckley *et al*. 2012), although there are cases of restrictions up to 70% (Puterflam *et al.* 2006). This level of restriction can equate to a feed restricted bird

eating 53g/day at 42 to 49 weeks old whereas a bird of the same age fed *ad libitum* can eat 161.6g/day (de Jong *et al.* 2002). These levels of feed restriction are very effective at reducing hen bodyweight, with restricted hens up to 50% smaller in bodyweight than hens given access to *ad libitum* feed (Mench 2002).

A smaller bird is beneficial to the industry as reproductive traits such as fertility, egg number and egg quality are all negatively impacted by weight gain (Decuypere *et al.* 2010). Restricting hens therefore allows for a lighter bird that will maintain a higher rate of lay and will lay eggs that are of higher hatchability. Whilst there are industry benefits to feed restriction, there are benefits to the hen herself. Feed restriction maintains birds at a lighter bodyweight and prevents disorders associated with rapid weight gain such as, metabolic disorders, cardiovascular disease, lameness and skeletal problems (D'Eath *et al.* 2009, Mench 2002).

While there are significant benefits to feed restriction of broiler breeder hens, selection for high growth rate and high feed intake means feed restriction denies hens their natural feeding behaviours and nutrient supply. Several studies have demonstrated indications of hunger in feed-restricted hens such as increased pecking, hyperactivity increased feed motivation (Sandilands *et al.* 2006, Mench 2002, Savory 1993), increased drinking to compensate for decreased feed (Tolkamp 2005) and higher glucose to non-esterified fatty acids (NEFA) ratio (de Jong *et al.* 2003). This supports a state of chronic hunger in hens and it is therefore likely they are also experiencing stress due to feed restriction.

Developmental Programming and Hen Stress

Evidence of chronic stress in breeder hens under feed restriction has been demonstrated in several different ways, including behavioural and physiological

measures. The behaviour of feed-restricted hens is largely indicative of negative affective states. Feed-restricted hens display reduced comfort behaviours, such as preening which is suggested to be a 'positive' behaviour in hens, (Hocking 2006, Hocking 1993) as well as reduced time standing and sitting (de Jong *et al.* 2003). Increases in other negative behaviours such as stereotypies, including object pecking, have also been seen in restricted hens (Hocking 2006, de Jong *et al*. 2003), with stereotypic behaviours believed to be used by birds as a way of relieving stress (Kostal 1992). Other behaviours shown to increase in feed-restricted hens have been hyperactivity and pacing (Sandilands *et al.* 2006). Reduced time spent on activities associated with comfort and increases in stereotypic behaviours and hyperactivity, suggest feed-restricted birds are stressed. However, physiological measures also need to be considered when determining stress levels.

Physiological stress in birds can be quantified by corticosterone levels in yolk (Babacanoğlu *et al.* 2013) plasma (Henriksen *et al*. 2011), serum (Cook *et al.* 2009) and heterophil: lymphocyte cell (H:L) ratios in the blood (Wein *et al.* 2017). There is conjecture as to the reliability of both measures as they can be influenced by other factors, such as circadian rhythms in the case of corticosterone or infections in the case of H:L ratios (Wein *et al.* 2017). However, H:L ratios and corticosterone levels are believed to be highly correlated in low stress situations (Wein *et al.* 2017) and although issues exist with both measures, if used together with behavioural measures can give an indication of avian stress. Both measures have been shown to be negatively correlated with increased feed restriction in breeder hens (Hocking *et al.* 1996), suggesting as feed intake is reduced, corticosterone and H:L ratio increase, indicating stress is increased in feed restricted breeder hens.

There is increasing evidence that this chronic stress in breeder hens can lead to changes within the egg, impacting on the embryo. One change consistently measured is hormone deposition, particularly corticosterone. Stressed hens are shown to have elevated corticosterone levels (de Jong *et al.* 2002) and corticosterone has been shown to transfer to eggs in poultry (Royo 2008). This then has the potential to impact their offspring through developmental programming.

Hypothalamic-Pituitary-Adrenal Axis Reprogramming in Meat Birds

Hypothalamic-Pituitary-Adrenal Axis Development in Poultry

In chickens, development of the HPA axis takes place in several stages, with a fully functional HPA axis apparent by embryonic day fourteen (Jenkins and Porter 2004). This makes the HPA axis particularly susceptible to development changes before this time. As with other avian species, the chicken embryo is exposed to only what was present in the egg when it was formed and laid, and the embryo will go on to absorb the hormones and nutrients within the egg (von Engelhardt *et al.* 2009). This includes corticosterone, which, if elevated, may impact on several areas of HPA axis development, such as the glucocorticoid receptors. The HPA axis is not 'set' until later in development so is vulnerable to changes throughout embryonic development, with negative feedback not present until day eleven and production of glucocorticoids from the adrenals not present until day 16 (Jenkins and Porter 2004), shown below in Figure 4.

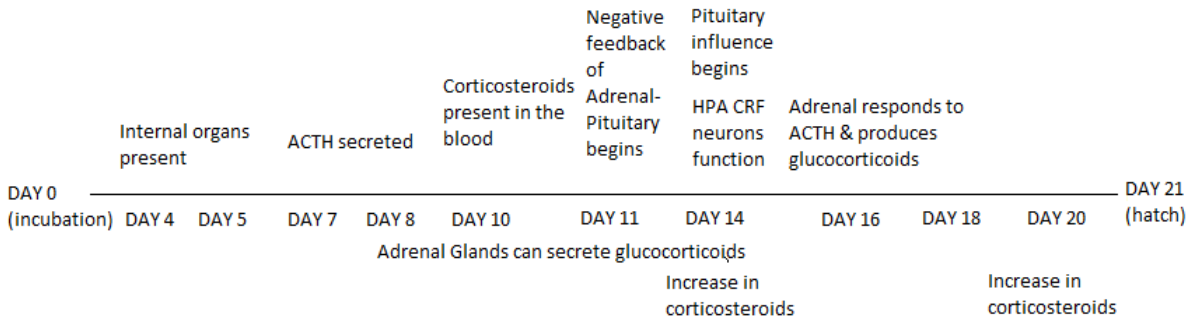

Figure 4**:** Hypothalamic-pituitary-adrenal (HPA) axis development from lay (day 0) to hatch (day 21) in meat birds. Adrenocorticotropic (ACTH) secretion begins as early as day 7, with corticosteroids present in the blood by day 10. In the second half of incubation, negative feedback begins and by day 14 the HPA axis is functional, with pituitary, adrenal and hypothalamic neurons functioning. By day 16 the adrenals are responsive to ACTH levels, producing glucocorticoids in response to increased ACTH levels.

Hypothalamic-Pituitary-Adrenal Axis Reprogramming in Poultry

There is an increasing body of research on avians and developmental programming, including maternal stress and developmental programming. Some studies in avians have imitated rodent studies by using restraint stress in birds such as quails, to study the effects of maternal stress on chicks. There is good evidence of reduced fertility after increased corticosterone exposure (Schmidt *et al.* 2009). Other studies have focused on wild birds, such as European starlings and the influence of increased *in ovo* corticosterone, with reductions in HPA axis response in chicks exposed to elevated corticosterone (Love *et al.* 2008). Corticosterone and the HPA axis have also been studied in other species such as Japanese quail and shown increased yolk corticosterone, reduced growth and increased HPA axis responsiveness to capture stress (Hayward and Wingfield 2004).

The results seen in avian species suggest that developmental programming does occur in chicks from hens exposed to maternal stress and can occur across species. However, there are some conflicting results, especially around the HPA axis response, with some studies reporting increases in HPA axis response (Hayward and Wingfield 2004) and others, a decrease (Love *et al.* 2008). This suggests that there may be some variation in bird species, level of corticosterone exposure, timing of stress exposure, method of stress exposure and the age of the chick when measurements are taken.

Meat birds and Maternal Stress Reprogramming

As well as wild avian species, developmental programming and maternal stress has been researched in poultry. Some research has focused on the nutritional composition of poultry feed, including betaine levels (Gudev 2011), low protein diets (Rao *et al.* 2009) and varying vitamin and mineral levels (Rebel *et al.* 2006, Surai 2000). Stress placed on hens has also been demonstrated to impact on the composition of the egg. Reductions in egg, yolk (Rozenboim *et al.* 2007) and albumen mass (Downing & Bryden 2008) have been found after exposure of hens to heat or handling stress. These reductions in yolk, albumen and overall egg mass then have the potential to impact on the amount of nutrients available to the developing chick (Henriksen *et al.* 2011). The effects of developmental programming on meat bird chicks has been demonstrated in chicks fed either the same ration as their mother or a different ration, with changes in growth and abdominal fat found (van der Waaij *et al.* 2011).

In ovo manipulation has been used to try and better understand how hormone deposition by hens can impact the embryo (Ahmed *et al*. 2014b). As with other bird species, elevated corticosterone levels resulted in smaller progeny (Henriksen *et al.*

2013), behaviour changes (Ahmed *et al.* 2014a) and reduced immunocompetence in poultry (Henriksen *et al.* 2013). Elevated corticosterone *in ovo* has also resulted in downregulation of the corticotropin releasing hormone (CRH) gene and protein content of the GR in the hypothalamus (Ahmed *et al.* 2014b). Downregulation of the GR gene protein may mean decreased sensitivity of the receptor, resulting in reduced negative feedback and increased corticosterone.

Maternal stress can go on to have differing effects, as seen in humans with PTSD, with reduced cortisol levels in children from mothers with PTSD (Yehuda 2002). Pregnant women present at the September 11 attacks who developed PTSD had children with lower salivary cortisol during the first year of life and in children of holocaust survivors these effects were found to be longer lasting, with reduced urinary cortisol in these children into adulthood (Yehuda 2005). Reduced cortisol in these children is believed to be due to glucocorticoid receptors becoming more sensitive as a result of decreased cortisol exposure *in utero*. These children can therefore have an oversensitive response to cortisol levels, resulting in an overly efficient negative feedback loop, reducing cortisol levels. The varying maternal stress levels may also be an important factor to consider in poultry as to how chicks will be affected by the hen's stress response.

Developmental Programming, Stress and Sex Specific Effects

Maternal Stress and Sex Specific Effects

It is becoming increasingly clear that the effects of maternal stress and developmental programming on animals, are also sex specific. In rats, dams stressed during pregnancy, have male and female offspring with different HPA axis programming. Female rats have been shown to have changes within the

hypothalamus and increased serum corticosterone when born from a mother under increased stress, while males from stressed mothers did not (McCormick 1995, Weinstock 1992). Effects of exposure to stress hormones during pregnancy have also shown sex specific effects on metabolism in rats. Males from a stressed prenatal environment have been found to have hyperglycaemia and hyperinsulinemia later in life (O'Regan *et al.* 2004). Therefore, it is likely that there is a sexual dimorphism effect in offspring from mothers with increased corticosterone. In rats, the increased stress response of females suggests that negative feedback to the HPA axis is less sensitive in females and corticosterone levels remain elevated (Tilbrook & Clarke 2006). It has also been previously shown that corticosterone levels are lower in males and male corticosterone pulses do differ to females (Lightman *et al.* 2002). These physiological differences demonstrate the possibility that males and females will respond differently to changes in corticosterone levels if exposed during HPA axis development.

Maternal Stress and Sex Specific Effects in Avian Species

In avian species, elevated corticosterone *in ovo* has been shown to effect males and females differently. In quails, males showed decreased growth after exposure to elevated corticosterone, while female growth was unaffected (Hayward *et al.* 2006). Females exposed to increased corticosterone showed reduced corticosterone after a stress series test, whereas exposed males did not differ to control males, suggesting a decreased response to stress in these females (Hayward *et al.* 2006). This is opposite to what was found in rodents, with increased corticosterone in female rats. These differences could be due to variations in the HPA axis between species. While there is limited research into the sex specific effects of maternal stress and increased corticosterone in avians, there is the

potential that there could be significantly different effects on males and females exposed to increased corticosterone *in ovo*.

Relevance to the Poultry Industry

It is well established that broiler breeder hens are chronically hungry due to feed restriction practices (de Jong *et al.* 2003) and are likely in a state of chronic stress (De Jong 2011). With evidence of elevated corticsoterone in feed-restricted breeder hens (de Jong *et al.* 2002) and corticosterone depositon into the egg possible in poultry (Royo 2008), it seems possible that this is influencing offspring of feed-resticted hens. Feed restriction of breeder hens, while necessary to maintain rate of lay and fertility of eggs, is a welfare issue for these birds (Mench 2002, D'Eath *et al.* 2009). The possibility to reduce the level of feed restricion in breeder hens may lead to improved welfare and reduced stress without compromissing productivity.

Possible flow-on effects from reduced stress in breeder hens are changes to meat birds through developmental programming. Decreases in circulating corticosterone concentrations, and changes in gene expression within the HPA axis leading to lifelong changes to poultry exposed to differing corticosterone levels *in ovo* have already been shown (Ahmed *et al.* 2014b). Chicks exposed to decreased circulating corticosterone have higher growth, decreased aggressive behaviours (Ahmed *et al.* 2014a), are larger at hatch and have better immunocompetence (Henriksen *et al.* 2013). Decreased corticosterone exposure in birds during development from less feed-restricted hens, and the consequential improvements in growth, behaviour and immunity would be of significant benefit to the poultry industry.

Substantial research is still needed to understand if reductions in feed restriction in hens will change egg corticosterone levels to a point that will then go onto lead to

these changes. Another point of consideration is how these changes will affect males and females, with evidence in other species of differing impacts on growth (Hayward *et al.* 2006) and stress response (McCormick 1995). It is therefore possible that male and female meat birds may be differently affected by changes to corticosterone levels within the egg, and in future this could lead to management changes of poultry based on their sex.

In conclusion, manipulation of the diet and environment of breeder hens and subsequent effects on her progeny have significant implications for the chicken meat industry. Alternative management strategies of breeder hens by reducing the level of feed restriction could lead to a reduction of stress in these hens. While this could mean improved welfare for hens, it has the potential to have significant impacts on their offspring. It is possible that reduced feed restriction in hens may mean reductions in egg corticosterone and embryo exposure, leading to mis- or reprogramming of the HPA axis. This then has the potential to improve growth and immunocompetence of these offspring but may occur in a sex-dependent way. Elevations in corticosterone levels in feed-restricted hens and the possible ongoing effects on the physiological responses of their progeny, is of significant scientific and commercial interest to the poultry industry. This thesis explores the evidence and impacts of developmental programming in avian species, particularly the relevance and possible impacts to the meat bird industry, through feed-restriction stress and elevations in corticosterone levels in breeder hens and the impacts on the growth and development of their progeny.

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Chapter 2:

Effect of Restricted Feed Intake in Broiler Breeder Hens on Their Stress Levels and the Growth and Immunology of Their Offspring

Chapter Introduction

The following manuscript was formatted for The Journal of Translational Animal Science and was accepted for publication (2018).

This chapter paper was the first to investigate how feed restriction stress in broiler breeder hens can impact on the growth, development and immune response of their offspring with sex differences also described.

Hen feed restriction is known to cause chronic hunger stress. However, the downstream effects on progeny is unknown so growth performance, organ weights, circulating plasma corticosterone and immune response was measured.

Hens were maintained at three separate bodyweights through feed restriction practices for 10 weeks. Progeny from hens were then grown out to 42 days old and their growth, organ weights and immune response were measured

Statement of Authorship

Statement of Authorship

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Contribution to the Paper	Project design, experimental work, data collection, analysis and interpretation, main author of manuscript
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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Effect of restricted feed intake in broiler breeder hens on their stress levels and the growth and immunology of their offspring[1]

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ABSTRACT: The prenatal environment has been shown to have significant effects on the lifelong health of offspring in humans and other species. Such effects have not been studied extensively in avian species but could prove important, especially in the case of severe feed restriction imposed on broiler breeder hens to prevent obesity and reduce rate of lay. Feed restriction can potentially affect not only nutrient supply to the embryo but stress hormone levels within the hen. This study investigated the impact of nutrient restriction of the breeder hen on growth rate and immune responses in the progeny with the objective to measure the impact of feed restriction of broiler breeder hens on growth and immune response of the progeny. Broiler breeder hens were feed restricted from 24 wk of age and maintained at three bodyweights; 3.4, 3.6, and 4.0 kg until 43 wk of age and behavioral and physiological measures of stress recorded. Chicks were hatched from each hen treatment and at day 7 vaccinated for infectious bronchitis virus (**IBV**) and at 16, 18, and 20 d old given an immune challenge of lipopolysaccharide. Growth and immune responses of these birds were then recorded. Sex ratio was affected by hen bodyweight, with a significantly increased proportion of males hatched from heavy hens. Growth rate from 35 to 42 d of age was reduced in male progeny from low bodyweight hens. Female progeny from heavy hens responded to an immune challenge by reduced live weight and increased heterophil: lymphocyte ratio, suggesting a more robust immune response in these birds than in the progeny from lower bodyweight hens. Overall, progeny from heavy hens had increased antibodies at day 35 to the vaccination of IBV compared with progeny of low bodyweight hens, also suggesting an improved immune response in these birds. Breeder hens restricted to the lowest feed level showed behaviors indicative of increased stress (object pecking) and an increased heterophil: lymphocyte ratio. Feed restriction of broiler breeder hens increased indices of stress in hens and resulted in offspring that have reduced growth rate and immune response in a sex-dependent way.

Key words: broiler breeder, feed restriction developmental programming, immune response, meat chicken, stress

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INTRODUCTION

The concept that early life events affect health was first demonstrated during the Dutch famine (Barker, 2004). Early "events" occur during embryonic/fetal development, or early postnatal life and "impacts" can be metabolic, physiological, behavioral, and immunological (Chmurzynska, 2010). The major environmental factor studied to date has been maternal nutrition using intrauterine growth-restriction (**IGUR**) (Wu *et al.*, 2004) and dietary composition and level (Armitage *et al.*, 2005; Langley-Evans, 2006), with studies confirming the reprogramming effects of maternal diet on progeny growth and health. Animals reprogrammed by early nutrition display "metabolic syndrome," which can include diabetes and hypertension (Langley-Evans, 2006; Elahi *et al.*, 2009). Recently attention has been directed to the effects of maternal stress reprogramming on immunological functioning, with evidence of alterations in immunity in offspring of stressed mothers in multiple species, from rodents (Kay, 1998) to livestock (Couret *et al.*, 2009; He *et al.*, 2014)

Although developmental programming stress studies have been carried out in several species, there is limited research in meat chickens. Mothers of meat chickens, breeder hens, are selected for rapid, efficient growth of their progeny, leading to detrimental effects on their production of fertile eggs. The subsequent management practice of feed restriction of hens leads to chronic hunger and stress (de Jong *et al.*, 2002; Mench, 2002; de Jong *et al.*, 2003). Breeder hens are exposed to both under-nutrition and stress, likely affecting growth and immunity of their offspring, through developmental programming. The aim of this study was to measure the impact on the growth and immunity of progeny from breeder hens feed restricted to differing bodyweights. We hypothesized that hens kept at the highest level of feed restriction and lowest bodyweight would show increased stress and have offspring with reduced growth and immunity.

MATERIALS AND METHODS

Animal Husbandry

All animal use was approved by the animal ethics committees of The University of Adelaide (S-2014-121) and The Department of Primary Industries and Regions, South Australia (PIRSA) (#14/14). A total of 36 Cobb 500 broiler breeder hens were maintained in groups of 12 at a commercial breeder facility in group pens. Hens were maintained at three different bodyweights, low, medium, and high, through feed restriction on a commercial breeder hen diet. Hens were separated into treatment groups at 24 wk of age and remained in these groups for the duration of the experiment until 43 wk of age. Birds were fed once a day at a level that would allow for a difference in bodyweight between groups to be maintained. Feed intake for hens was increased from 21 wk of age from an average of 112 g/bird/d to 31 wk of age where birds were maintained at the maintenance needed to maintain the desired bodyweights from each treatment. Low bodyweight' hens were maintained on an average at 3.4 ± 0.1 kg and fed 140 g of feed/hen/d, "medium-bodyweight" hens were maintained on an average at 3.5 ± 0.1 kg and fed 145 g of feed/hen/d and "high-bodyweight" hens were maintained on an average at 3.9 ± 0.1 kg and fed 160 g of feed/hen/d. Hens were weighed weekly to ensure that correct bodyweights were maintained and feed intake of each group was adjusted to maintain bodyweight differences between groups.

Eggs were collected over a 2-wk period from each group of breeder hens. Eggs from each hen treatment collected over the 2-wk collection period were randomized and incubated at 38° and 55% humidity from days 0 to 15, and then 36.7°C and 60% humidity until hatch at day 21 at Roseworthy Campus, The University of Adelaide. After hatch viable chicks were weighed, ID tagged and placed into group-rearing pens. Birds from each hen treatment group were placed in pens together with chicks from the same hen group over three replicates.

Standard husbandry procedures were followed with chicks placed in a shed at 25°C with heat lamps in each pen and a light cycle of 23 h light for the first 24 h. From the second day, the light cycle was changed to 16 h light and the shed temperature adjusted accordingly. Chicks were fed ad libitum a standard commercial meat bird starter diet (Ridley Turkey and Meat Chicken Grower) until 3 wk of age and a commercial meat bird finisher diet (Ridley Turkey and Meat Chicken Finisher) until 6 wk old. All birds were given an ocular vaccination for infectious bronchitis virus (**IBV**) at day 7, weighed weekly, and feed intake recorded.

Hen Behavior Analysis

Breeder hens were observed daily over 1-h periods in the morning during the 2-wk egg collection. Behaviors were recorded using an ethogram at 30-s intervals and the number of birds displaying each

behavior at each time point was recorded. The total numbers of observations for each behavior and hen group were then added to give an overall total. The behaviors used (Table 1) were adapted from a previous study on breeder hen behavior (de Jong *et al.*, 2003).

Heterophil:Lymphocyte Counts

After blood collection, a drop of blood was placed onto a glass slide and smeared across the slide to create a monolayer of cells. The slide was then fixed in 70% methanol for 60 s and allowed to dry and later stained with Wright-Giemsa Stain using a Hematek Stain Pak (Bayer) Automatic Stainer. All slides were counted at 40× magnification three times by the same counter, under a blinded analysis. Cells counted were lymphocytes, heterophils, monocytes, basophils, and eosinophils until a total of 100 cells were counted. A ratio of heterophils to lymphocyte cells was then calculated for each slide.

Sampling

A blood sample was collected from 18 broiler progeny of each hen treatment group (n = 54). Samples were collected when birds were 21, 35, and 42 d old. Birds were sampled via the brachial vein using a 23-gauge needle and 2-mL syringe into 4-mL lithium heparin tubes and stored on ice. Samples were centrifuged at 2000 g for 5 min and plasma collected and stored at −20°C.

Meat birds were grown until 42 d old and at day 42, 69 birds (23 per hen group) were euthanized. Birds were dissected and gross organ weight recorded for duodenum, jejunum, ileum, liver, heart, proventriculus, gizzard, spleen, and bursa. Duodenum, jejunum, ileum, gizzard, and proventriculus samples were opened and flushed of digesta prior to weighing and sampling. Tissue samples of the duodenum, jejunum, ileum, liver, spleen, and bursa were then collected, snap frozen in liquid nitrogen, and stored at −80°C.

Table 1. Recorded broiler breeder hen behaviors using an ethogram, at 30-s intervals, for 1 h, daily over 2 wk of lay

Behavior category	Observed behaviors
Walking	walking/running
Sitting	sitting still
Standing	standing still
Peck drinker	peck drinker (not drinking)
Foraging	pecking/scratching litter
Comfort	preening, nibbling, stretching, and wing flapping
Peck object	peck object, peck cage (not including drinker)
Aggression	peck another bird/fighting
Other	any other observed behavior

Lipopolysaccharide Injections

Half of the remaining chicks (n = 72) were given three injections of a lipopolysaccharide (**LPS**) injection on alternate days, at 16, 18, and 20 d of age. Birds in each treatment group received an immune challenge injection of LPS *Escherichia coli* O55:B5 (Sigma-Aldrich) at a dose rate of 0.5 mg/kg bodyweight. Dose was determined after a review of the literature and the protocol was taken from Tan *et al.* (2014). The dose, however, was reduced to 0.5 from 1.0 mg/kg to reduce the risk of making the birds ill. Prior to injection, birds were weighed to determine dose rate and the injection site was disinfected using 70% ethanol. The birds were given an intra-peritoneal injection beneath the keel bone, 1 to 2 mm under the skin using a 23-gauge needle and 1-mL syringe. The remaining chicks (n = 74) in each hen treatment group did not receive the injection of LPS.

Yolk Immunoglobulin Y

Both egg yolk and serum samples were tested for Immunoglobulin Y (IgY) concentration. Egg yolk samples were collected from 20 eggs from low medium and heavy bodyweight hens (n = 60) over 1 wk. Medium hens were excluded from analysis and only low and heavy hen yolks were tested, due to the larger difference in the hen feed intake between these two groups. The eggs were weighed and the yolk collected and stored at −20°C. To test IgY concentrations in the yolk and serum samples, a 96 well IgG (Chicken) ELISA kit was used (Abnova, Sapphire Bioscience) and standard kit procedure was followed.

For the egg yolk ELISA, IgY was first extracted using an adapted method (Hamel *et al.*, 2006). Briefly, the yolk was defrosted and twice the amount of Dulbecco's PBS (Sigma-Aldrich) was added to the yolk and shaken vigorously. An equal amount of chloroform (Sigma-Aldrich) was then added and mixed thoroughly to produce a thick emulsion which was centrifuged at 1,000 g for 30 min at room temperature. After centrifuging, three distinct layers were visible with IgY present in the watery top layer, which was then removed and aliquoted into 1-mL tubes and stored at −20°C until analysis.

Yolk and Plasma Corticosterone

Corticosterone was extracted from yolks (n = 40) 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. The samples were centrifuged the next day and the supernatant taken and dried once more. The samples were then thawed, resuspended in 500 µL of phosphate buffer saline, and analyzed.

Extracted yolk and plasma samples from progeny birds at 23 (n = *53*) and 42 d old (n = 54) were analyzed for corticosterone concentrations. Samples were analyzed at the University of Western Australia, Animal Biology Department using a validated Radio Immuno Assay Corticosterone 125I RIA KIT (MP Biomedical, Orangeburg, NY).

IBV Antibody Analysis

Plasma samples collected at 35 d of age from offspring of heavy (n=15) and low (n =17) bodyweight hens were analyzed for IBV antibodies, after vaccination at day 7. Samples were analyzed by ACE Laboratory Services (Bendigo, Victoria) using an IBV (Ab) ELISA (BioCheck).

Statistical Analysis

Statistical analysis was performed using the IBM SPSS statistical program version 21. Hen behavior, yolk IgY concentration, plasma IBV titres, yolk and plasma corticosterone, and H:L cell counts were analyzed using a general linear model (GLM) with fixed effects of hen bodyweight, gender, and LPS treatment and interactions fitted into the model. Bodyweight was analyzed for each bird using repeated measures with the same parameters fitted into the model. Organ weights were analyzed using a GLM, with day 42 bodyweight fitted into the model as a co-variate. A Fisher's exact test was used to determine the sex ratio of the progeny from each hen bodyweight group. A P value of <0.05 was deemed significant, with a P value of >0.05 and <0.10 considered a trend. All data presented are the mean ± SEM.

RESULTS

Hen Behavior

Forage and pecking behaviors differed between hen bodyweight groups (P < 0.05) (Table 2). Hens kept at the lowest bodyweight were observed foraging the least number of times (4.8 ± 2.7) compared with medium (23.6 ± 2.8) and high bodyweight hens (13.4 ± 3.1). Low bodyweight hens also pecked objects in the cage more (184 ± 6.6) than medium (137.7 ± 6.8) and high bodyweight hens (132. 8 ± 7.5) (P < 0.05).

Hen Heterophil:Lymphocyte Counts

The H:L counts of white blood cells at 31 wk of age approached statistical significance (P = 0.06). Low bodyweight hens had a greater H:L ratio (1.03 ± 0.1) compared with hens maintained at medium (0.67 ± 0.1) and high bodyweights (0.59 ± 0.1).

Yolk and Progeny IgY

No statistical significance (P > 0.05) was found in egg yolk IgY concentration (ng/mL) in eggs taken from heavy (64.6 ± 6.6) and low bodyweight hens (66.5 ± 6.7). There was also no statistical difference (P > 0.05) in blood serum IgY (ng/mL) taken on day 23 from progeny of low (12.6 ± 3.0) and high bodyweight hens (13.9 ± 3.0).

Progeny Sex Ratio

The sex ratio of progeny from low and heavy bodyweight hens was significantly different

Table 2. Total number of times hens maintained at a low, medium, and heavy bodyweight were observed displaying foraging and pecking behaviors, using an ethogram with observations taken every 30 s, over 1 h, daily for 2 wk

Hen weight	Forage	SEM	*P*-value	Peck	SEM	*P*-value
Low	4.8[a]	2.7	0.011	184.0[a]	6.6	<0.001***
Medium	23.6[b]	2.8	0.007	137.7[b]	6.8	0.156
High	13.4[c]	3.1	0.011	132.8[b]	7.5	<0.001***

Values are means ± SEM, n = 36. Means in a column with superscripts differ, P < 0.05

($P < 0.048$). A greater proportion of males were hatched from heavy bodyweight hens ($n = 26$), compared with lower bodyweight hen offspring ($n = 18$). Female hatch rates were opposite and were greater in lower bodyweight hens ($n = 19$) and reduced in heavy bodyweight hen progeny ($n = 11$) (Figure 1).

Yolk and Plasma Corticosterone

A trend towards significance ($P = 0.086$) was found in yolk corticosterone (ng/g) between offspring of low (90.8 ± 17.7), medium (74.8 ± 21.3), and high (87.3 ± 18.1) hens. The yolk corticosterone concentrations (ng/g) between low and medium bodyweight hens was significantly different ($P = 0.045$).

At 23 d of age, there was no significant difference ($P > 0.05$) in plasma corticosterone (ng/mL) in offspring from low (89.4 ± 10.3), medium (95.8 ± 11.2), and high (120.4 ± 17) bodyweight hens. There was a trend towards a significant difference in plasma corticosterone (ng/mL) ($P = 0.065$) in the interaction between males and females from high and low bodyweight hens. Females from high bodyweight hens had increased corticosterone (139.8 ± 31) compared with females from low bodyweight hens (71.4 ± 13.9). In males, the opposite was seen with decreased corticosterone in males from high bodyweight hens (101 ± 9.8) compared with males from low bodyweight hens (107.5 ± 13.9).

At 42 d of age, there was also no significant difference ($P > 0.05$) in plasma corticosterone levels (ng/mL) between offspring of low (70.7 ± 7.1), medium (57.3 ± 9.3), and high (55.8 ± 8.3) bodyweight hens. There was a trend towards significance ($P = 0.094$) in the interaction of hen bodyweight*sex, in plasma corticosterone (ng/mL) in the offspring at 42 d old between males and females from high and low bodyweight hens. The opposite was found to day 23, with corticosterone levels were elevated in females from low bodyweight hens (82.5 ± 11.8) compared with females from high bodyweight hens (50.3 ± 13.6). In males, plasma corticosterone levels were reduced in those from low bodyweight hens (58.8 ± 7.8) compared with male from high (61.3 ± 9.6) bodyweight hens (Figure 2).

Progeny Growth

A significant three-way interaction ($P < 0.001$) was found in bodyweight of the progeny, of hen bodyweight, sex, and age. This interaction showed an effect of decreased bodyweight (g) in male progeny from low bodyweight hens (2678.9 ± 52.9) compared with males from heavy bodyweight hens (2906.3 ± 46.1) at 42 d of age (Figure 3).

Immunity Challenge and Response to LPS

A significant difference ($P = 0.05$) was found at day 35 in plasma antibodies to IBV between progeny from heavy ($n = 15$) and low bodyweight hens ($n = 17$). Antibody titers to the vaccination at day 7 were elevated in progeny from heavy hens (492.7 ± 76.1) compared with progeny from low bodyweight hens (287.3 ± 70.1), as seen in Figure 4.

Heterophil:lymphocyte (H: L) ratios at 23 d of age showed significant differences between males and females from heavy hens ($P < 0.05$). Females from heavy hens had a significantly greater H: L ratio (0.79 ± 0.15) than males from

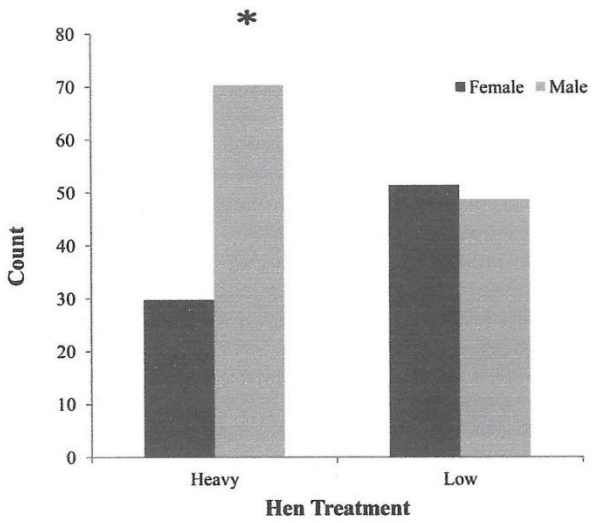

Figure 1. Sex ratio of broiler progeny at hatch from low and heavy bodyweight breeder hens. Significance was evaluated using a chi-square, Fischer's exact test with significance at $P < 0.05$.

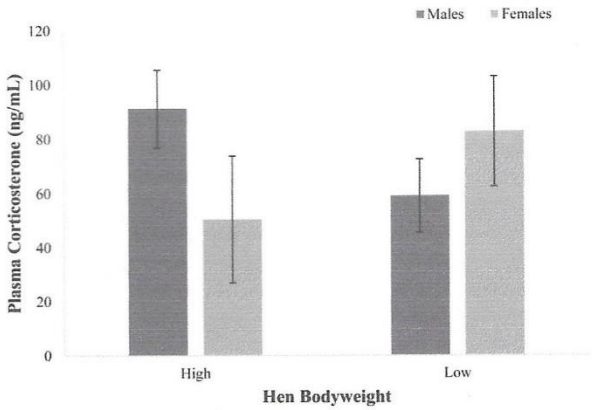

Figure 2. Plasma corticosterone (ng/mL) at 42 d of age in males and females from low ($n = 13$) and high ($n = 11$) bodyweight hens. Values are mean ± SEM. Significance was evaluated using a two-way ANOVA.

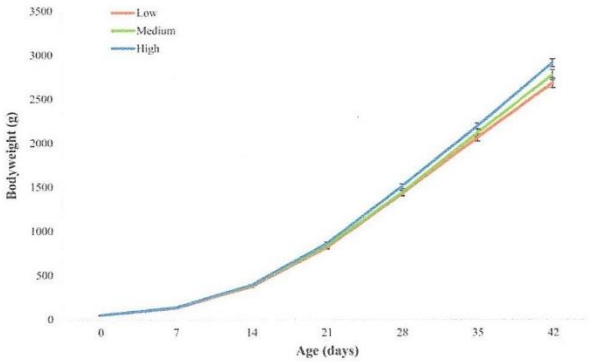

Figure 3. Bodyweight (g) of male progeny from low, medium, and heavy hens from hatch (day 0) until 42 d of age. Weight is mean ± SEM. Significance was evaluated using a repeated measures model with significance at $P < 0.05$.

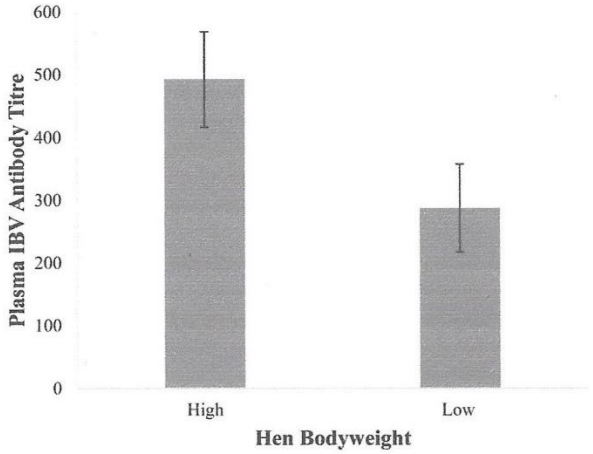

Figure 4. Plasma IBV antibody titers at 35 d old in progeny of low ($n = 17$) and heavy hens ($n = 15$). Values are means ± SEM. Significance was evaluated using a one-way ANOVA with significance at $P < 0.05$.

heavy hens (0.32 ± 0.07) as well as females from both low (0.38 ± 0.11) and medium bodyweight hens (0.28 ± 0.11). There was no difference seen between males from heavy (0.32 ± 0.07), medium (0.32 ± 0.08), and low bodyweight hens (0.42 ± 0.08) (Figure 5).

There was a significant difference ($P < 0.05$) in bodyweight (g) on day 21 between all progeny injected with LPS (798 ± 8.7) and controls (835.7 ± 8.7). After injection with LPS, a significant difference in bodyweight was observed between males and females on day 21 ($P < 0.05$). Female bodyweights (g) were lower after LPS injection (786.45 ± 11.7) compared with males that received LPS (842.8 ± 9.2).

There was also a significant interaction of sex-*hen bodyweight on day 21 bodyweight ($P < 0.05$). Females hatched from heavy hens were affected by the LPS challenge, with those given the LPS injection weighing less (755.3 ± 27.2) than control

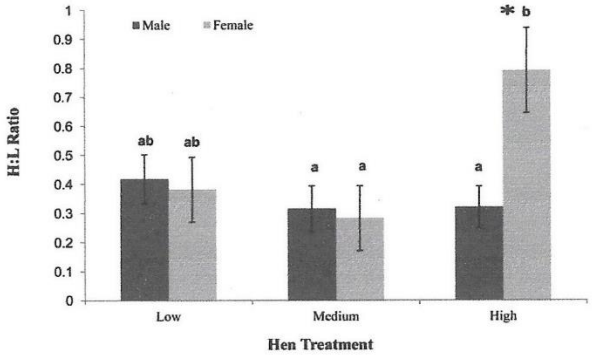

Figure 5. Heterophil:lymphocyte ratio of males and females from low, medium, and heavy bodyweight hens. Values are means ± SEM ($n = 36$). Significance was evaluated using a one-way ANOVA with significance at $P < 0.05$. Labeled means without a common letter differ, $P < 0.05$.

females (860 ± 27.2). Males from heavy hens were not affected in the same way, with males injected with LPS weighing the same (848.5 ± 18.5) as male controls (873.3 ± 19.3).

At dissection at 42 d old, there was a significant difference in spleen weight ($P < 0.05$) with bodyweight at day 42 fitted as a co-variate. Progeny from heavy bodyweight hens had significantly heavier gross spleen weight at day 42 (3.0 ± 0.2) compared with progeny of medium (2.4 ± 0.2) and low bodyweight hens (2.3 ± 0.2).

DISCUSSION

Hen Feed Restriction and Stress

Hens maintained at a low bodyweight from 24 wk old displayed decreased foraging behavior and increased object pecking. Increased pecking and reductions in comfort behaviors (such as foraging) can indicate chronic hunger in breeder hens, with correlations between these behaviors and other measurements of hunger such as feed intake motivation tests and glucose/NEFA ratio (de Jong *et al.*, 2003). These behaviors, indicative of chronic hunger, were accompanied by changes in the ratio of heterophil to lymphocytes in the blood, an accepted indicator of stress in poultry (Müller *et al.*, 2011). We cannot directly attribute stress in the hens to low feed intake per se as there may be some other component of low bodyweight that is the trigger to stress, but whatever it is, it must ultimately relate to low intake.

Yolk corticosterone levels can reflect plasma corticosterone levels in hens (Henriksen *et al.*, 2011). Surprisingly, hens maintained at a medium level of feed restriction had the lowest yolk corticosterone

concentration. These same hens also showed increased foraging behavior, and this together with the yolk corticosterone suggests that medium bodyweight hens were the least stressed, followed by heavy hens. This is likely due to an increased level of feed given to these birds, compared with the lowest bodyweight hens.

IgY Maternal Transfer

No difference was found in yolk IGY antibodies between hen treatments. There was also no significant difference between progeny of hens in plasma IgY, and therefore, it can be assumed that differences in progeny were not due to the transfer of IgY antibodies from the hen to the chick.

Progeny Sex Ratio

Sex ratio of chicks hatched was affected by hen bodyweight, with an increase in males hatched from heavy bodyweight hens. One possible reason behind this is the ability of avian species to modify hatchling sex ratio via changes in production of gonadal steroids, resulting in changes in egg hormone concentrations (Henriksen *et al.*, 2011). Testosterone is thought to be one such hormone behind this ability to manipulate sex ratios, with increases in yolk testosterone linked to increases in male born offspring (Veiga *et al.*, 2004; Rubolini *et al.*, 2005b). As testosterone and corticosterone levels can be influenced by one another; corticosterone levels can be opposite to testosterone in chicken egg yolk (Henriksen *et al.*, 2011). The decreased corticosterone within the yolks from heavy hens may therefore suggest that testosterone levels were elevated within these eggs, resulting in increased male hatchlings; although testosterone was not measured in this study, it should be considered in future work.

Progeny Growth

Hen bodyweight did significantly affect the growth of their offspring, but only in males during the final week of growth, with males from low bodyweight hens significantly lighter than males from heavy mothers. Yolk corticosterone was increased in low bodyweight hens, and elevated corticosterone in hens has been previously linked to decreased growth in their offspring (Janczak *et al.*, 2006; Shini *et al.*, 2009; Ahmed *et al.*, 2014), and could explain the reduced growth in males from low bodyweight hens. Previous avian research has also found reduced growth only in males after corticosterone exposure (Hayward *et al.*, 2006) as well as behavior changes in males (Goerlich *et al.*, 2012). It is possible that males may be more sensitive to changes in corticosterone levels during development and other maternal changes (Rubolini *et al.*, 2005a), and perhaps, this is why only male growth was affected.

Progeny Immunity and Response to LPS Challenge

Hen bodyweight had a significant effect on the antibody titer at 35 d of age of their progeny to the IBV vaccine at day 7. Antibody levels were found to be elevated in progeny from high bodyweight hens, compared with those from low bodyweight hens. This is a significant result as it demonstrates the impact of the hen bodyweight on the ability of the progeny to mount an immune response to a vaccination. The increased antibody titers in progeny of high bodyweight hens suggest an enhanced immune response of these birds in response to the vaccination, compared with progeny of low bodyweight hens.

Spleen weight relative to bodyweight was also increased in progeny from heavy hens and although spleen weight as a measure of immunity is debatable may have affected the antibody titers to IBV. A reduced spleen weight can be an effect of increased stress and corticosterone (Post, 2003) and although not significant, corticosterone within the yolk was slightly elevated in the low bodyweight hens. There is therefore the potential that there was an elevated corticosterone or other glucocorticoid elevated exposure within the low bodyweight eggs. These hens did also have elevated H:L counts and behaviors indicative of stress, which could have affected the immunity of their offspring, including spleen size and weight. This reduced spleen weight may then have gone on to reduce their immune ability as the spleen in avians is a storage organ of lymphocytes (Smith and Hunt, 2004) and their ability to produce the same amount of antibodies as birds from high bodyweight hens.

The impact of maternal stress and elevated glucocorticoids has been demonstrated in multiple species. In rodents, stressed mothers have given birth to offspring with decreased leukocyte counts, reduced B cell proliferation (Kay, 1998; Llorente *et al.*, 2002), and reduced antibodies (Gorczynski, 1992; Sobrian *et al.*, 1997), as seen in this study. Similar findings have also been shown in primates (Coe *et al.*, 1999), with decreased T cell response to antigens in offspring of stressed mothers and decreased white blood cells in piglets from stressed sows (Couret *et al.*,

2009). Therefore, the impact of maternal stress on the immunity of the offspring has been demonstrated across other species and is likely to occur in meat birds, as shown in this study and although the link between larger spleens and increased immunity has not been definitively shown in avians (Smith and Hunt, 2004). Spleen size could have been affected by maternal stress and possibly affected the antibody production in response to the vaccine. Overall, progeny from less feed-restricted heavy bodyweight hens was exhibiting an expected response and may be more sensitive to an immune threat than birds from lower bodyweight hens, under increased feed restriction.

Overall LPS-injected birds had a reduction in bodyweight at day 21, which might be expected as the birds partitioned nutrients to mount an immune response (Klasing, 2007). Voluntary feed intake did not appear to alter between treatments; however, the challenge was at a low dose and showed no effect on bird behavior or feeding. If the challenge dose was elevated, it is likely that feed intake would decrease. Differences in response between birds from low and heavy bodyweight hens were also found, with increased plasma corticosterone in LPS-challenged birds from heavy hens. An increased plasma corticosterone concentration might also be expected as pro-inflammatory cytokines are elevated, along with corticosterone (Lu *et al.*, 2008).

Differences in immune response were also found within gender between progeny from different bodyweight hens. Females from heavy hens challenged with LPS were significantly different from other LPS females, with reduced growth at day 21 after the challenge, increased H:L ratio, and increased plasma corticosterone. Males from heavy hens given LPS showed a similar increase in corticosterone later at day 42, suggesting they may be slower to respond than the females.

Reduced bodyweight and increased corticosterone are part of the immune response to the LPS. The elevation in H:L ratio also suggests the mounting of an immune response, as heterophils are part of the avian immune response (Shini *et al.*, 2010) and are also elevated with increased corticosterone (Shini *et al.*, 2008). These changes were not seen in females from low and medium bodyweight hens, which indicates that these birds were not mounting the same immune response. Therefore, the heavy hen females seem more able to respond to immune problems and although the LPS was low and did not cause any visible illness, these birds may be more likely to be able to defend against an immune challenge.

Reduced bodyweight through feed restriction in broiler breeder hens appears to induce a stress response with changes in behavior and H:L counts, affecting the progeny, possibly related to elevated corticosteroid levels in the yolk of the developing embryo. Progeny from these hens was affected in several ways in a sex-specific way. Males from less feed-restricted hens grew at a greater rate later in life and females from these hens mounted a stronger immune response to an LPS challenge compared with progeny from hens with heavier feed restriction. The results of this study have serious implications for the chicken meat industry both in terms of the welfare of feed-restricted hens but also for the health and productivity of the progeny meat bird. Maintaining breeder hens at a heavier bodyweight may therefore mean that hens are less stressed and could result in improved growth in male broilers and immunity in females as well as overall immune response to vaccination.

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Chapter 3:

Injection of Corticosterone *In Ovo* Leads to Reduced Growth and Sex-Dependent Effects on Organ Weights of Meat Birds

Chapter Introduction

This experiment followed on Chapter 2 where hen bodyweight and stress were shown to impact on their progeny. The aim of this experiment was to select a possible driver for the effects seen in Chapter 2 and measure the impact on meat birds. The stress hormone corticosterone was selected due to the stress seen in hens restricted to a lower bodyweight and evidence of the effects of maternal stress on offspring through changes in stress hormones found in the literature. Corticosterone was injected directly into meat bird eggs to mimic maternal stress, removing the factor of the hen. Progeny were then grown again to 42 days old as in the first experiment, and similar measurements collected.

As significant differences were found between males and females in Chapter 2, this was also further investigated in this experiment. Birds were sexed and the differing responses of males and females to the injection of corticosterone also investigated.

Abstract

Exposure to increased levels of the stress hormone corticosterone can significantly impact poultry, including meat birds, with potential lifelong changes in growth and development after corticosterone exposure *in ovo*. In this study, eggs were injected at embryonic day eleven with either 1µg/mL corticosterone in phosphate buffered solution (PBS) or PBS alone, via the chorioallontoic membrane. Birds then continued through incubation and at hatch were allocated to pens on the basis of injection treatment and sex. They were then fed a commercial diet *ad libitum* and weighed weekly until 42 days old. Organ weights and tissue samples were collected at 4, 21 and 42 days of age. At 21 days old average daily gain was reduced in corticosterone males compared to control males. At 42 days old corticosterone-injected birds had a lower bodyweight and average daily gain (ADG) compared to controls. At day 42 liver weight was reduced in corticosterone birds. A sex effect was also found in organ weights at 42 days of age. Jejunum weights were reduced in corticosterone treated females compared to control females. Spleen weight was increased in corticosterone males compared to control males. Increased corticosterone exposure during embryonic development can therefore reduce growth later in life and variably affect organ weights in meat birds, depending on sex and age.

Introduction

Corticosterone, a glucocorticoid hormone, is released during times of stress via the hypothalamic-pituitary-adrenal (HPA) axis (Kandel *et al.* 2013). Release of corticosterone from the adrenals serves an important purpose, as this hormone can elevate circulating glucose levels (Lynn *et al.* 2003, Lin *et al.* 2007), providing energy for stressful situations and restoring homeostasis. Elevation of corticosterone within

fertile avian eggs at point of lay, can however, have significant impacts on lifelong growth (Hayward and Wingfield, 2004), behaviour (Ahmed *et al.* 2014a) and immunity (Henriksen *et al.* 2013).

Breeder hens are exposed to chronic stress due to lifelong feed restriction, as shown by increased 'negative' behaviours such as object pecking (Bowling *et al*. 2018), and increased circulating plasma corticosterone levels (de Jong *et al.* 2002). As circulating plasma corticosterone levels are increased within the hen, corticosterone levels deposited into her eggs also increase (Hayward and Wingfield, 2004). The developing embryo within the egg is then exposed to this increased level of corticosterone which potentially reprograms the hypothalamus and HPA axis during development.

In meat chickens exposed to increased corticosterone levels *in ovo*, reprogramming of the HPA axis has already been demonstrated, with decreases in the glucocorticoid receptor (GR) protein within the hypothalamus (Ahmed *et al.* 2014b). A flow-on affect to another area of the HPA axis pathway was also shown, with a downregulation in corticotropic-releasing hormone (CRH) gene expression in meat birds exposed to increased corticosterone *in ovo* (Ahmed *et al.* 2014b).

While, it has been shown that corticosterone exposure can lead to changes to the HPA axis in meat birds, the possibility of differences between males and females exposed to increased corticosterone *in ovo* has not been extensively studied. Previous studies indicate that male and female meat birds responded differently to stress, with increased serum corticosterone and central benzodiazepine receptors (part of the $GABA_A$ complex) in males compared to females after a stressful stimulus (Marin *et al.* 2002). Sex differences have also been shown in quails exposed to

elevated corticosterone *in ovo*, with reduced growth in males, while female growth remained unaffected (Hayward *et al.* 2006).

Males and females show variations in the sensitivity of the HPA axis to developmental programming. Female rats from mothers with elevated corticosterone, have been shown to also have increased corticosterone while males did not (Tilbrook and Clarke, 2006). This suggests these females have a reduced HPA axis feedback mechanism, elevating corticosterone. Therefore, the effects of maternal corticosterone levels on offspring differ between male and female rats and the same may be true in poultry, including meat birds.

Reprogramming of the HPA axis and subsequent effects on growth and development of meat birds has potentially important implications for the poultry industry. Variations in growth can impact on production and processing of birds, so any growth differences between sexes imparted by stress hormone exposure, could have significant economic effects. In this study, the impact of elevated corticosterone *in ovo* on growth and organ development in male and female chickens was quantified. The hypothesis was that embryonic exposure to elevated levels of corticosteroid *in ovo* differentially affects the growth and organ development of male and female meat birds.

Methods

In Ovo Injections

All animal use was approved by The University of Adelaide (S-2015-170) and The Department of Primary Industries and Regions, South Australia (PIRSA) (#23/15) ethics committees. A total of 144 Ross 308 fertilised eggs were collected (Baiada

Poultry Pty. Ltd) and incubated for 21 days at The University of Adelaide, Roseworthy Campus.

Eggs were incubated at 38ºc and 55% humidity from day 0 to 15, then 36.7ºC and 60% until hatch at day 21. At embryonic day eleven 86 eggs were injected with a previously validated dose of 1µg corticosterone (Sigma-Aldrich) in 100µL phosphate buffered solution (Ahmed *et al.* 2014b) and remaining 58 eggs were injected with the phosphate buffered solution (PBS) only as controls. The dose was determined from previous studies (Ahmed *et al.* 2014b), with a higher dose than normal biological levels chosen to elicit a stronger response as a proof of concept.

More eggs were injected with the corticosterone injection because it was unknown if there would be a higher embryo loss in this group. Solutions were injected into the chorioallontoic membrane (CAM) although injecting into the yolk to mimic maternal stress has been a previously validated method. However, distribution of injected corticosterone into the yolk may remain close to the embryo and not be evenly distributed within the yolk up to embryonic day 6, therfore not mimicking the natural corticosterone deposition into the egg during formation (Engelhardt *et al.* 2009). This site was therefore chosen after reviewing previous research, where it was found to be a safe site with low mortalities and allowed rapid absorption of the injected substance into the developing embryo (Rodricks *et al.* 2006).

The timepoint of embryonic day 11 was chosen as the injection timepoint after both reviewing previous literature and experience of other injection studies conducted by researchers. A pre-incubation or early embryonic development injection timepoint would be most suitable as an indication of the impacts of maternal stress. However, due to concerns of high embryo losses, a later timepoint was chosen. Embryonic

day 11 was used based on previous cortiocosterone injection studies in poultry (Ahmed *et al.* 2014b; Rodricks *et al.* 2006; Lay and Wilson 2002).

To locate the injection site, eggs were candled to ensure the air cell was at the top, and the air cell location marked with a pencil. The injection site (through the air cell) was swabbed with 70% ethanol and a sterile 23G needle and hub used to puncture the egg, to a depth of 2 mm. The tip of an insulin syringe was inserted into the hole and the solution injected into the CAM (Figure.1). Once the needle was removed, the hole was sealed with (Selly's Glass silicone sealant aquarium safe) and the egg returned to the incubator as soon as possible.

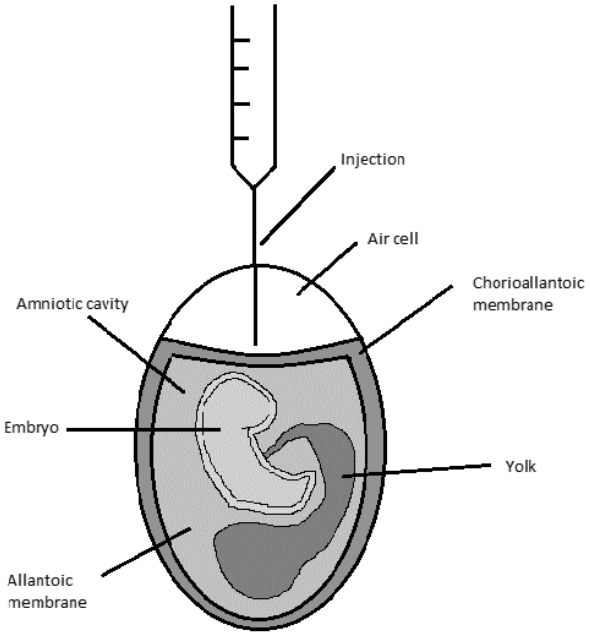

Figure 1: *In ovo* injection into the chorioallontoic membrane (CAM) at embryonic day 11 through the air cell of the egg.

Animal Husbandry

At hatch chicks were weighed weekly, feather-sexed and given individual identification tags. Chicks were then allocated to group rearing pens based on the *in ovo* injection they received and by their sex. A total of 85 birds were placed from

the corticosterone injected treatment (41 males, 44 females) and 37 for the PBS treatment (16 males, 21 females). This difference was due to a higher number of eggs injected with corticosterone, due to the possibility of losing embryos due to this treatment.

Chicks were housed in standard husbandry conditions, in a poultry shed at 25ºC with heat lamps in each pen and a light cycle of 23 hours light for the first 24 hours. After the second day the light cycle was altered to 16 hours light and the shed temperature monitored throughout the trial. Birds had *ad libitum* access to feed and water and were fed a commercial meat bird starter diet from hatch to day 21 (Ridley Turkey and Meat Chicken Starter) and a finisher diet from day 21 to 42 (Ridley Turkey and Meat Chicken Finisher). At seven days post-hatch all birds were given an intra-ocular vaccination against Infectious Bronchitis Virus (IBV).

All birds were weighed weekly from hatch until 42 days of age (6 weeks). The feed intake of birds for each group rearing pen was recorded and a weekly feed conversion ratio (FCR) calculated. The average daily gain of the birds was recorded using the weekly body weight recording with week 1 from hatch (day 0) to day 7, week 2 from day 7 to 14, week 3 from day 14 to 21, week 4 from day 21 to 28, week 5 from day 28 to 35 and week 6 from day 35 to 42.

Sample Collection

Blood samples were collected from corticosterone treated males *(n=9)* and females *(n=5)* from and PBS males *(n=6)* and females *(n=6)* at day 4. Blood was also collected at day 21 from corticosterone treated males *(n=6)* and females *(n=12)* and PBS treated males *(n=6)* and females *(n=6).* A final blood collection was taken at 35 days of age from corticosterone males *(n=11)* and females *(n=12)* as well as

PBS males *(n=3)* and PBS females *(n=6)* (see Table 1). Blood samples were collected via the jugular vein at all time points for analysis of heterophil/lymphocyte (H:L) ratio counts. Blood was collected from birds using a 23-gauge needle and 2mL syringe into 4mL lithium heparin tubes and stored on ice.

As seen in Table 1, at day 4, organ weights were collected from a section of the previously blood sampled birds including, males *(n=9)* and females *(n=5)* from corticosterone birds and PBS males *(n=6)* and females *(n=6).* At 21 days of age, another sub-set of birds were euthanised and organ weights recorded for corticosterone injected males *(n=13)* and females *(n=11)* as well as PBS males *(n=6)* and PBS females *(n=6).* Finally, at day 42, birds were euthanised and organ weights collected from corticosterone males *(n=38),* females *(n=30)* and PBS males *(n=9)* and females *(n=6)* (Table 1). Numbers vary between treatments and sex as sex was confirmed after euthanasia during dissection and where it was incorrect another bird was sampled. Also, as there were more corticosterone birds to begin with, more were sampled when available. Organ weights of duodenum, jejunum, ileum, proventriculus, gizzard, liver, heart, spleen and bursa were recorded. Samples of jejunum, ileum, liver, spleen and bursa were collected, frozen in liquid nitrogen and stored at -80ºC.

Table 1**:** Number of birds sampled from each *in ovo* injection treatment and sex. Values are number of birds used for blood sampling and organ weight collection.

	Day 4		**Day 21**		**Day 35**	**Day 42**
	Blood	Organ	Blood	Organ	Blood	Organ
Corticosterone Males	9	9	6	13	11	38
Corticosterone Females	5	5	12	11	12	30
Phosphate buffered saline Males	6	6	6	6	3	9
Phosphate buffered saline Females	6	6	6	6	6	6

Heterophil: Lymphocyte Counts

After blood collection a drop of blood was placed onto a slide and smeared to create a monolayer of cells. The slide was then fixed in 70% methanol for 30 seconds and allowed to dry. Slides were later stained with Wright-Giemsa Stain using a Hematek Stain Pak (Bayer) Automatic Stainer and counted at 100X magnification. Cells were counted one microscope field at a time until a total of 100 cells were counted, and from this a ratio of lymphocyte to heterophil cells was calculated.

Statistical Analysis

Analysis was performed using the IBM SPSS program version 21 with a P-value < 0.05 considered significant. A general linear model (GLM) with a LSD test was used to determine differences in weekly bodyweight, feed eaten, feed conversion ratio (FCR), average daily gain (ADG) and cell counts. Organ weights were also analysed using a GLM. The bodyweight was fitted into the model as a co-variate for organ weights on the corresponding day.

Results

Bird Growth

At 21 days of age, there was a significant interaction of *in ovo* injection*sex ($P < 0.05$). In males, corticosterone *in ovo* significantly reduced ADG (69.1 ± 1.2) compared to PBS males (77.4 ± 2.5), as seen in Table 2.

At day 42 there was a significant effect of the *in ovo* injection on bird weight ($P =0.032$) and average daily gain ($P < 0.001$). At day 42, corticosterone-injected birds had a significantly lower ADG (95.9 ± 2.5) and bodyweight (3112 ± 32.4) compared to PBS-treated birds ADG (122.7 ± 5.1) and bodyweight (3376.2 ± 73.3). Feed eaten and feed conversion ratio (FCR) were non-significant ($P > 0.05$) throughout the study from day 7 to 42 (data not shown).

Table 2: Average daily gain (ADG) (g/day) from day 7 until day 42 of male and female meat birds injected with corticosterone or phosphate buffered saline *in ovo*.

Day	Corticosterone	Phosphate Buffered Saline	P value	Corticosterone Males	Phosphate Buffered Saline Males	Corticosterone Females	Phosphate Buffered Saline Females	P value
D7	17.2 ± .3	17.5 ± .5	n.s	17.8 ± .4	17.6 ± .7	16.5 ± .4	16.5 ± .6	n.s
D14	49.3 ± .5	49.4 ± .8	n.s	51.1 ± .6	52.2 ± 1.3	47.6 ± .7	46.6 ± 1.0	n.s
D21	66.7 ± .9	70.1 ± 1.6	n.s	69.1 ± 1.2	77.4 ± 2.5	64.2 ± 1.2	62.7 ± 2.0	P = 0.009
D28	97 ± 1.6	98.3 ± 3.2	n.s	97 ± 1.6	107 ± 5.0	89.4 ± 2.2	89.7 ± 4.1	n.s
D35	107.1 ± 1.6	109.8 ± 3.3	n.s	118.7 ± 2.3	122.6 ± 5.1	95.6 ± 2.2	97 ± 4.1	n.s
D42	95.9 ± 2.5	122 ± 5.1	P< 0.001	106.8 ± 3.6	126.8 ± 7.9	85 ± 3.3	118.4 ± 6.5	n.s

Table 3: Bodyweight from hatch (day 0) until day 42 of male and female meat birds injected with corticosterone or phosphate buffered saline *in ovo*

Day	Corticosterone	Phosphate Buffered Saline	P value	Corticosterone Males	Phosphate Buffered Saline Males	Corticosterone Females	Phosphate Buffered Saline Females	P value
D0	47.2 ± .3	47 ± .5	n.s	47.1 ± 0.5	46.4 ± 0.8	47.2 ± 0.4	47.7 ± 0.6	n.s
D7	168.4 ± 1.7	167.8 ± 3	n.s	169.9 ± 2.4	169.7 ± 4.6	166.9 ± 2.5	165.8 ± 3.9	n.s
D14	512.1 ± 3.7	511.5 ± 7	n.s	521.9 ± 5.3	537.5 ± 11.5	502.2 ± 5.2	495.5 ± 7.8	n.s
D21	977 ± 8.6	1000 ± 16.8	n.s	1003.6 ± 12.5	1062 ± 27.8	950.4 ± 11.9	938.1 ± 18.9	n.s
D28	1672.8 ± 18.6	1731 ± 40.6	n.s	1752.7 ± 27	1860 ± 67.9	1593 ± 25.7	1601.9 ± 44.5	n.s
D35	2425.3 ± 29.5	2516.8 ± 67.5	n.s	2619 ± 42.7	2734 ± 113	2231.6 ± 40.8	2299.6 ± 74	n.s
D42	3112 ± 32.4	3376.2 ± 73.3	P = 0.032	3375.2 ± 46.4	3611 ± 122.7	2850.1 ± 45.3	3141.4 ± 80.3	n.s

Values are means ± SEM, with $P < 0.05$ considered significant.

Organ Weights

At day 4, there were no significant differences (P > 0.05) in organ weights between injection treatments. At day 21, heart weight was significantly reduced (P = 0.042), in corticosterone males (5.6 ± .2), and increased in females (5.9 ± 1.9), compared to PBS males (6.4 ± .3) and females (5.6 ± .3). There were no significant differences in jejunum, ileum, liver, spleen or bursa weights at day 21, between treatment groups or in the interaction of treatment*sex.

At day 42, the interaction of *in ovo* injection*sex had a significant effect on jejunum weight (P = 0.041), with corticosterone decreasing jejunum weights in females (28.8 ± 1.0) compared to PBS females (33.8 ± 1.6). Spleen weight was also significant for *in ovo* injection*sex (P < 0.001), with significantly increased spleen weight in corticosterone males (3.3 ± .2) than in PBS males (1.7 ± .4), shown in Table 3.

At day 42 liver weight was reduced significantly in corticosterone-injected birds (P = 0.013). Liver weight in corticosterone birds was reduced (63.6 ± 1.5) compared to PBS birds (72.7 ± 3.2). There were no significant effects (P > 0.05) of injection or the interaction of injection*sex on ileum, gizzard, proventriculus, heart and bursa at 42 days old.

Table 4: Weights (g) of jejunum, spleen and liver relative to body weight of 42-day old males and females injected with corticosterone or phosphate buffered saline *in ovo*

Organ	Corticosterone	Phosphate Buffered Saline	P value	Corticosterone Males	Phosphate Buffered Saline Males	Corticosterone Females	Phosphate Buffered Saline Females	P value
Jejunum	29.1 ± .6 (0.93%)	32.2 ± 1.3 (0.95%)	P = 0.039	30.1 ± 1.1 (0.91%)	29.3 ± 2.1 (0.81%)	28.8 ± 1.0 (1.01%)	33.8 ± 1.6 (1.08%)	P =0.041
Spleen	3.1 ± .1 (0.10%)	2.5 ± .2 (0.07%)	P = 0.014	3.3 ± 0.2 (0.10%)	1.7 ± 0.4 (0.18%)	2.8 ± 0.1 (0.10%)	3.2 ± 0.3 (0.10%)	P < 0.001
Liver	63.6 ± 1.5 (2.04%)	72.7 ± 3.2 (2.15%)	P = 0.013	62 ± 2.6 (1.80%)	60.2 ± 5 (1.70%)	67 ± 2.1 (2.40%)	73.7 ± 3.5 (2.30%)	n.s

Values are mean ± SEM, with P < 0.05 considered significant\

Heterophil: Lymphocyte Ratio

There was no significant difference (P > 0.05) in heterophil: lymphocyte ratio between injection treatments at day 4. On day 21 there was a significant interaction between *in ovo* injection and sex (P = 0.044) on heterophil: lymphocyte (H:L) ratio. Females showed a decrease in H:L ratio (0.52 ± 0.04) when injected with corticosterone *in ovo,* compared to PBS females (0.67 ± 0.07), shown in Fig. 2. There were no significant differences with either male or female birds at day 35.

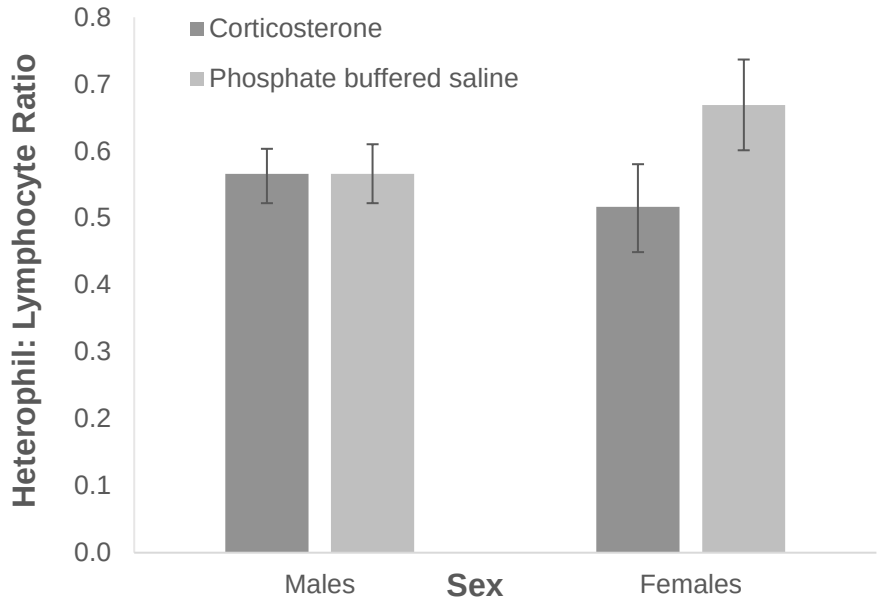

Figure 2: Heterophil: lymphocyte ratio at 21 days old of male and female birds injected with corticosterone (*n=17*) and phosphate buffered solution (*n=10*). Significance was evaluated using a 1-way ANOVA with significance at $P < 0.05$. Values are means ± SEM.

Discussion

The original hypothesis that growth will be affected by *in ovo* corticosterone injection was supported in the study. Bodyweight gain was depressed at day 42 post-hatch in birds injected with corticosterone, in accord with the findings of Ahmed *et al.* (2014a). The reduction in growth and average daily gain could be due to metabolic changes in the bird as it matures. In both this study and that of Ahmed *et al*. (2014b), the effect of corticosterone on growth appears to begin later in life, perhaps reflecting physiological or metabolic adjustments at this time. At 28 days post-hatch, meat bird growth dynamics, specifically fat deposition, undergoes a switch from adipocyte hyperplasia (increases in cell numbers) to adipocyte hypertrophy (increases in cell size) (Cherry *et al.* 1984). The rapid weight gain of meat birds and this change in metabolism from day 28, is reminiscent of obesity seen in humans in middle age (Palmer and Kirkland, 2016). This increase in fat deposition can then go on to also cause oxidative, inflammatory and metabolic stress (Rudich *et al.* 2007) along with increases in stress

kinases, intracellular toxins, proinflammatory adipokines and hypoxia (Klöting and Blüher, 2014).

Increases in oxidative, inflammatory and metabolic stress due to increasing fat deposition from day 28, may potentially led to increased stress on the birds as they continued to rapidly gain weight. Corticosterone males had a reduction in average daily gain at 21 days old compared to control males and are therefore potentially affected by this change in metabolism earlier than females, leading to a reduction in weight. This could be due to males growing faster than female meat birds. However, as fat deposition was not measured in this study, it is unknown if these birds did have increased fat deposition leading to stress. This would need to be investigated in future studies.

A reduced liver weight relative to bodyweight may also be indicating changes in metabolism of corticosterone-injected birds as the liver is the source of insulin-like growth factor-I (IGF-I) (Sjögren *et al.* 1999). It has been suggested that changes in maternal diet and increases in glucocorticoids, such as corticosterone, can reduce hepatocyte development and liver growth, impacting on IGF-1 levels (El Khattabi *et al.* 2003; Scanes 2011). As IGF-1 is important in the regulation of growth, reduced levels can lead to reductions in growth (Sjögren *et al.* 1999), and these changes in the liver may have altered growth potential in corticosterone birds, leading to an overall reduced bodyweight in the final week.

The original hypothesis relating to male and females, was that male and female growth and organ weights would be affected differently by exposure to increased corticosterone *in ovo*. This hypothesis was also proven, in relation to organ weights. In female birds, corticosterone *in ovo*, decreased jejunum weight, at day 42, while in males it was heavier, although not significantly. This could be a result of the earlier

metabolic changes seen in male corticosterone birds at day 21, where they had a reduced average daily gain. The jejunum of these birds may have compensated to increase absorption, resulting in a heavier jejunum relative to body weight at day 42.

Heart weight relative to bodyweight was also differentially affected between males and females at 21 days old. Females exposed to corticosterone had an increased heart weight, while for males the opposite was found. Heart weight has been found to be reduced in meat birds at day 35, due to corticosterone exposure from 21 days of age (Lin *et al.* 2006) and in laboratory rats, differences in cardiovascular physiology, such as hypertension, have been seen in females exposed to glucocorticoids *in utero (*O'Regan *et al.* 2004). This could be similar in female meat birds, with increased heart weights after corticosterone possibly indicating an increased work rate of the cardiovascular system. However, there was no increases in female mortality in corticosterone-treated birds due to heart failure in this study, which may possibly be due to the short lifespan of meat birds. This could be an area investigated in future research, with detailed measures, of the cardiovascular system of meat birds after exposure to corticosterone, such as histology.

At 42 days old, male and female spleen weight was also affected differently, with the *in ovo* corticosterone injection increasing spleen weight in males and decreasing spleen weight in females. This could act as an advantage in these males, as increased spleen weight may indicate increased immune cell capacity (Smith and Hunt 2004). This could also be investigated further with detailed measurements of the spleen such as lymphoid density of the spleen, to gain a better understanding of the impacts on the spleen and immunity of elevated corticosterone *in ovo*.

Overall, the organ weight results showed significant differences between males and females. In corticosterone treated males an increased jejunum weight may have been advantageous, allowing a potential for improved nutrient absorption, reduced heart

weight possibly reducing the risk of heart failure and increased spleen size increasing immune capacity. However, in corticosterone treated females the opposite may be said as reduced jejunum weight could reduce growth potential, increased heart weight may perhaps increase cardiovascular problems and reduced spleen weight could impact on immunity.

Sex differences were seen in H:L ratio counts at day 21. In females, there was a reduction in H:L ratio count in females injected with corticosterone, which may indicate a reduction in stress, as H:L ratio counts can be indicative of stress in poultry (Müller *et al*. 2011). As mentioned previously, adrenocorticoid responses have been shown to be sex-dependent in meat birds, with increased corticosterone in males after an induced stress (Marin *et al.* 2002). While information is still limited in meat birds, there is mounting evidence that males may be more susceptible to changes in stress response (Marinm*et al.* 2002). However, in rats, females showing reduced corticosterone are believed to have a less sensitive HPA axis and reduced negative feedback (Tilbrook and Clarke 2006). Therefore, it is still difficult to understand how the male and female meat bird HPA axis differs and how they will respond to elevated corticosterone *in ovo.*

The differences in male and female stress and HPA axis responsiveness are not yet completely understood, but one possible explanation is an effect of estrogen on corticosteroid response. Estrogen can increase the enzyme 11β-hydroxysteroid dehydrogenase (11ß-HSD), responsible for converting corticosterone to inactive cortisone (Kaludjerovic and Ward 2012). Females, with their higher estrogen levels, have increased levels of 11ß-HSD and an increased ability to break down corticosterone (Kaludjerovic and Ward 2012), possibility enabling them to have an increased resistance to increased corticosterone levels.

The inability of males to break down the increased *in ovo* corticosterone as effectively as females, due to lower estrogen levels, may mean male birds are exposed to higher corticosterone levels for longer, resulting in increased glucocorticoid receptor sensitivity as well as flow on affects to other areas of the HPA axis pathway, such as CRH (Ahmed *et al.* 2014b). To further understand these changes, the effects of corticosteroids on expression of hypothalamic genes such as GR, CRH and 11β-HSD need to be measured in male and female birds. Such studies will elucidate the mechanisms generating the sex-dependent effects of steroids on chicken development and growth.

Conclusion

Administration of corticosterone *in ovo* can result in reductions in growth and organ development later in the life of meat birds. Reductions in growth later in the life of meat birds exposed to increased corticosterone could have significant implications for the poultry industry. Stress in the breeder hen and subsequent elevated corticosteroid levels in the eggs will reduce growth and production in meat birds from these hens. The differences between males and females is also important to the industry due to sex variations in stress response, and organ development and the resulting differing effects this could have on the growth, stress and immune response. These discrepancies may mean management practices in breeder hens need to be modified to reduce stress, and male and female meat birds managed differently, to optimise their production and health.

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Chapter 4

Effects of Corticosterone Injection at Embryonic Day Eleven on Broiler Growth and Tonic Immobility

Chapter Introduction

This chapter is a conference paper that was presented at and formatted for the Australian Poultry Science Symposium (APSS) in Sydney Australia, in 2017. The work in this chapter details part of the previous experiment, not included in Chapter 3.

The focus of this chapter was behavioural stress response measured through the tonic immobility test. This was researched because of previous work detailing changes to the hypothalamic-pituitary-adrenal (HPA) axis that impacted on behaviour (Ahmed *et al.* 2014a) and behavioural stress response (Wang *et al.* 2013). It was not known however; how male and female meat birds would respond and how increased corticosterone had programmed their HPA axis behavioural stress response. Therefore, the speed of male and female behavioural stress response was measured using the tonic immobility test.

EFFETCS OF CORTICOSTERONE INJECTION AT EMBRYONIC DAY ELEVEN ON BROILER GROWTH AND TONIC IMMOBILITY

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Summary

Elevated corticosterone *in ovo* can have significant lasting impacts in avian species, including poultry. Stress in broiler breeder hens can lead to elevated corticosterone in the egg, exposing the developing embryo, leading to lifelong changes. This study aimed to mimic these effects by injecting broiler eggs with corticosterone or phosphate buffered solution at embryonic day eleven, into the chorioallontoic membrane. Differences were seen in growth during the final week, with corticosterone injected birds lighter. Behavioural changes were also observed in males, using tonic immobility testing, at day 14 ($P < 0.05$). These changes show that exposure to elevated corticosterone at embryonic day eleven has long-term impacts on chick growth and behaviour. Further research into this area and larger trials using this injection time point would are needed to be conducted to further look at these changes.

Introduction

Hens exposed to an environment of increased stress can have significantly elevated circulating corticosterone levels (de Jong *et al.* 2002), increasing deposits of corticosterone within the egg (Saino *et al.* 2005). Embryos within the egg are exposed to this increase in corticosterone during development, and changes to the hypothalamic-pituitary-adrenal (HPA) axis can occur (Ahmed *et al*. 2014b). These changes can then go onto to impact the lifelong health of the embryo with reductions in growth (Hayward and Wingfield, 2004) immune response (Henriksen *et al*. 2013) and changes in behaviour (Ahmed *et al.* 2014a). In humans and other species these changes occur through a process known as developmental programming and have been proven to have significant impacts later in life (Barker *et al.* 2006). This area is still being researched and understood, and this study aimed to further understand the lifelong impacts of *in ovo* exposure of elevated corticosterone in chicken embryos on lifelong growth and behaviour.

Methods

In Ovo Injections

A total of 144 eggs were collected from Baiada hatchery and incubated at the Roseworthy Campus, University of Adelaide. At embryonic day eleven 96 eggs were injected with corticosterone (1µg in 100µl phosphate buffer solution) and the remaining eggs injected with the phosphate buffered solution (PBS) only. This dose rate was chosen as it was used in previous studies (Ahmed *et al.* 2014a; Ahmed *et al.* 2014b) as a high dose and has therefore been demonstrated to have measurable effects throughout the lifetime of the embryo. Also, corticosterone concentrations can vary substantially between eggs and hens (Janczak *et al.* 2006) and as we had no control

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over the hens, we wanted to ensure the dose was high enough that the corticosterone group was significantly elevated compared to the control group. Solutions were injected into the chorioallontoic (CAM) membrane using a 1mL insulin syringe and needle after a hole was made using a 23G needle. After the injection, the hole was sealed, and the egg continued through incubation as normal.

Blood Sampling

At 35 days of age, 32 birds were blood sampled via the jugular vein. Samples were centrifuged at 3500rpm for five minutes, and the plasma collected and stored at -20^{0}C. Samples were then sent to the School of Animal Biology, University of Western Australia where they underwent Radioimmunoassay testing for corticosterone using a Corticosterone 125I RIA KIT (MP Biomedical, Orangeburg, NY).

Animal Husbandry

After hatch, chicks were weighed, feather sexed and placed into treatment pens. Chicks from each treatment (corticosterone or PBS) and sex were placed together. Chicks were monitored daily for signs of ill health and unwell birds were culled. Throughout the trial chicks had *ad libitum* access to water and a commercial broiler diet and were individually weighed weekly for the duration of the trial (6 weeks).

Tonic Immobility Test

All birds undertook a tonic immobility test (TI) at 14 days of age. Birds were placed onto their back and restrained for 20 seconds, where after the restraint was removed and birds allowed to flip over onto their front. The time taken for birds to do this was recorded in seconds, with a maximum score of 60 seconds possible. Results from the test were then placed into categories of slow or fast responses, with fast being 0-29 seconds and slow being 30-60 seconds.

Statistical Tests

Statistical tests were performed using the IBM SPSS program, version 21 with a P value < 0.05 considered significant. A Fischer's exact test was used for the tonic immobility data.

Results

Average Daily Gain

During the six week grow out trial, birds that were injected with corticosterone and PBS *in ovo,* a maintained similar average daily gain (ADG) throughout the first five weeks of the trial. However, a significant split is seen between the two groups from week five to week six, whereby week six corticosterone injected birds had a significantly lower ADG (96.45 ± 3.82) compared to PBS treated birds (121.82 ± 6.47).

Plasma Corticosterone

At day 35, plasma corticosterone (ng/mL) was significantly ($P < 0.05$) different between corticosterone and PBS injected birds. Corticosterone treated birds had significantly lower levels (66.6 ± 4.03) compared to PBS birds (88.7 ± 4.8).

Tonic Immobility

At day 14, no significant difference was seen between response times in the tonic immobility test between *in ovo* injection treatments ($P > 0.05$). There was however a significant difference in speed between males injected with corticosterone and PBS ($P < 0.05$). A higher number of corticosterone injected males were slow to flip over (*n=23)* than were fast (*n=11)*.

Discussion

Average Daily Gain

Average Daily Gain (ADG) was unaffected for the majority if the trial but was significantly impacted by treatment during the final week of growth. Corticosterone treated birds grew less during this final week than PBS birds, with other trials reporting similar reductions in growth after exposure to increased corticosterone *in ovo* (Ahmed *et al.* 2014a). Corticosterone can have many lasting impacts on the body, and if elevated can mean decreased IGF-1 (Scanes, 2011), which may lead to a reduction in growth. Unfortunately, IGF-1 levels were not recorded in this trial, so it is unclear if there were differences between treatments.

Also, broilers grow rapidly, with a 400% increase in broiler growth in the last 50 years (Zuidhof *et al.* 2014) and therefore develop much faster than other species. In humans and other species exposed to altered uterine environments, metabolic diseases including diabetes can arise (Hales, 2001), but are often not seen until later in life (Barker *et al.* 2006). This may explain why these changes in the broilers were not observed until the final week of growth. However, metabolic measures were not recorded in this trial so future work would need to include these measures on birds that have been exposed to elevated corticosterone *in ovo* to better understand the impacts on metabolism and growth and why they are occurring.

Plasma Corticosterone

In this study plasma corticosterone was significantly different between *in ovo* treatments at day 35. However, the kit used read the results at an elevated level, impacting on the final results although the results were verified by those conducting the test. Surprisingly, the birds injected with corticosterone had lower levels than the PBS injected birds which may mean that there is a down-regulation of corticosterone occurring later in the life of the bird. Impacts on growth were still seen from day 35, with corticosterone still supressing growth as in other trials with increased corticosterone exposure *in ovo* (Hayward and Wingfield 2004). This suggests that there may be an interaction effect of corticosterone, but this would need much more study to understand.

Tonic Immobility

TI is used to assess the fear response in birds, with the speed of the response indicating the level of fear/stress the bird is experiencing (Wang *et al.* 2013). At day 14 only males showed a significant difference in speed, with corticosteroid treated males responding slower, while females remain unaffected. Other studies have shown similar results with corticosterone treated birds responding slower to the test (Ahmed *et al.* 2014a). Males were affected while females were not, and this may mean that they are more sensitive to the corticosterone elevation. A similar study where birds were

exposed to an early life stress resulted in a dampened response to the stress (Goerlich *et al.* 2012). Male offspring of these birds also had a dampened stress response, suggesting males may be more sensitive to corticosteroid changes during development. However further research into understanding mechanisms behind these behavioural changes and differences between males and females.

Conclusion

Injection into the CAM at embryonic day 11 can be used in poultry to mimic the stress levels of feed restricted breeder hens. Using this technique, we found reductions in average daily gain of corticosterone treated birds in the final week of growth, which may be due to changes in growth factors and metabolic disorders but more work in this area is need. Behaviour was also altered, with males impacted at day 14 and responding slower to the test. The reasons for this sex effect are unclear but may be due to increased sensitivity in males to corticosterone but more work to investigate this is needed. Elevated corticosterone *in ovo* can have significant impacts on broiler growth and behaviour but the reasons for these changes needs to be researched further to better understand the mechanisms behind them.

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Chapter 5

Embryonic Hypothalamic Expression of the 11ß Hydroxysteroid Dehydrogenase Type 1 Gene Differs Between Male and Female Meat Birds and Administration of Corticosterone *In Ovo* Reduces Embryonic Hypothalamic Gene Expression of Multiple Genes

Chapter Introduction

The third and final experiment is presented below in chapter 5. In experiment 2, (chapter 3), differences were found in birds after a corticosterone injection *in ovo*, with reduced growth later in life. There were also differences between sexes, in corticosterone levels and organ weights.

Also, in chapter 4, further evidence was presented for differences between males and female hypothalamic-pituitary-adrenal (HPA) axis programming in response to elevated corticosterone *in ovo.* The delay in response of males exposed to corticosterone in the tonic immobility test demonstrated a reduced ability to respond to a stress and indicated reprogramming of their HPA axis compared to control males. Females, however, were not affected and because of this sex differences in gene expression within the hypothalamus was investigated**.**

In this experiment, the effects of corticosterone on the HPA axis pathway and negative feedback were measured. This meant measuring the gene expression of target genes in the hypothalamus of male and female birds. To understand the earlier impacts of corticosterone on meat birds, samples were collected from embryonic day 11 through to 21 days old. From this, it was hoped that an understanding of the effects of corticosterone on the HPA axis regulation of target genes in meat birds would be gained, including the differences between males and females. This could then help to explain differences found in the first and second experiments.

Abstract

The importance of corticosterone during a stressful event is well documented. The impact of extended elevations of corticosterone are also well understood, with documented evidence of reduced immunity and growth. The impact of exposure to increased corticosterone during embryonic development on the hypothalamic-pituitary-adrenal (HPA) axis is also now beginning to be understood. However, there is limited understanding on the impacts on meat birds, particularly differences between males and females. The aim of this study was to investigate the effects of elevated corticosterone *in ovo* on male and female meat bird hypothalamic gene expression.

Meat bird eggs were injected at embryonic day 11 with corticosterone (1µg corticosterone in 100ul in phosphate buffered solution via the chorioallanatic membrane. Hypothalamic gene expression of the glucocorticoid receptor (GR), corticotropic releasing hormone (CRH), arginine vasotocin (AVT), 20-Hydroxysteroid dehydrogenase (20HSD) and 11ß Hydroxysteroid dehydrogenase type 1 (11ßHSD1) were measured at embryonic day 14, hatch, day 7 and day 21. At embryonic day 14 gene expression of GR, CRH, AVT and 20HSD were significantly reduced in corticosterone birds, compared to PBS birds and 11ßHSD1 was reduced in males compared to females.

The reduction in expression of genes of the HPA axis pathway (GR, CRH, AVT) in birds exposed to increased corticosterone indicate that increased exposure to corticosterone during development can impact on the avian brain. This in turn has the potential to permanently alter the HPA axis function of these birds and create lifelong impacts on growth and immunity. The reduction in 11ßHSD1, an enzyme involved in converting glucocorticoids into an active form, in males could indicate a decreased ability in males to cope with increases in corticosterone during embryonic development. To further

understand these impacts, further research is needed to truly understand the lifelong impacts of increased cortiocosterone on meat birds and potential sex differences.

Introduction

Avians, like all vertebrates, when exposed to environmental stressors, can rapidly increase circulating glucocorticoids, including corticosterone (Herman *et al.* 2012). Short term elevations in corticosterone and cortisol can help survival and the ability to cope with stressful situations. This is due to increases in circulating glucose, insulin release and mobilisation of stored energy (Lin *et al.* 2007; Jiang *et al.* 2008). Elevated corticosterone also increases heart rate, respiration rate and blood pressure (Silverman *et al.* 2005) and these physiological changes allow a rapid response to stress as part of the 'fight or flight' response.

Corticosterone and cortisol are therefore very important in responding to stress and increasing the ability to survive in dangerous situations. However, if corticosterone levels are prolonged for extended periods of time, detrimental effects can result. This can include decreased growth (Lin *et al.* 2006) and reduced immunity through the suppression of cytokine release from immune cells and diversion of nutrients from the immune and digestive systems (Silverman *et al.* 2005).

Elevated circulating glucocorticoids in avian species including poultry, can lead to increased corticosterone deposition into the yolk of eggs (Royo 2008, Almasi *et al.* 2012). Increased exposure to corticosterone *in ovo* has been shown to negatively impact avian post-hatch development, including reduced growth, (Hayward and Wingfield 2004), increased aggression, delayed stress-response behaviour (Ahmed *et al*. 2014b) and reduced adaptive (humoral) immune response (Henriksen *et al.* 2013). Such lifelong effects have been linked to alterations during embryonic development in

receptors involved in the hypothalamic-pituitary-adrenal (HPA) axis negative feedback (Seckl 2007).

The two major receptors for glucocorticoids are the mineralocorticoid receptor (Type 1) and glucocorticoid receptor (Type 2) (Bossis *et al*. 2004). The glucocorticoid receptor (GR), is located throughout the brain including the paraventricular nucleus (PVN) (Kloet *et al.* 2008) where it responds to high levels of corticosterone, activating the HPA axis negative feedback response (Bossis *et al.* 2004). The HPA axis feedback loop then allows the restoration of homeostatic levels of corticosterone (Herman *et al.* 2012). This negative feedback loop reduces the release of corticotropic-releasing-hormone (CRH) and arginine vasopressin (AVP) (vasotocin in avians) via neurons within the PVN of the hypothalamus (Bossis *et al.* 2004). Ultimately, the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary is reduced due to CRH and AVP reductions, and in turn corticosteroid release is also reduced, maintaining homeostasis (Bossis *et al.* 2004).

Continual exposure of GRs to corticosterone can lead to dysfunction of the HPA axis stress response. Long term elevated circulating corticosterone reduces the sensitivity of the receptor, leading to a disruption in the negative feedback loop, resulting in corticosterone levels remaining elevated (Seckl, 2007). Long-term exposure to stressful events can increase receptor sensitivity and HPA axis negative feedback and significantly decrease corticosterone levels (Grossman *et al.* 2003). This is seen in people experiencing Post Traumatic Stress Disorder (PTSD) (Yehunda 2001) where exposure to high stress trauma, results in an increased GR sensitivity and a ‘blunted’ HPA axis response (Algamal *et al.* 2018).

As evidence increases as to the effects of stress hormones on the HPA axis in mammals, there is also evidence in avians, particularly when exposed to elevations

within the egg. In European Starlings, HPA axis response was reduced after exposure to increased corticosterone *in ovo* (Love *et al*. 2008), while HPA axis response was increased in Japanese Quails (Hayward and Wingfield, 2004). Differences between sexes have also been shown, with exposure to elevated corticosterone resulting in reduced growth in male Japanese quail, while female growth was unaffected (Hayward *et al.* 2006).

In chickens, elevated corticosterone *in ovo* has resulted in smaller progeny (Henriksen *et al.* 2013; Ahmed *et al.* 2014b*)*, behavioural changes, such as increased aggression (Ahmed *et al.* 2014a), reduced tonic immobility response (Henriksen *et al.* 2013), as well as reduced immunity (humoral immune response to a challenge) and reduced weight of the Bursa of Fabricius (Henriksen *et al.* 2013). The HPA axis pathway has also been shown to be impacted by increased corticosterone *in ovo*, with downregulation of the CRH gene and protein content of the GR in the hypothalamus (Ahmed *et al.* 2014b). Sex differences have been shown after early life stress in chickens, with improved associated learning in male chicks (Goerlich *et al.* 2012).

The impacts on the embryonic gene expression of genes along the HPA axis pathway, after increased exposure to corticosterone *in ovo*, has not been demonstrated in meat birds. Sex differences have also not been studied in meat birds embryonically. Therefore, the aim of this experiment was to investigate the effects of elevated corticosterone *in ovo* on HPA axis development in male and female meat birds.

It was hypothesised that increased corticosterone exposure *in ovo* would decrease glucocorticoid receptor sensitivity, affecting the expression of CRH, AVT and enzymes 11ß-HSD (Type 1 and 2) and 20-HSD. It was also hypothesised that hypothalamic gene expression of genes in the HPA axis pathway would be different between male and female birds after exposure to increased corticosterone *in ovo*.

Methods

In Ovo Injections

Fertilised chicken eggs (Ross 308) were collected from Baiada Poultry Pty. Ltd and incubated at the Roseworthy Campus, The University of Adelaide. All animal use was approved by The University of Adelaide (S-2016-132) and The Department of Primary Industries and Regions, South Australia (PIRSA) (#18 / 16) ethics committees.

Eggs (288) were left to pre-warm at room temperature (25ºC) for two hours before being set at day zero in the incubator at 38ºC and 55% humidity until embryonic day 15. From embryonic day 18 to hatch (day 21), eggs were incubated at a reduced temperature of 36.7ºC and increased humidity of 60%. On embryonic day seven, all eggs were candled, and 20 eggs removed due to infertility/early embryo death and 268 eggs remaining in the experiment.

At embryonic day eleven, 134 eggs were removed from the incubator and injected with 1µg corticosterone (Sigma-Aldrich) 100ul in phosphate buffered solution (PBS). The remaining 134 eggs acted as controls and were injected with the PBS solution only. Both solutions were injected through the air cell at the top of the egg, into the chorioallontoic membrane (CAM) using a 25G 1 ½ inch gauge needle and 1ml insulin syringe. The injection site was sealed with silicon and the egg returned to the incubator.

Animal Husbandry

At hatch 105 chicks were weighed and feather-sexed, chicks were placed into group rearing pens based on treatment (corticosterone or PBS) and sex, with a maximum of 10 birds per pen. Standard poultry husbandry procedures were followed, with temperature, water, feed and general chick condition monitored daily. The initial light period was 1 hour of darkness and 23 hours of light during the first 24 hours and from day two onwards was kept at 8 hours of darkness and 16 hours of light. All birds had

ad libitum access to water and a commercial meat bird feed (Ridley Turkey and Meat Starter). Each week birds were weighed (days 7, 14 and 21), feed intake per pen recorded and feed conversion ratio (FCR) calculated.

Sampling

A sub-section of birds were culled and tissue samples and weights collected at embryonic day fourteen, hatch (day zero), day seven and day twenty-one. At embryonic-day fourteen, 40 embryos were euthanized, weighed, dissected and samples of yolk, hypothalamus, taken and frozen in liquid nitrogen. The remaining embryos continued as normal through incubation. At hatch (day 0), and day seven, 24 chicks (6 per treatment and sex) were weighed, blood sampled and euthanised. Tissue samples collected of the brain (hypothalamus), collected and frozen in liquid nitrogen. On the final day of the experiment at day 21, the remaining 80 birds from each treatment, corticosterone males (*n*=18) and females (*n*=22), PBS males (*n*=13) and females (*n*=27) were weighed, blood sampled and euthanized. Again, samples were again collected of the brain (hypothalamus) from 6 birds per treatment and sex and frozen in liquid nitrogen.

Corticosterone Extraction and Analysis

Yolk samples were collected at embryonic day eleven (n=36), from corticosterone (*n=17*) and PBS injected (*n=16)* eggs. Samples was collected at the same time as embryos were sampled, using a 2mL syringe, stored on ice and later at 2ºC until analysis.

Extraction of cortiocosterone from yolks was carried out as by Cook *et al*. 2009. In this method, ~0.1 g of yolk was taken and added to 0.5 mL of distilled water and vortexed until mixed. For extraction 3mL hexane: diether (30:70 ratio) was added and vortexed. After being left to settle, the mixture was snap frozen in an ethanol/dry ice bath. Later,

after thawing, the supernatant was removed, dried and 1mL of ethanol added, and the solution frozen overnight at -80ºC. The following day the samples were centrifuged, and the supernatant again collected and dried again. For analysis, samples were thawed and resuspended in 500µL of phosphate buffered saline. Samples were analysed using a validated Radio Immuno Assay Corticosterone 125I RIA KIT (MP, Biochemical, Orangeburg, NY) at the University of Western Australia, Animal Biology Department.

Hypothalamus Collection

Hypothalamus tissue was also collected at embryonic day fourteen, hatch, day 7 and day 21. To collect the tissue, the brain was removed from the skull with a cut made horizontally along the skull from ear to ear in a circle, at which point the skull was removed. The brain was then carefully removed from the skull, beginning with the cranial end and slowly edged out until past the cerebellum and then the brain stem was cut, and the intact brain removed. The hypothalamus and surrounding tissue were removed by using a serious of four incisions, two vertical on either side of the cerebellum and cerebrum and two horizontal above and below the cerebellum to ensure the hypothalamus was removed whole and intact, as shown in Figure 1. To ensure removal of the entire hypothalamus, surrounding tissue was also captured, due to the difficultly in visualising the hypothalamus, particularly at embryonic day 14. The collected tissue was also trimmed before gene expression testing as much as possible to remove surrounding tissue.

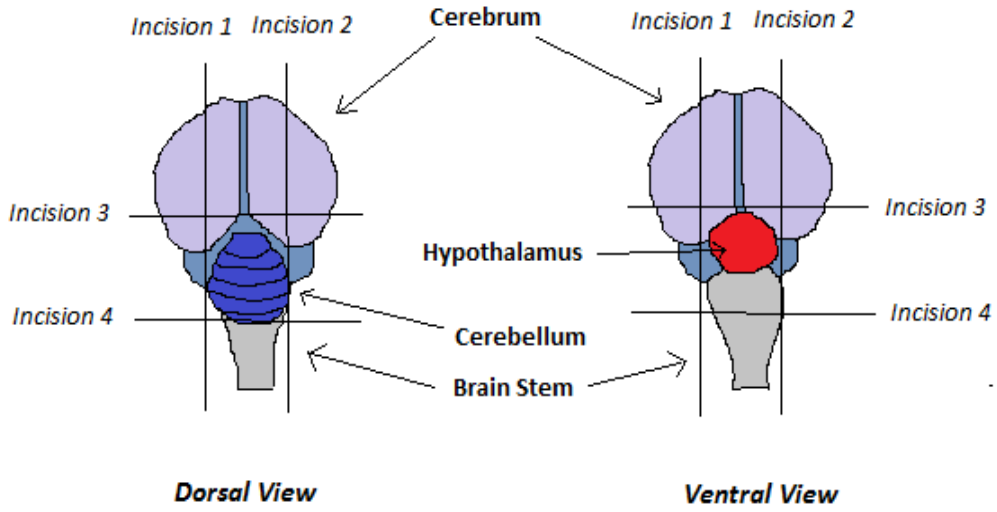

Figure 1: Ventral and dorsal view of the dissection of the hypothalamus from the brain, using a serious of four incisions, two vertical on either side of the cerebellum and cerebrum and two horizontal above and below the cerebellum to ensure the hypothalamus was removed whole and intact.

Isolation and Quantification of Total RNA From Hypothalamic Samples

Total RNA was extracted using a RNeasy Plus Universal Kit (Qiagen, Hilden, Germany). Approximately 100-200 mg (age dependent) of frozen (-80ºC) hypothalamic tissue was homogenized in 2mL of QIAzol Lysis Reagent (Qiagen, Hilden, Germany), aliquoted (1 mL) and stored at -80ºC. Thawed QIAzol homogenates were combined with gDNA eliminator solution (100 µL) and chloroform (180 µL) then centrifuged at 12,000g for 15 minutes at 4ºC. The upper aqueous solution (300 µL) and 70% ethanol (300 µL) were mixed and transferred onto a RNeasy column. The remaining wash and collection steps were run to the manufacturer's specifications. RNA was eluted in 60 µL of RNase free water.

RNA purity and concentration were measured using UV spectrophotometry (Nanodrop 1000; Thermo Scientific, Wilmington, DE). The 260/280 ratios ranged between 2.04-2.15 and final concentrations between 324 ng/µl – 1272 ng/µl. RNA integrity was confirmed on a subset of samples (n=12) by 1% agarose gel electrophoresis with clear

28S and 18S bands visible. A further subset of samples (*n=9)* were validated using a Tapestation 2200 (Aligent). Values for all samples were above the acceptable RIN range (>8.0) and ranged between 8.6 and 9.4.

Design of Real-Time qPCR assays

Oligonucleotides for quantitative PCR assays were designed for reference genes; Beta Actin (ACTB), TATA-binding protein (TBP) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and genes of interest (GR, 20-HSD, CRH, 11ßHSD & AVT*).* (Table 1) using the GenBank database (National Centre for Biotechnology Information; NCBI) database and the Ensembl chicken genome browser (*Gallus gallus*)*.* Two sets of exon-intron spanning primers were designed per gene using Primer 3 design software (version 4.0) (http://bioinfo.ut.ee/primer3-0.4.0/). Amplicon sizes were between 80 and 250 bp in length and primers spanned exon-intron boundaries greater than 500 bp in length. Suitability of primers for qPCR assays were validated by the following criteria: slope between (3.3-3.5) and R^2 > 0.93-0.99.

Table 1: Real-Time qPCR Primers

RNA Target	Gene Name	Direction[1]	Oligonucleotide Sequence (5'-3')	Accession no.[2]
GR	Glucocorticoid receptor	F	GGTGTTCTTACTTGTGGCAGC	NM_001037826.1
		R	GTTCCTTCCAGCGCAGAGAT	
20-HSD	20-Hydroxysteroid dehydrogenase (carbyl reductase)	F	GAAGGTGTGTGGTGCTGGAG	XM_015299436.1
		R	ACAATCGCCAATCCAATCCCT	
CRH	Corticotropic-releasing hormone	F	CAGAGGCAGAGGAAGGACG	NM_001123031.1
		R	GCTGCTGAGGGAAGAAATCG	
11β-HSD1	11ß Hydroxysteroid dehydrogenase type 1	F	GGTTCTCATCCCCTTGCTGG	XM_417988.5
		R	TCCCGTCACGATCACTCTCT	
AVT	Arginine Vasotocin	F	TGCTACATCCAGAACTGCCC	NM_205185.2
		R	AAGCAGCGACCCCTGTTC	
ACTB	Beta Actin	F	AATGGCTCCGGTATGTGCAA	NM_205518.1
		R	GGCCCATACCAACCATCACA	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F	TGTGACTTCAATGGTGACAGC	NM_20435
		R	GCTATATCCAAACTCATTGTCATACC	
TBP	TATA-binding protein	F	TCAGCAGCTATGAGCCAGAA	NM_205103
		R	CTGCTCGAACTTTAGCACCA	

[1]F = forward primer; R = reverse primer

[2]GenBank accession number

Synthesis of cDNA and Real-Time qPCR

RNA concentrations were normalised to 40n/μL using a liquid handling robotics system (EpMotion 5075; Eppendorf, Hamburg, Germany). Complementary DNA was synthesised from 400ng of total RNA using the High Capacity cDNA synthesis kit (Applied Biosystems, Carlsbad, CA) and carried out to the manufacturer's specifications. Reactions were incubated for 2 hours at 39ºC and the reverse transcriptase was inactivated at 65ºC for 20 minutes. Stock cDNA was diluted 1:4 with 10mM Tris (pH 8.0; Ambion) and kept at -80ºC.

Prior to running qPCR, stock cDNA (1:4) was diluted (1:20) with PCR grade water. SYBR-based (PowerSyber Green; Life Technologies) PCR reagent (19μL) was combined with 10μL of 1:20 cDNA. The SYBER/cDNA (5μL) was transferred in triplicate onto a 384-wellMicroAmp plate (Applied Biosystems). For each gene, a 5-point standard curve of pooled cDNA (1:4) was diluted in nuclease-free water 4-fold to; 1:8, 1:32, 1:128, 1:512 and 1:2048 was ran with a no template control (NTC). Quantitative PCR was performed on a 384-well real-time PCR machine (7900HT; Applied Biosystems).

qPCR Data Normalisation

Gene expression analysis and normalisation was conducted in accordance with the method described in Vandesompele *et al.* 2002. Prior to analysis of the genes of interest, reference genes were selected. Reference genes analysed were Beta Actin (ACTB), TATA-binding protein (TBP) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Following the method describe in Vandesompele *et al.* 2002 a M-value was generated for each gene. The M value is a reflective of the stability of the reference gene compared to the other reference genes, with a lower M-value is indicative of increased

stability (Vandesompele *et al.* 2002). To calculate the M values, the delimited data text files from each PCR run were used and exported to the geNorm spreadsheet. The reaction efficiency of each gene assay was determined from the standard curve and applied to a ΔCt quantification model to calculate the relative quantities between samples. The non-normalised data was then transferred to the GeNorm software to calculate the M value of the reference genes and normalisation of the genes of interest.

The M values were then used to rank genes in order of their stability, with the most stable genes used for normalisation. A pairwise variation analysis was used between the normalisation factor Vn/n+1, to determine the number of reference genes needed for accurate normalisation. The Vn/n+1 measures the effect of adding more genes to the normalisation factor (the effect is calculated as the geometric mean of the expression values of the selected reference genes), as described in Vandesompele *et al.* 2002.

The qPCR run output data was entered into the geNorm spreadsheet (geNorm) for normalisation. After analysis of the reference genes using the M Value, all reference genes were used for normalisation. The reference genes were then used to analyse the genes of interest measurements using geNorm, using geometric averaging (Vandesompele *et al.* 2002). Statistical analysis of the normalised relative PCR data was performed using a general linear model in the IBM SPSS program version 21 with a P-value < 0.05 considered significant. The embryonic and post hatch reference genes and genes of interest were analysed separately.

Results

Reference Genes

It is recommended that at least three reference genes be used for normalisation in qPCR (Vandesompele *et al.* 2002). The three reference genes chosen for both

embryonic and post-hatch samples had acceptable M values (< 1.5), when analysed in geNorm (Table 2).

Table 2**:** Average expression stability (M values) of the reference genes for embryonic and post-hatch samples, determined using geNorm software

Reference Gene	**M value Embryonic**	**M value Post hatch**
Beta Actin (ACTB)	0.462	0.404
TATA-bind protein (TBP)	0.379	0.349
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	0.57	0.525

Hypothalamic Gene Expression: Embryonic Day 14

Relative gene expression was successfully measured for GR, CRH, AVT, 20HSD and 11ßHSD1 in day 14 embryos. Gene expression of GR, CRH, AVT and 20HSD were significantly reduced in corticosterone birds, compared to PBS birds (Figure 2).

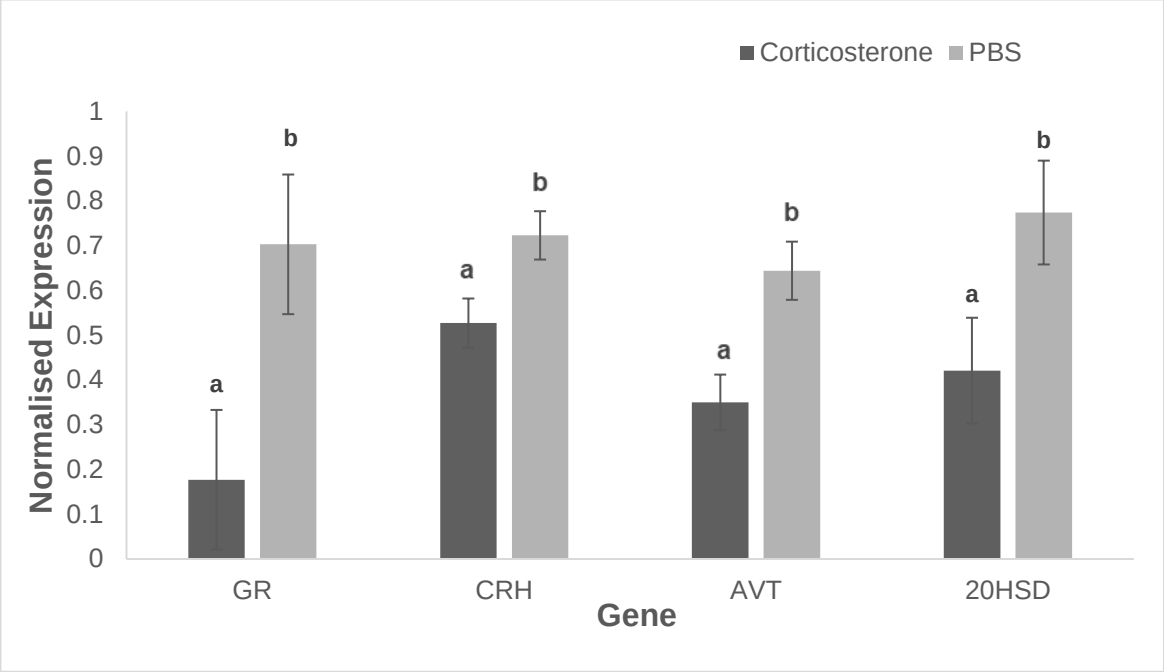

Figure 2: Normalised gene expression of glucocorticoid receptor (GR), corticotropic releasing hormone (CRH), arginine vasotocin (AVT) and 20-Hydroxysteroid dehydrogenase (20HSD) mRNA at embryonic day 14, of birds injected with corticosterone or phosphate buffered saline at embryonic day 11. Significance was evaluated using a 1-way ANOVA with significance at $P<0.05$. Labelled means without a common letter within a gene, differ $P<0.05$.

Expression of 11ß hydroxysteroid dehydrogenase type 1 (11ßHSD1) hypothalamic RNA was significantly greater in females, compared to males, irrespective of treatment ($P<0.05$; Figure 3).

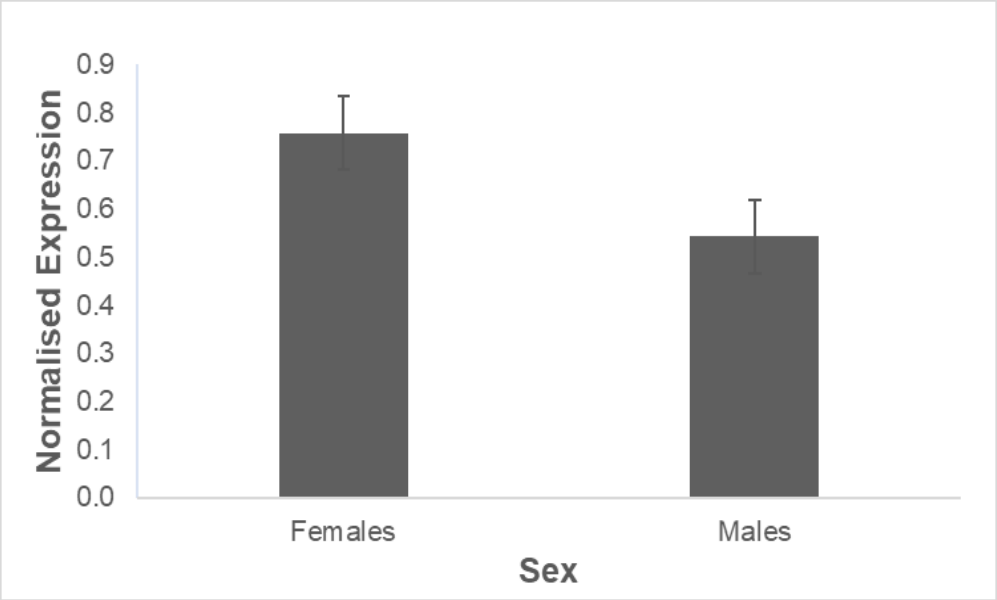

Figure 3: Normalised gene expression of 11ß hydroxysteroid dehydrogenase type 1(11ßHSD1) mRNA at embryonic day 14, of male and female meat birds. Values are mean ± SEM and were statistically significant, with significance evaluated using a 1-way ANOVA with significance at $P<0.05$.

Hypothalamic Gene Expression: Post hatch

Post hatch (hatch, day 7 and day 21) relative gene expression was successfully measure for glucocorticoid receptor (GR), corticotropic-releasing hormone (CRH) and 20-hydroxysteroid dehydrogenase (20HSD). There were no significant differences ($P >0.05$) between treatments or sex in gene expression for GR, CRH, 20HSD at hatch, day 7 or day 21.

RIA: Yolk Corticosterone

There was no significant difference ($P > 0.05$) in yolk corticosterone (ng/g) at embryonic day 14 between eggs injected with corticosterone or PBS. There was also no significant effect $P > 0.05$ of sex or the interaction of injection treatment and sex, in yolk corticosterone.

RIA: Serum Corticosterone

At hatch, there was no significant effect of injection treatment or sex on corticosterone levels ($P > 0.05$). On days 7 and 21 post hatch, serum corticosterone (ng/ml) remained similar for treatment and sex, with no significant differences ($P > 0.05$) found.

Discussion

Effects of Corticosterone Injection on Gene Expression

Hypothalamic expression of genes encoding the GR, CRH, AVT and 20HSD were reduced at embryonic day 14 in corticosterone treated birds. The reduction in expression of GR is potentially due to the exposure of increased corticosterone at embryonic day 11 and the HPA axis negative feedback response consequently activated.

The GR is part of corticosterone regulation and response to elevations of corticosterone via the HPA axis negative feedback response. The mineralocorticoid receptor (MR), located within the Hippocampus, is believed to be more involved in circadian rhythms of corticosterone (Bossis *et al*. 2004). Therefore, although both receptors are impacted by alterations in corticosterone levels, the GR responses to changes in short term corticosterone changes, such as the elevations in corticosterone experienced by the birds injected *in ovo*.

When the injection was given at embryonic day 11, the negative feedback loop responded as though it was a normal elevation in corticosterone from the adrenal glands. This in turn resulted in decreased gene expression of GRs as the response continued and corticosterone levels were reduced. Following the reduction in GRs, the gene expression of CRH and AVT were also reduced as they are also part of the HPA axis feedback loop (de Kloet 1995).

The expression of the ACTH gene was unfortunately not successfully measured but could be assumed to also be decreased as CRH and AVT were reduced and are directly involved in ACTH regulation (McEwin *et al.* 1998). Expression of the enzyme 20HSD, involved in deactivating corticosterone to its inactive form (20-dihydrocorticosterone) (Kučka *et al.* 2006), was reduced which indicates corticosterone regulation was implicated and levels reduced, through negative feedback.

However, yolk corticosterone was not shown to be significantly different in birds given the corticosterone injection. This may be due to the timing of the measurements which was three days post the injection, which is substantial in a 21-day incubation period. Hormonal regulation of corticosterone is rapid, and levels would have returned to normal within that time, while gene expression, however, is longer lasting (Kloet *et al*. 2008).

Differences in hypothalamic gene expression were not observed post hatch (day 0 to 21). However, the changes in embryonic gene expression could influence the ability of the birds to regulate the HPA axis negative feedback response later in life. Previous studies in chickens have shown decreases in the expression of GRs in older birds at 113 days of age (Ahmed *et al.* 2014a) exposed to increased corticosterone *in ovo.*

This may be due to embryonic exposure, resulting in an increased sensitivity or enhanced efficiency of the HPA axis response. Previous work found that piglets exposed to the increased maternal stress had reductions in hypothalamic GR binding sites, indicating a possible programming for an enhanced HPA axis response (Kanitz *et al*. 2003). It is therefore possible, that the later life increases in GR expression in birds exposed to corticosterone *in ovo* indicates the HPA axis and these receptors have been programmed to be more sensitive. Birds are then able to respond faster later in life under a short-term stress event, with increased expression. However, this response

may also prove detrimental under a chronic, sustained stress as the efficiency could lead to an overreaction of the HPA axis and altered ability to respond to stress.

To better understand the effects of corticosterone *in ovo* on long term development, a longer-term period of growth of the birds is needed. Measurements of gene expression and hormone levels (corticosterone, ACTH, CRH) within the embryo and later in life can then be better compared. It is also important to understand how stress later in life impacts the HPA axis of birds exposed to increased corticosterone *in ovo* and the effects of short versus long term stressors, including through behavioural measurements of stress such as tonic immobility.

The mechanism of corticosterone delivery also needs to be further investigated, to better mimic maternal deposition. The injection into the CAM was chosen as a proof of concept and to ensure delivery of the corticosterone and absorption and survival of the embryo. However, perhaps injecting multiple times may better reflect the sustained increased exposure to corticosterone by embryos naturally as the yolk is gradually absorbed by the developing embryo.

Hypothalamic Gene Expression Differences Between Males and Females

At embryonic day 14, there were sex differences in the gene expression of 11β hydroxysteroid dehydrogenase (11βHSD1), with increased expression in female birds, regardless of treatment. The role of 11ßHSD1 is to increase intracellular uptake of corticosterone (Wang *et al.* 2014). An increase in this enzyme could allow increased uptake of corticosterone into the cell to activate GR receptors, and in turn initiate a faster HPA axis negative feedback response. An increase in 11βHSD1 could therefore be advantageous in times of increased corticosterone and allow female birds to respond faster than males.

Although there was no effect of injection treatment, the increased levels of 11βHSD1 may have helped females exposed to corticosterone respond to the sudden increase in corticosterone. Females were potentially able to respond to the spike in corticosterone after the in injection at embryonic day 11 faster than males, due to the increase intracellular uptake of corticosterone activating GRs, as shown in Figure 4.

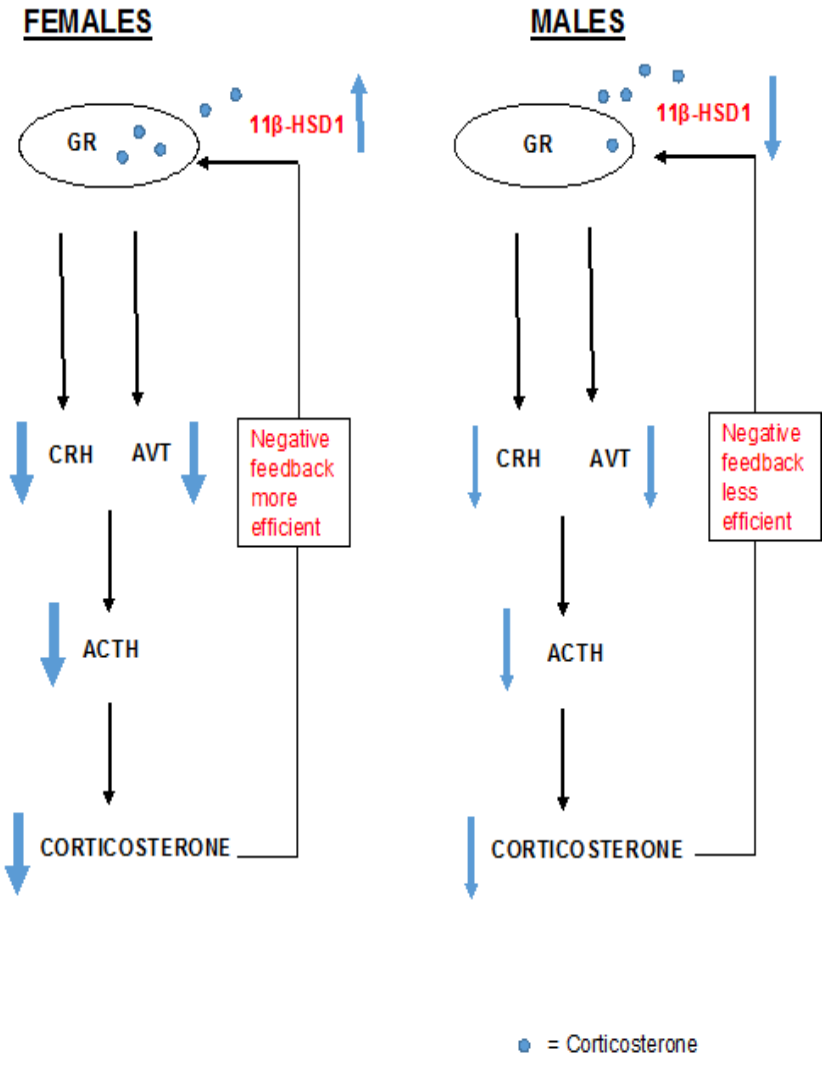

Figure 4: Proposed effect of elevated corticosterone exposure during embryonic development on the hypothalamic-pituitary-adrenal (HPA) axis of male and female meat birds. The elevations in 11β-hydroxysteroid 1 (11βHSD1) in females allows faster uptake of corticosterone into cell to activate the glucocorticoid receptor (GR). Females had a larger reduction in corticotropic-releasing–hormone (CRH) and arginine vasotocin (AVT). Adrenocorticotropic hormone (ACTH) and corticosterone release are possibly also reduced faster in the females, resulting in a more efficient negative feedback response.

In other avian studies, differences have also been found between male and female HPA axis responsiveness, with evidence suggesting a faster HPA axis response in

females. Female quails exposed to corticosterone as hatchlings, showed a reduced stress response to capture restraint stress at 22 days of age, compared to males (Marasco *et al.* 2012). In adult domestic chickens injected with CRH, female birds had a 50% reduced corticosterone response compared to male birds given the same injection (Madison *et al.* 2007). Together these results suggest that female birds are able to respond to elevated stress and corticosterone faster than males and restore corticosterone levels faster.

There is, however, limited evidence of the differences between male and female chickens after exposure to increased corticosterone *in ovo*. Studies conducted in other species have found differences between males and females after embryonic exposure to maternal stress and stress hormones. Male rats from mothers exposed to restraint stress in late gestation showed increased anxiety behaviour as adults compared to their female counterparts (Brunton *et al.* 2011). Postnatal stress of rats separated from their mothers during their first week of life also showed sex differences with attenuated plasma ACTH and corticosterone response to restraint stress in adult males but not females (Gehrand *et al*. 2018). Gene expression in male rats from mothers stressed in late pregnancy has also been shown to be different, with increased mRNA expression of the CRH Type 1 receptors (Brunton *et al.* 2011).

Although not avian studies, the results in rats also suggest differences between the male and female HPA axis, particularly after exposure to stress during gestation or early in life. The increase in anxiety behaviour in males indicates a more responsive or overactive HPA axis and stress response, while the attenuated ACTH and corticosterone may suggest the opposite. However, while ACTH and corticosterone levels were reduced in male rats, this was not reflected in hypothalamic, pituitary or adrenal mRNA expression (Gehrand *et al.* 2018). Therefore, there are still questions

around how gene expression can influence corticosterone release in males and females, not mammals, but avians too.

To better understand expression of genes involved in the HPA axis pathway, such as GR, CRH and 11βHSD1 and the advantages there may be for female meat birds, measurements of embryonic gene expression should be taken throughout development. The delivery of corticosterone should also be altered, with a more sustained exposure of the corticosterone throughout development to understand how longer-term exposure to elevated corticosterone effects males and females. Also, if females are indeed able to activate the HPA axis negative feedback sooner. Measurements of hormones, including corticosterone, CRH and ACTH would also give further understanding of the differences in the HPA axis of male and female meat birds during embryonic development.

There were no differences between male and female post hatch 11βHSD1 gene expression. This may have, as already discussed been due to the measurements only continuing until day 21. As already discussed earlier, the birds may not have undergone significant stress during this time. Therefore, it would be beneficial to measure the gene expression differences in 11βHSD1 past day 21 to understand if gene expression differs between males and females later in life.

However, the difference may only be present during embryonic development and benefit females at this time, allowing them to perhaps be programmed for later life. This could allow females to potentially be better able to cope with stressful situations, such as being in an outdoor production system. Further research is needed to understand differences in male and female HPA axis development in male and female meat birds.

Conclusion

The sensitivity of the avian HPA axis during embryonic development to changes in corticosterone levels was evident in this study. Key genes involved in the regulation of corticosterone levels were significantly downregulated at embryonic day 14 after exposure to elevated corticosterone *in ovo*. There is the potential for alterations in HPA axis gene expression during embryonic development to lead to an increased HPA axis sensitivity as shown in other species.

This altered state of HPA axis function and ability to respond to stressful events later in life could have wide ranging impacts on growth, development and performance of meat birds. Although birds may be able to respond to stress faster with an increased HPA axis sensitivity, if they are exposed to a sustained stress this could result in a malfunction of the HPA axis. If left unable to appropriately cope with stress, there would likely be ramifications in other physiological areas, leading to reduced growth and performance.

Male and females may also respond differently to stress, with this study showing differences in gene expression during embryonic development between sexes. While these differences were not seen post-hatch there is the possibility that if grown to full maturity there will be differences due to the HPA axis programming *in ovo.* There are also many other factors, such as hormonal differences, that could influence sex differences in growth and stress response. To fully understand this, birds need to be grown to full maturity and the differences between males and females investigated.

Once the differences between sexes and the impacts of increased exposure to corticosterone *in ovo* are better understood, practices of meat birds can be used to manage these differences. This could involve understanding how the life stage of the hen or living conditions may influence cortiocosterone deposition into the egg and the

level of exposure likely to her offspring. It could also mean managing males and females separately, such as placing females in a free-range system as they may cope with the increased stress better than males. More understanding, however, is needed to develop management strategies beneficial to male and female meat birds.

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Chapter 6

General Discussion

Developmental programming is a relatively recent scientific notion, and its importance first began to be understood during the Second World War. The role of maternal nutrition during pregnancy on lifelong health was revealed during the Dutch Famine, with children born at this time smaller due to restricted maternal nutrition. The famine ended with the war and these children then had access to normal nutrition and were able to catch up in growth. Later in their lives, however, they experienced issues with weight gain, cardiovascular disease and metabolic disorders, including increased insulin and glucose levels (Hales 2001). It has since been recognised that the problems experienced by people born during the Dutch Famine were from programming by their mother for a thrifty, efficient phenotype. This thrifty phenotype was not needed after the famine, however, and instead of being an advantage, it led to disease later in life.

As with maternal nutrition, maternal stress can affect offspring development across species. Stress during pregnancy has led to reduced birthweight in humans (Wadhawa *et al.* 1993), reduced immune cell numbers in pigs (Couret *et al.* 2009) and increased corticosterone levels in rats (Vallee *et al.* 1999). In avians, similar consequences of maternal stress to offspring have been observed with behavioural changes such as altered stress response and aggression, reduced growth (Janczak *et al.* 2006, Ahmed *et al*. 2014) and reduced humoral immunity (Henriksen *et al*. 2013).

Reductions in growth, immunity and altered stress response in avians is a major issue to the meat bird industry relying on the production of rapidly growing, healthy birds. Broiler breeder hen feed restriction leading to chronic stress (de Jong *et al.* 2002) potentially makes meat birds susceptible to the effects of maternal stress. The initial aim of this project was to determine if feed restriction and reduced bodyweight in broiler breeder hens leads to chronic stress in these hens, and whether this stress impacts on the growth and development of their progeny.

As discussed in chapter 2, the first hypothesis was that low bodyweight hens maintained under the highest level of feed restriction would show increased signs of stress, was supported. Hens maintained at the highest level of feed restriction and lowest bodyweight, demonstrated behaviours indicative of increased stress and had a higher H:L ratio, together indicating increased stress in these birds (Babacanoğlu *et al.* 2013).

The second hypothesis that hens with increased stress and reduced bodyweight would have offspring with reduced growth and immunity was also supported. Acquired immunity was reduced in offspring of low bodyweight hens, with reduced spleen weights and antibodies at day 35 to the earlier Infectious Bronchitis Virus (IBV) vaccine. Interestingly, differences in growth were sex dependent. It was found that growth rate was reduced in later life (days 35-42) only in males from low bodyweight hens, similar to previous avian studies (Goerlich *et al.* 2012). This reduction in growth in male meat birds impacts directly on production, with birds weighing less at day 42 than those from heavier hens.

Chapter 2 gives an overall picture of the potential impacts of maternal feed restriction and stress on progeny through developmental programming. Unfortunately, the broiler breeder hen treatments used were not replicated with only one group of hens per bodyweight treatment (low, medium & high). Hen treatments were not replicated because the research was carried out at a commercial facility with limited space available. Hens were also not selected until close to coming into lay and ideally should have been selected at hatch and their individual diets controlled from that point. More measurements from the hens, such as circulating corticosterone and other hormones throughout lay, would also have been informative to give a better understanding of hen corticosterone hormone fluctuations as they came into lay, reached peak lay and aged. Other circulating hormones involved in growth and metabolism that are transferred

from the hen to the egg, such as triiodothyronine (Ho *et al*. 2011) and IGF (Al-Musawi *et al.* 2012) would be useful to further understand influences of the hen on offspring growth hormones.

In terms of the offspring, the effects seen on the immune response were subtle and in future perhaps a stronger or different immune challenge could be used. The dose of LPS used in this trial was milder at 0.5mg/kg, compared to the previous study the dose and procedure was modelled on, at 1mg/kg (Tan *et al.* 2014). While this was used to ensure birds did not become ill and suffer, however, a slight increase in the dose may have led to a more obvious immune response. Also, using a different challenge, such as exposing birds to previously used 'dirty' litter could illicit a stronger immune response. A more detailed look at the immune response of the birds would have been beneficial, including spleen lymphoid composition and circulating lymphocyte cytokines, such as interferon-*γ* (IFN-*γ*) and tumour-necrosis factor-*α* (TNF-*α*), between treatment groups (Erf 2004).

Also, in future work, measuring a range of hormones such as, testosterone, throughout the growth of the birds, and genes involved in their regulation, would help in understanding the reasons behind sex differences. Testosterone was unfortunately not measured in this experiment as the sex differences seen were unexpected and yolk samples had already been used for previous testing. Testosterone should, however, be considered in future work in meat birds to determine the role it may play on growth and HPA axis responsiveness through developmental programming.

The effects of hen stress on their offspring were demonstrated by the first experiment. The next experiment followed on from this work by focusing on a model of maternal stress, employed to explore the effects on meat bird growth and development and differences between males and females in more detail.

In ovo corticosterone injection was chosen to mimic stress experienced by the breeder hen and the subsequent corticosterone elevation within the egg (Babacanoğlu *et al*. 2013). The advantage of injecting corticosterone directly into the egg, was that it eliminated the various other factors of the hen, such as age, diet, flock size, environment, feeding schedule, lighting schedule, social ranking within the flock and disease, allowing the focus to be directly on the effects of elevations in corticosterone. A disadvantage, however, was eggs were collected from an unknown and uncontrolled hen population, and so initial cortiocosterone levels within the egg were unknown. The effect of this was reduced, however, with the randomisation of eggs across treatments.

This model allowed us to assess the effects of elevated corticosterone on meat bird growth and organ development in both male and female birds. The hypothesis that embryonic exposure to elevated levels of corticosteroids *in ovo* would differently affect the growth and organ development of male and female birds was partially supported. Corticosterone treated birds followed the same trend as male birds from low bodyweight hens, with decreased growth from days 35 to 42, but not earlier in life.

These results were comparable with those found in chapter 2, validating the model of corticosterone injection as a mechanism of developmental programming changes in offspring from stressed hens. In chapter 2, birds were exposed to naturally occurring levels of corticosterone and although not statistically significant, at 42 days of age males from low bodyweight hens had decreased corticosterone compared to males from high bodyweight hens, along with reduced growth. In chapter 3, there was no sex difference, with growth supressed in both male and female corticosterone exposed birds.

Further evidence of the different effects on males and females exposed to elevated corticosterone exposure was observed when behaviour and stress response were

tested. Males injected with corticosterone *in ovo* had an increased response to a stressful stimulus (being placed onto their back) during the tonic immobility test, compared to control males. In females there was no difference between treatments. The mechanisms behind differences between male and female stress response were further explored, in chapter 5.

The evidence of altered responses to corticosterone between males and females was shown in chapter 2, with male growth reduced and stress response altered in chapters 2 and 3, respectively. To understand the reason behind these sex differences to corticosterone exposure, the hypothalamic-pituitary-adrenal (HPA) axis was considered. Previous literature around the HPA axis has shown differences between male and females in mammalian HPA axis and stress responses (Tilbrook and Clarke 2006). There is, however, limited research into meat bird sex specific HPA axis and the effects of elevated corticosterone on HPA axis development and lifelong stress response. Chapter 5, therefore aimed to address this gap by investigating differences between male and female meat bird HPA axis development after exposure to elevated corticosterone *in ovo.*

Similar to chapter 4, eggs were injected with corticosterone, and gene expression of genes associated with the HPA axis including, glucocorticoid receptor (GR), corticotropic releasing hormone (CRH), arginine vasotocin (AVT) and the enzymes 11β hydroxysteroid dehydrogenase (11ß-HSD) and 20-hydroxystreroid dehydrogenase (20HSD) were measured. Gene expression was measured at embryonic day 14, when the HPA axis is functional, at hatch, at 7 days of age and 21 days old. These time points were chosen to determine the initial expression during embryonic development and later in life to understand if programming of the HPA axis *in ovo* affects HPA axis gene expression post hatch.

It was hypothesised that increased corticosterone exposure would decrease GR sensitivity, affecting the expression of CRH, AVT and enzymes 11ß-HSD (type 1) and 20-HSD. Decreased expression of GR, CRH and AVT expression in corticosterone injected birds at embryonic day 14 was observed. No differences in gene expression, however, were found post-hatch, partly disproving the hypothesis. This may highlight the short-term changes in gene expression *in ovo* but the impacts of these changes in gene expression later in life when birds are under stress, needs to be further researched.

It was also hypothesised that GR sensitivity would also be increased in males compared to females. Interestingly, differences in hypothalamic gene expression were observed in male and female birds at embryonic day 14. Gene expression of 11ß-HSD1 was increased in female birds by 2-fold compared to males. 11ß-HSD1 is involved in increasing intracellular uptake of available corticosterone to activate the GR, stimulating a negative feedback response leading to reductions in CRH, ACTH and corticosterone release (Wang *et al.* 2014). Therefore, if females have higher levels of this enzyme at embryonic day 14, they may be able to cope with an increase in corticosterone and increase intracellular uptake of corticosterone. Corticosterone release from the adrenals is then reduced with the increased activation of the GR and increased negative feedback.

It could therefore be hypothesised, based on the results of the experiments presented in thesis, that the increased uptake of corticosterone to activate GR's may have resulted in an increased number of GR receptors in females during embryonic development. Male GR's, however, potentially became over-sensitised as they have decreased 11ß-HSD1 enzyme gene expression during embryonic development, and uptake is slower.

The altered GR expression could result in decreased GR numbers, which would need to be measured using immunohistochemistry. The sensitivity of the GR would then set the birds with different abilities to cope with stress during post-hatch growth and development. Therefore, male birds are not able to respond as efficiency to increases in stress. This may prove to be a significant issue as meat birds undergo metabolic changes at approximately 21 days of age onwards, with increased fat deposition (Cherry *et al.* 1984), potentially leading to increased metabolic and physiological stress. Females that have been programmed with potentially increased GR's, can respond to increased levels of corticosterone and restore homeostasis relatively quickly. Males, who may have fewer GR receptors to respond with, take longer to begin to restore homeostasis. This could account for the elevated plasma corticosterone levels in males in chapter 2 at day 21. The increased sensitivity of the male GR's could have resulted in an over-response to the increased corticosterone levels, leading to a decrease in corticosterone, as seen at day 35 in males in chapter 2. The fluctuating and delayed response of males to the increased metabolic stress then potentially resulted in the decreased growth in males from low bodyweight hens, reduced immunity in males from heavy hens compared to females in chapter 2 and the increased stress response time in chapter 4 (see Figure 1).

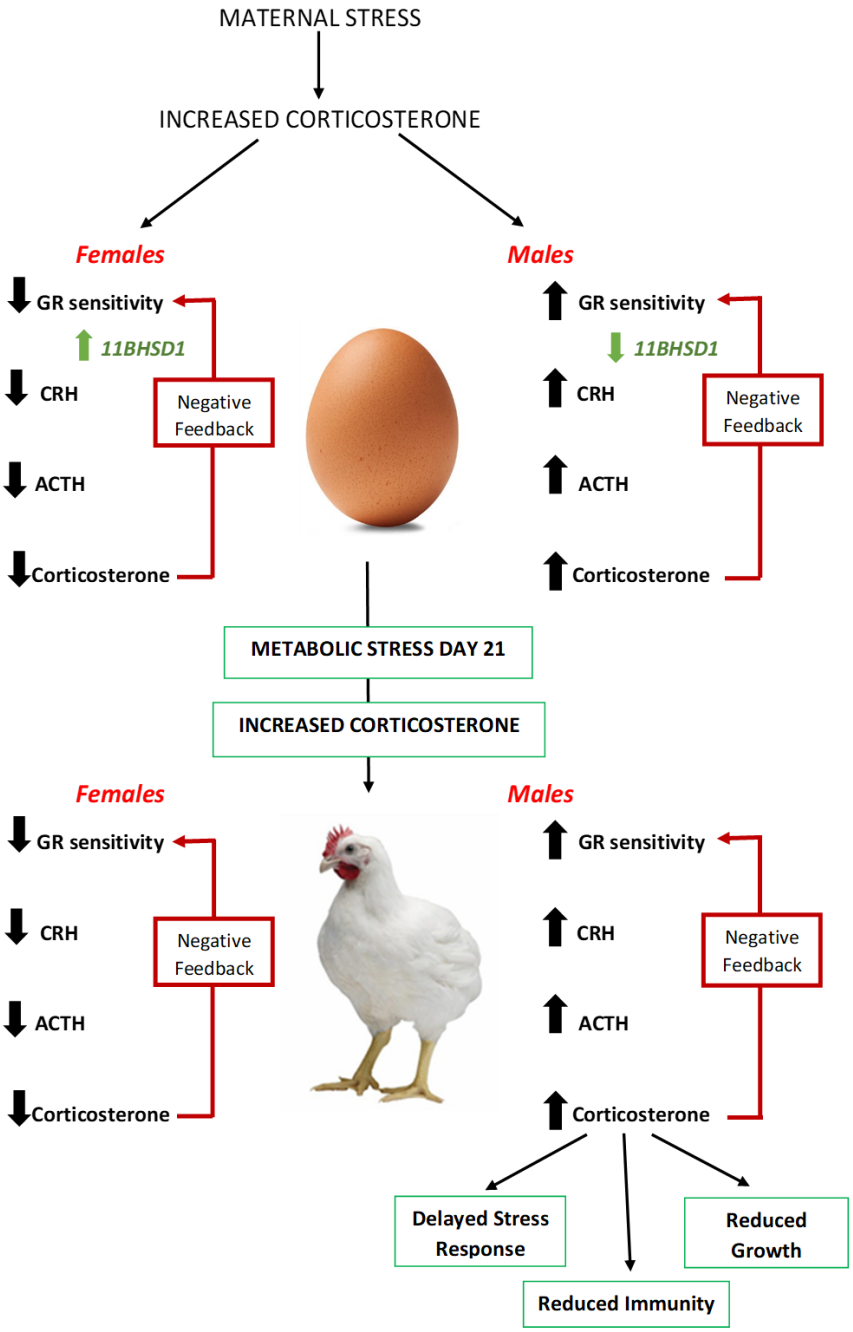

Figure 1: Hypothesis of the impact of increased maternal stress and increased corticosterone *in ovo* on hypothalamic-pituitary-adrenal (HPA) axis development in male and female meat birds. Within the egg, during embryonic development, females have increased expression of the 11ß-HSD1 enzyme and uptake the excess corticosterone faster and are then programmed with increased glucocorticoid receptors. Males have slower uptake with fewer receptors but have increased receptor sensitivity. At 21 days old, birds undergo metabolic changes and have increased stress levels. The increased receptors in females allows then to cope with the stress efficiently, while males take longer due to a reduction in receptors. Males then overcompensate because their receptors have increased sensitivity, resulting in a dysfunctional HPA axis. This may lead to reduced growth, decreased immunity and a reduced ability to respond to stress.

The differences found between males and female GR and HPA axis development after exposure to corticosterone is a significant step towards understanding chronic feed restriction in broiler breeder hens and how maternal stress can impact on growth and development of progeny. Continued research into male and female meat bird GR numbers throughout embryonic and post-hatch life, using methods such as immunohistochemistry is warranted.

Further investigation is needed into the change in gene expression of the GR from pre-hatch embryonic life through to the final week of post hatch production at 42 days of age. The effect of increased corticosterone on GR expression has been demonstrated here at embryonic day 14 to day 21 but measuring changes beyond this point is required. Similarly, CRH and ACTH expression also need to be further investigated. Adrenal gland weights and cell histology, particularly the glucocorticoid secreting layer (zona fasciculate) (Mescher 2010) could also be measured to understand potential differences between males and female abilities to secrete corticosterone hormone. Together, this would give a more thorough understanding of the entire HPA axis pathway in male and female meat birds.

The effects of sex hormones, testosterone and estrogen also need to be further considered. Unfortunately, due to limited samples and resources they were not measured in this study. However, a further understanding of changes in these hormones in both male and female meat birds and their possible interaction with corticosterone expression and uptake is important.

The effects on growth of birds found suggest further investigation and measurements of metabolism and body composition is necessary. Measurements of hormone levels involved in growth, metabolism and appetite such as, thyroid-releasing hormone (TRH), triiodothyronine, IGF, insulin and leptin throughout the life of the birds, including

embryonic levels, would help further understand growth of meat birds after exposure to increased corticosterone *in ovo.* Measurements of fat deposition and percentage would also be needed to understand metabolism changes and where weight is being lost or gained in birds.

Another important measurement that needs to be considered in future studies is the impact of maternal stress and elevated cortiocosterone on nutritional composition of the egg. Previously, maternal stress has been shown to reduce not only overall egg mass, but yolk (Rozenboim *et al.* 2007), albumen and shell mass as well (Dowing & Bryden 2008). The high fat yolk and albumin contain important proteins vital for energy during early chick development (Romanoff & Romanoff 1949). Reductions in yolk and albumen mass through maternal stress and corticosterone may impact on the lifelong development of the chick. Future research into the impacts of corticosterone elevations *in ovo* due to maternal stress need to not only consider the impacts on cortiocosterone and other hormones, but egg nutritional composition as well. Alternations in yolk and albumen mass due to corticosterone elevations may impact on the early growth and development of chicks and the lifelong growth potential of meat birds.

Corticosterone exposure, either maternally or deposited via an *in ovo* injection to the developing avian embryo can affect embryonic gene expression, lifelong development and thus reduce growth, decrease organ weights, decrease circulating corticosterone levels and decrease immune response later in life. These effects are possibly due to reprogramming of the HPA axis in meat birds. Management of broiler breeder hens may therefore, need to be evaluated and the levels of feed restriction kept at a level that maintains hen health and production, as well as reduces stress. The level of hen feed restriction could be reduced to a point where hens continue to lay eggs and maintain a suitable weight but are not as chronically hungry and therefore, as stressed.

Due to the genetics of broiler breeder hens they will always need to be maintained under a level of feed restriction but finding the best possible balance to reduce their stress levels and maintain production will benefit the hens, their offspring and the industry. Not only would increases in hen feed intake benefit hen welfare, by reducing chronic stress experienced by broiler breeder hens, the negative effects seen in meat bird progeny of feed restricted stressed hens would be reduced. This would benefit the meat bird industry with birds showing increased growth and higher final bodyweights at processing, as well as an increased ability to cope with stress and pathogens, also improving growth and reducing moralities.

Significant differences between males and females were also shown across experiments. Males were more susceptible to the effects of both feed restriction stress in their mothers and increased corticosterone exposure during embryonic development. The reduced growth in males from feed restricted stressed hens, from day 35 to 42 may mean it is benefical to cull an process males at day 35, to combat the cost of feeding for the final week if growth is reduced.

Management of female meat birds could also be altered, with female stress reponse and immunity not as affetcted by maternal feed restriction stress as males. Female meat birds may be considered more suitable to a free-range environment as they are less susceptible to the negative effects of increased corticosterone exposure *in ovo* than males. Female meat birds were not affected by the stress test and could perhaps cope better with the potenital increased stress of being outdoors, due to factors such as changing climate condtions. They also had improved immunity when from heavy hens and may therefore be better suited to the increased pathogen exposure of being in an outdoor system.

The effects of developmental programming continue to significantly affect lifelong health across species. The impacts on avians, particularly meat birds are only beginning to be understood. Within this thesis, further evidence of the effects of developmental programming have been shown through maternal stress and increases in corticosterone *in ovo.* The reduction in growth and organ development in meat birds due to developmental programming may have significant effects in the poultry industry. Reduced growth during the final week due to maternal stress could result in reduced profit, with smaller birds produced by day 42.

The sex effects shown could also impact on production. Male birds seemed to be more sensitive to the effects of developmental programming through increased corticosterone, with reduced growth, immunity and stress response. Males affected in this way would also cost the meat bird industry with smaller birds produced and potentially more birds becoming sick and even dying due to reduced immune and stress response capabilities.

Males in Chapter 2 from high bodyweight, low feed restricted hens, were on average 225g heavier at 42 days of age, than those from lighter, high feed restricted hens. In Chapter 1, it was shown that where one pure bred female can equate to over three million meat birds and 5.7 million kg of meat at 1.86kg chicken meat per bird (Australian Chicken Meat Federation 2019). If it as assumed that the extra 225g liveweight equated to approximately 125g of increased chicken meat in males, there is the potential for 1.96 chicken meat per bird. If this was applied to half the birds prodcued from the single less feed restricted hen as male offspring, it would equate to an increase of up to 5.9 million kg of chicken meat, as shown in figure 2.

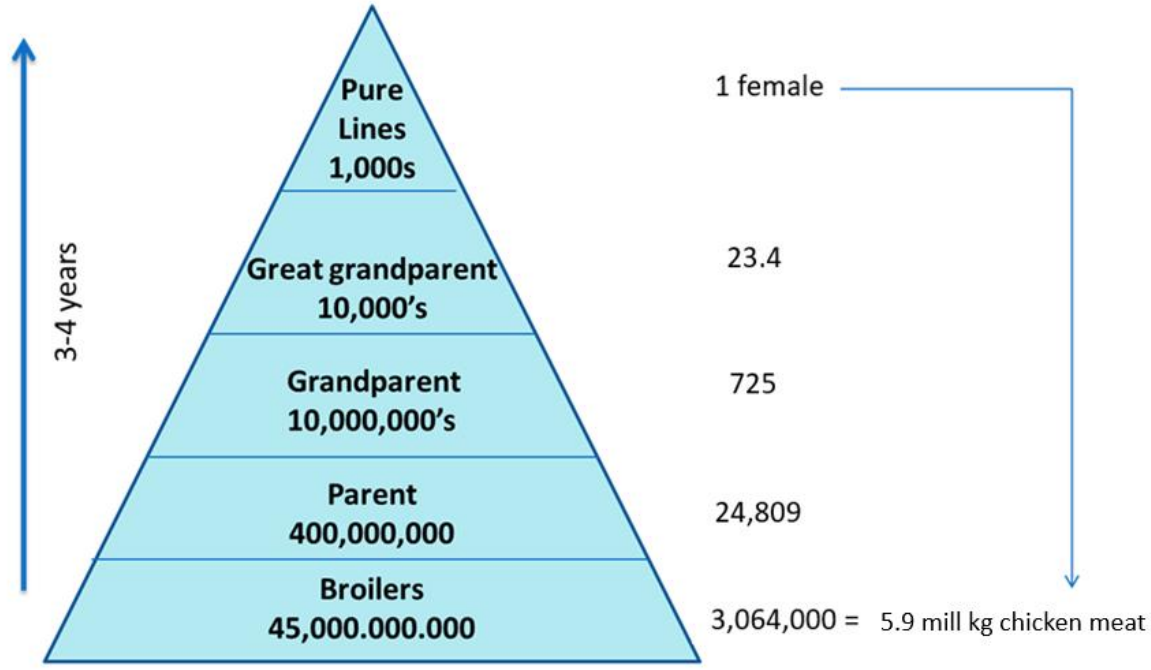

Figure 2: Generations of meat birds used for genetic selection from pure lines, through to parent/breeder flocks and the final broiler meat bird. Also shows the increase in chicken meat production by increased weight of males from less fed restricted hens at an increase to 1.96kg chicken meat per bird in males and 1.86 kg in female birds. Adapted from (Eenennaam *et al.* 2014).

Through this thesis, the effetcs on breeder hens and their progeny through corticosterone have been shown. Breeder hen welfare and stress coping ability are reduced with increased feed restriction and the offsrping's growth, development, immune and stress response are also reduced. This also impacts on the welfare of the hens and their progeny and production, with increased stress and reduced immunity potentially leading to increased morbiity and mortality.

Together these effects only represent the impacts of increased corticosterone due to maternal stress on meat birds, through developmental programming. There is likely to be many more effects of maternal stress on meat birds through other mechanisms and pathways such as epigenetic changes and other hormone changes within the egg. Therefore, continuing research in developmental programming in meat birds is important to understand how treatment of the hen effects meat birds and how to limit these effects and continue to improve bird health, welfare and production.

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Appendix 1: Supporting Publications – Conference Paper

The following is work undertaken during the PhD and presented at an international scientific conference. The presenting author is underlined.

Prenatal Programming of Broiler Growth and Immunity through Maternal Bodyweight

Combined Meeting of the Incubation and Fertility Research Group (IFRG) Workshop on Fundamental Physiology and Perinatal Development in Poultry (PDP) 2015 Meeting, Berlin Germany

Prenatal Programming of Broiler Growth and Immunity through Maternal Bodyweight

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Perinatal programming occurs in many species, causing lasting effects on progeny health and development. Progeny immunity can also be influenced by programming effects on the mother and may be of significance in broiler birds. Broiler breeder hens are fed restricted to 50% of their *ad libiutm* feed intake, leading to a state of chronic hunger throughout their lives. This persistent hunger can cause stress to hens which may lead to a perinatal programming effect on their offspring. Changes to broiler immunocompetence through perinatal programming could have significant impacts on the broiler industry as broilers are prone to poor immunity.

This study examined the link between maternal stress caused by feed restriction in hens and the ability of their offspring to respond to an immune challenge.

To achieve this 36 Cobb 500 broiler breeder hens were selected and maintained, through fed restriction at three separate body weights; 3.4kg, 3.6kg and 4.0kg. Hen behaviour was observed daily using an ethogram over two weeks of lay. 200 eggs were collected, and 170 viable chicks were hatched, weighed and placed into group rearing pens of ten birds from the same hen treatment group, with three replicates of each group.

Half of the chicks from each hen were given a series of three injections of lipopolysaccharide (LPS) *E.coli* O55:B5 at 16, 18 and 20 days old. Birds were injected at a dose rate of 0.5 mg/kg bodyweight, intraabdominally. Blood samples were collected from three birds per pen on days 21 and 35 for heterophil/lymphocyte (H/L) counts. H/L counts were completed by counting one hundred cells per slide, three times. Birds were grown until 42 days old and 70 were euthanised, sexed and dissected with organs and breast muscle weighed.

Behaviour results from breeder hens maintained at a lower bodyweight showed increased pecking behaviour ($P>0.05$) compared to those hens at a higher bodyweight. Increased object pecking in breeder hens is thought to indicate an unfulfilled hunger drive and may indicate stress in these birds. Progeny from the hens also demonstrated differences in growth from day 35 to 42 ($P=0.59$) but in male birds only. Males from heavy hens grew heavier in this week, followed by medium progeny and finally low bodyweight progeny which were the lightest. Differences between males from each hen treatment show perinatal programming effects on chick growth did occur and can influence bird growth.

Sex effects were also observed on day 23 H/L counts ($P>0.05$) with female progeny from heavy hens having a higher H/L ratio than male birds from all hens and females from low and medium bodyweight hens. These results also show a perinatal effect with females from heavy progeny more sensitive to the LPS immune challenge and increased immune cell numbers.

From this study, a link between hen bodyweight and progeny growth and immunity via perinatal programming, was demonstrated through differences in growth and H/L counts. The mechanism behind these differences needs to be investigated further as well as the differences between males and females observed in this study.

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Appendix 2: Supporting Publications - Published Paper

Developmental programming: a new frontier for the poultry industry?

The following manuscript was published in the Journal of Animal Production Science and includes work undertaken during the PhD.

Reference:

P.I Hynd, S. Weaver, N.M Edwards, N.D Heberle and M. Bowling (2016) Developmental programming: a new frontier for the poultry industry? *Journal of Animal Production Science*, **56,** 1223-1238

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Animal Production Science, 2016, **56**, 1233–1238
http://dx.doi.org/10.1071/AN15373

Perspective

Developmental programming: a new frontier for the poultry industry?

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Abstract. Increasing evidence that the maternal environment influences the programming of developing embryos and fetuses through epigenetic mechanisms has significant potential application in the broiler industry. The broiler breeder hen is subjected to restricted-feeding regimes to maximise egg quantity and quality, but the genetically high-intake potential of these birds makes this regime a stressful one. We propose that this stress is signalled to the developing embryo via changes in yolk composition as an evolutionary adaptation to changing environments, and that exposure to high levels of corticosteroids *in ovo* is associated with developmental reprogramming, which has effects on the behaviour, health and growth of the progeny. The present paper describes some preliminary results from a series of trials designed to elucidate the relationship between breeder hen diet and egg composition, and the growth, behaviour and immune function of the progeny. We conclude that manipulation of the breeder hen diet is an untapped opportunity to maintain the competitiveness of the chicken meat industry and further, that achieving improved productivity by this means may be compatible with improved animal welfare outcomes for the hen and her progeny.

Additional keywords: epigenetics, environment, maternal, yolk.

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Introduction

The impact of maternal environment on subsequent physiological function and health of the resulting offspring has been well demonstrated in humans and other mammals for more than 50 years now, but it was the discovery that low-birthweight babies were more susceptible to cardiovascular pathologies, metabolic syndrome and cancers that ignited interest in the so-called 'developmental origins of health and disease' (Barker and Martyn 1992; Seckl 2004; Symonds *et al.* 2007). Interestingly, while most attention was initially paid to the pathophysiological consequences of maternal undernutrition and its effects on endocrine, cardiovascular and metabolic function, it is now clear that developmental programming also influences brain development and behaviour (Sarkar *et al.* 2008). Such changes mean the maternal environment can also be reflected in the behaviour of progeny. Given increasing interest in the welfare of animals, developmental programming events with subsequent effects on the behaviour, stress sensitivity and immune function of progeny are of great interest in animal production systems. Studies in pigs, which have large within-litter variances in birthweight, reflecting the uterine micro-environment, demonstrate that many of the changes in the development of the progeny take place in embryonic life (see review by Foxcroft *et al.* 2009). The mechanisms of this epigenetic programming are the subject of intense scrutiny in laboratory rodents and sheep (e.g. see review by Murgatroyd and Spengler 2011; Gatford *et al.* 2010), with increasing focus on the role of specific nutrients and their so-called 'nutrigenomic' effects (Burdge *et al.* 2007).

Developmental programming has enormous potential application in the poultry industry, perhaps more so than in any other. Broiler chickens now spend almost 40% of their lives *in ovo*, a consequence largely of faster growth to market weight with little change in time *in ovo*. Recent studies have highlighted the profound effect of the environment *in ovo* on the phenotypic development of the resulting chicken (Ho *et al.* 2011). These authors demonstrated a dramatic effect of the embryonic environment on embryonic body mass, heart rate and development rate. In an elegant design, the effect of breed-specific yolk composition on chicken phenotype was investigated by transferring broiler and layer chicken embryos from their natural yolk environments to reciprocal swap yolk environments (broiler embryo to layer yolk; layer embryo to broiler yolk) and comparing these with homologous transfers. Broilers transferred to layer yolks exhibited heart rates similar to layers raised on layer yolks. Similarly, development rate and body mass of embryos were significantly affected by swapping to a different yolk environment. In other words, the yolk source influenced embryo development more than did embryo genotype, at least for the traits measured. This is an intriguing finding because it elevates the importance of maternal environment relative to genotype in determining phenotype. Testosterone concentrations were higher and triiodothyronine levels were

lower in broiler versus layer yolks, leading these authors to conclude that at least part of the yolk effect may relate to the effect of these hormones on development. These exciting results suggest that avians provide a useful model of developmental programming. The avian embryos are readily accessible, they can be cultured *ex-ovo* (as per Ho *et al.* 2011), the embryonic environment can be manipulated readily by *in ovo* injection of prospective agents into the yolk sac, albumen or chorioallantoic membrane, and the developmental stages are rapidly traversed. Perhaps more importantly, it also demonstrates a very significant opportunity for the poultry industry to apply epigenetic or biochemical agents with programming potential via the diet of the broiler breeder hen and transferred to the embryo by transfer to the yolk. The continued presence of potential programming 'agents' within the egg throughout the entire embryonic development phase (unlike placental animals where transfer of nutrients and hormones can be variable and moderated), makes avians particularly susceptible to reprogramming events.

Broiler breeder hens that carry the genes for potential high *ad libitum* intakes, high growth rates and low feed conversion ratios are restricted-fed to as little as 60% of their *ad libitum* intake to increase reproductive efficiency, egg-shell quality and hatchability, and to reduce costs. Such regimes might be expected to impose significant stress on the hens and have come under scrutiny from an animal welfare perspective (Hocking *et al.* 2001). Indeed, these authors reported behavioural and hormonal indices of stress in the restricted hens compared with *ad libitum* fed hens. Given the demonstrated impacts of yolk hormone composition (Ho *et al.* 2011) on embryo development and, particularly, the role of corticosteroids in reprogramming embryos (Edwards and McMillen 2002), the potential impact of feed restriction and stress in breeder hens on avian embryo programming and subsequent health and production is evident. Many of the programming events are associated with changes to the sensitivity of the hypothalamo–pituitary–adrenal (HPA) axis, which is reset by various stress conditions in the mother during pregnancy (Edwards and McMillen 2002; Seckl 2004; Glover *et al.* 2010; Zhang *et al.* 2011).

The present paper explores the impacts of the intake and composition of broiler breeder hen rations on organ development, growth and immune status of their progeny. We posed the following questions: (1) do restricted-fed hens demonstrate behavioural and hormonal indices of stress; (2) does feed restriction in breeder hens have an impact on the growth of organs in the developing embryo; (3) does feed restriction in breeder hens have an impact on indices of immune function in their progeny; (4) does feed restriction in breeder hen have an impact on growth performance of their progeny; (5) is there an effect of breeder hen diet composition on the performance of her progeny when they are swapped to a diet of different composition?

(1) Do feed-restricted breeder hens demonstrate behavioural and hormonal indices of stress?

A wide range of bodyweights in a flock of broiler breeder hens at a commercial breeder facility (HiChick Pty Ltd, Kapunda, SA, Australia) was achieved initially by different stocking density and then by allocating the birds to three groups on the basis of bodyweight, and feeding them to maintain these differences (Fig. 1). The three groups (L = low bodyweight; M = medium bodyweight; and H = high bodyweight) comprised 12 birds/group and were differentially fed from Week 23 to Week 48.

Various behaviours were scored in the hens as indicators of positive welfare (foraging = pecking and scratching at litter) and negative welfare (object pecking). Serum samples were assayed for corticosteroid concentrations by using an avian-specific ELISA assay.

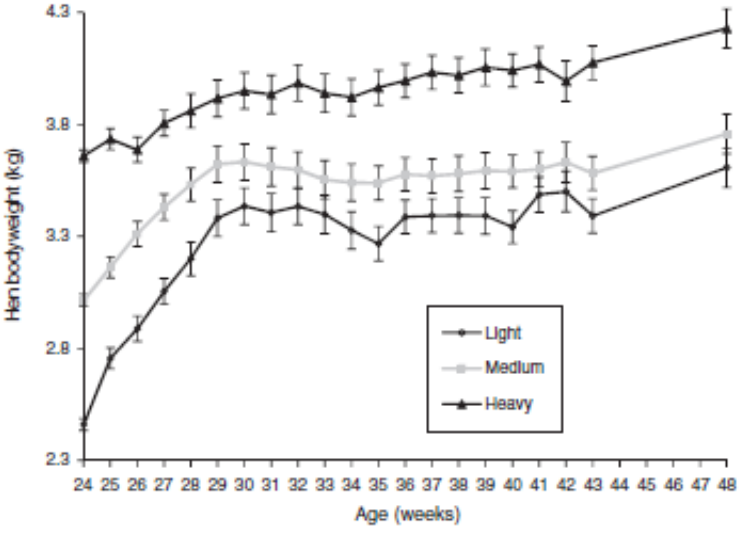

Fig. 1. Bodyweights (kg) of hens maintained at low (L), medium (M) and high (H) bodyweights throughout sexual development (Weeks 24–30) and during egg laying (Weeks 30–48). Values are means ± s.e.m.

Differential feeding of the breeder hens was associated with changes in the frequency of object pecking (Fig. 2) and foraging behaviours (Fig. 3). Hens maintained at medium or high bodyweights had lower frequencies of object pecking and higher frequencies of foraging behaviours, than did those maintained at low bodyweights.

Hen feed-restriction treatments were also reflected in differences in serum corticosteroid conceentrations at 31 weeks of age (Fig. 4).

Hens restricted-fed to low bodyweights (industry practice) had circulating concentrations of corticosteroid almost double those of hens fed at higher rates. We are currently assaying the corresponding concentrations of corticosteroid in the yolks of the eggs from the three bodyweight groups.

(2) Does fed restriction in hens have an impact on the growth of organs in the developing embryo?

The embryos of the differentially fed hens had similar patterns of organ growth (heart, gizzard, duodenum, jejunum, ileum, proventriculus), although there was some evidence of differential programming in liver weights (Fig. 5) and a tendency ($P = 0.12$) for spleen weights of progeny of L hens to be lighter (Fig. 6).

For some traits such as breast muscle weight, there was evidence of a 'sex-by-hen feeding level' interaction ($P < 0.05$; Fig. 7).

(3) Does feed restriction in breeder hens have an impact on immune function in their progeny?

Challenging the growing progeny with bacterial lipopolysaccharide (LPS) depressed bodyweight, averaged across hen treatment and sex at Day 21, by 6% ($P < 0.05$). Hen feeding level affected the LPS response, but there was a significant interaction with sex (Fig. 8). The bodyweight at Day 21 of the female progeny of the H level hens was significantly reduced relative to controls.

Hen feeding level also affected the ratio of heterophil to lymphocytes, again with a significant interaction with sex. Female progeny of H level hens had significantly ($P < 0.01$) more heterophil numbers and reduced lymphocyte numbers, such that the heterophil:lymphocyte ratio was markedly increased (Fig. 9).

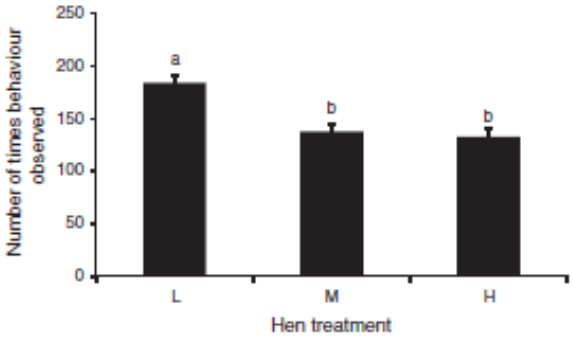

Fig. 2. Effect of bodyweight of breeder hens on pecking frequency (pecking at cage or objects = a 'negative' behaviour). Hen treatments were feed restriction to maintain bodyweights at low (L), medium (M) or high (H) levels. Values are means ± s.e.m. Different superscripts indicate significant ($P < 0.001$) differences.

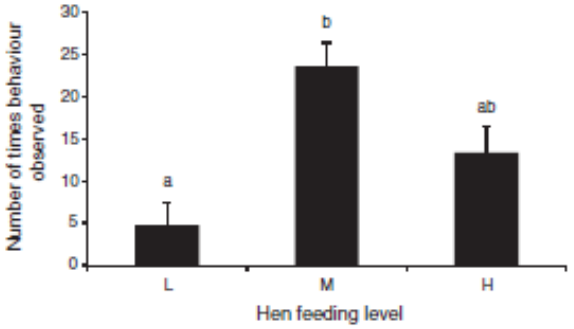

Fig. 3. Effect of bodyweight of breeder hens on the frequency of 'foraging' behaviours (litter scratching or pecking at litter = a 'positive' behaviour). Hen treatments were feed restriction to maintain bodyweights at low (L), medium (M) or high (H) levels. Values are means ± s.e.m. Different superscripts indicate significant ($P < 0.001$) differences.

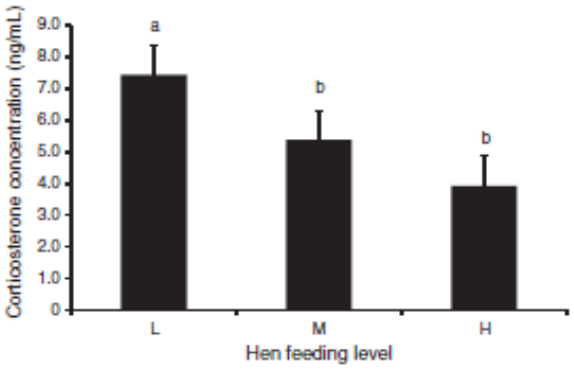

Fig. 4. Serum corticosteroid concentrations (ng/mL) in breeder hens maintained at low (L), medium (M) and high (H) bodyweights from Week 23 to Week 31. Values are means ± s.e.m.

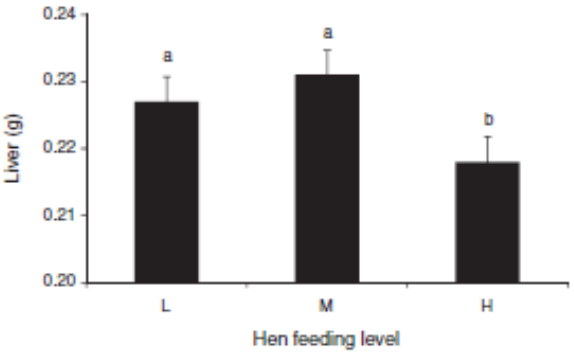

Fig. 5. Effect of differential hen feeding on liver weight of embryos at Day 14. Values are means ± s.e.m. Different superscripts indicate significant ($P < 0.05$) differences.

(4) Does feed restriction in hens alter the growth performance of their progeny?

There was a significant interaction between progeny sex and the impact of hen bodyweight treatment on growth to Week 42 ($P < 0.05$). Male progeny of hens on medium and high bodyweight regimes were significantly heavier than those from the low hen bodyweight group (Fig. 10). The differences among all groups were significant ($P < 0.05$).

(5) Is there an effect of breeder hen diet composition on the performance of her progeny on a diet of different composition?

Broiler breeder hens were placed onto two diets differing in the major grain component (corn versus wheat) but similar in all other nutritional specifications. The progeny of these hens were then placed onto four diets in a reciprocal cross-experiment such that the four groups reflected hen and progeny rations, respectively, as follows: (1) corn/wheat; (2) corn/corn; (3) wheat/wheat; and (4) wheat/corn. The growth performance of progeny that changed their diet type from that of their mothers, compared with those that stayed on the same ration, was assessed (Fig. 11). Progeny that were allocated to the same grain type as maternal grew faster than those allocated to a different majority grain source than maternal.

Progeny on a different major grain to the maternal diet had almost double the mortality rate of those fed the same major grain as the maternal diet.

Discussion

Our hypothesis is that feed restrictions in breeder hens as a means of maximising reproductive performance is a practice that imposes significant stress on the breeder hen. This stress, in turn, will be reflected in increased circulating concentrations of corticosteroids, which will be transferred to the yolk of the developing eggs. The embryos exposed to these enhanced corticosteroids will be reprogrammed to survive the harsh

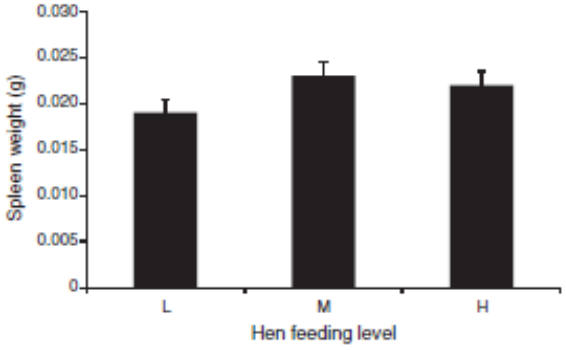

Fig. 6. Effect of differential hen feeding on spleen weight of embryos at Day 20. Values are means ± s.e.m. No significant ($P = 0.12$) differences were detected.

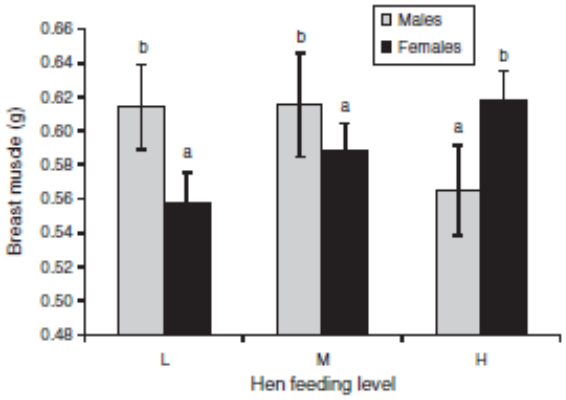

Fig. 7. Effects of differential hen feeding on breast muscle weight of male and female embryos at Day 20. Values are means ± s.e.m. Different superscripts indicate significant ($P < 0.05$) differences.

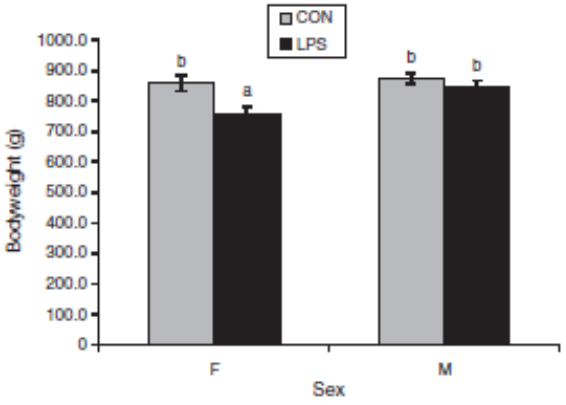

Fig. 8. Effect of lipopolysaccharide (LPS) injections on bodyweights of progeny of the H level hens at Day 21. Values are means ± s.e.m. Different superscripts indicate significant ($P < 0.05$) differences. CON, controls (no LPS injections).

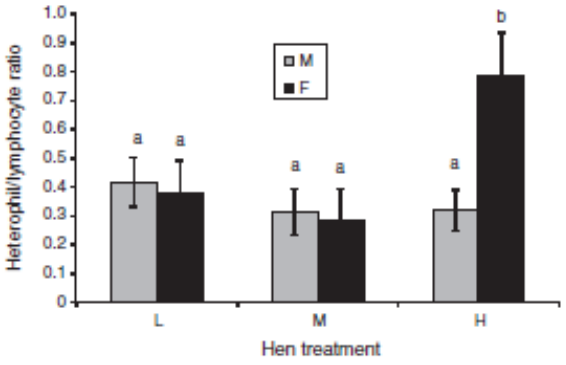

Fig. 9. Effect of breeder hen bodyweight (low (L), medium (M), high (H)) and sex of progeny on heterophil : lymphocyte ratio. Values are means ± s.e.m. Different superscripts indicate significant ($P < 0.05$) differences.

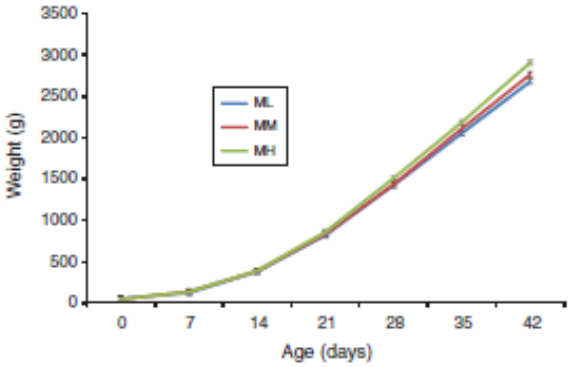

Fig. 10. Growth curves for male progeny of hens maintained at low (ML), medium (MM) or high (MH) bodyweight. Values are means ± s.e.m.

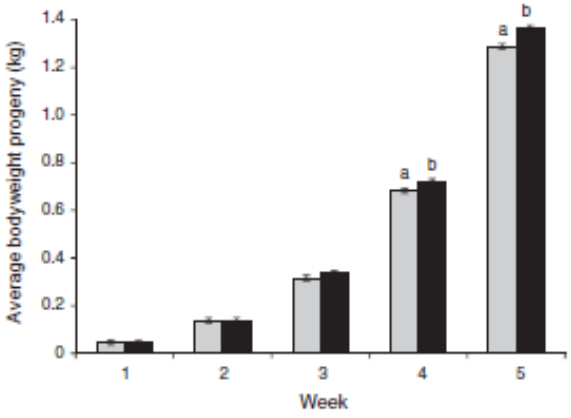

Fig. 11. Changes in bodyweight with time for progeny that remained on the same grain source as maternal ration (black bars) versus those that swapped to the alternative grain from their mothers (grey bars). Different superscripts indicate significant ($P < 0.01$) differences.

environment 'anticipated' by the hen. This reprogramming will have variable impacts on the progeny, depending on the extent to which the posthatch environment is correctly (or incorrectly) anticipated. In the case of the broiler chicken, the posthatch nutritional environment does not reflect that anticipated by the restricted-fed hen. Whereas some of the outcomes of this 'misprogramming' may be beneficial (e.g. enhanced efficiency of growth), other outcomes may be negative, particularly in relation to the functioning of the immune system and possible longer-term effects on cardiac and metabolic function. It is likely that many of the pathological effects of undernutrition during embryonic or fetal development that are seen in mammals (hypertension, hyperglycaemia, increased HPA axis activity and anxiety behaviour; Seckl 2004) apply also to chickens. Leg weakness, cardiac problems and aggressive behaviour may at least in part be associated with underfeeding of the breeder hen. These pathologies may be particularly prevalent in broiler chickens because of the rapid growth environments to which they are exposed posthatch. In mammals, growth restriction *in utero*, subsequent low birthweight, and rapid catchup growth postnatally, is predictive of the risk of cardiovascular disease in adult life (Irving *et al.* 2000).

The preliminary data presented here provide some support for this hypothesis. Restricted-fed hens showed increased frequency of negative behaviours such as spot pecking, in agreement with Hocking *et al.* (2001), and reduced behaviours indicative of positive welfare. The serum corticosteroid concentrations in restricted-fed hens were almost double those of better-fed hens. Hocking *et al.* (2001) similarly showed elevated corticosteroid concentrations in restricted-fed hens, compared with hens fed *ad libitum* and those on a less-severe restriction. Restricted feeding of the hens appears to be a practice that produces stress in the hens.

We are yet to measure the corresponding concentrations of corticosteroid in the yolk of differentially fed hens. If they are higher in restricted-fed hens, as we would expect, we would anticipate from evidence in mammals, that this would be reflected in effects on behaviour of the resulting progeny (Van den Bergh *et al.* 2005). High concentrations of cortisol during embryonic and fetal development in mammals are associated with a reprogramming of the hypothalamo–pituitary–adrenal axis such that downregulation of GR and MR receptors in the hippocampus results in reduced sensitivity to circulating cortisol, and, hence, a reduction in negative feedback (Levitt *et al.* 1996; Welberg and Seckl 2001). The circulating concentrations of cortisol remain high in these animals and this has an impact on behaviour and other aspects of physiology (see review by Seckl 2004).

Reprogramming of the HPA axis in non-avian species often differs between sexes. Weinstock *et al.* (1992), for example, demonstrated sex-specific responses to prenatal stress. The preliminary data on sex differences in response to maternal nutrition reported in the present paper suggest sex-specific programming effects in avians. Only male progeny were influenced by maternal nutrition in terms of growth rate, while females showed responses to maternal nutrition in terms of heterophil : lymphocyte ratio and response to LPS challenge. There was also a sex-specific response of development of certain organs, such as the breast muscle, to maternal nutrition, implying different programming events between the sexes.

Developmental programming in chickens appears to be influenced not only by feed restriction in the breeder hens, but also by the composition of the diet. Changing the predominant grain in the ration of the progeny compared with the ration of the hen appeared to influence the response of the progeny to a disease challenge. Chickens that were exposed to a grain type different from what their mothers were fed were more prone to the debilitating effects of an infectious challenge. This supports the notion that specific nutrients can act as programming agents to influence chicken health and paves the way for extensive studies of the interaction between maternal diet and progeny health.

The mechanisms underpinning fetal and embryonic programming have yet to be elucidated. The prevailing paradigm is that the nutritional environment influences methylation reactions and microRNA expression, which in turn alter chromatin structure, gene expression and protein production with consequent phenotype changes (see review

by Chango and Progribny 2015). We (Dr T. Crowley, Ms S. Weaver, Mr A. Keyburn, Dr P. Hynd) plan to measure the microRNA profiles of the yolks of the differentially fed hens from the experiments described in the present paper, to determine the extent to which the microRNAs are altered by dietary intake. At present, our hypothesis on the impact of hen dietary intake is focussing on stress induction by restricted feeding and the well known effects of corticosteroids on embryonic development. However, the preliminary data on the grain-swapping experiment imply a potential effect of specific nutrients on phenotype programming that may not relate to glucocorticoid exposure.

Tremendous gains have been made in the broiler industry over the past few decades through the application of genetic selection, nutritional science and management. Developmental programming and epigenetics have the potential to contribute to ongoing progress in the broiler industry to achieve improvements not only in production efficiency, but also in the health and welfare of the breeder hen and her offspring.

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