The avian maternal environment: Its influence on the physiological mechanisms contributing to progeny production efficiency in chicken meat birds

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- **GH/IGF-I axis** – growth hormone/insulin like growth factor I axis.
- **HPA axis** – hypothalamic pituitary adrenal axis.
- **HPT axis** – hypothalamic pituitary thyroid axis.
- **CORT** – corticosterone
- **CON** – control
- **SC** – *saccharomyces cerevisiae*
- **ED –** Embryonic day
- **ACTH –** adrenocorticotropic hormone
- **Bwt –** body weight
- **BC –** body composition
- **FCR –** feed conversion ratio
- **PR –** Progesterone receptor
- **AR –** Androgen receptor
- **ER –** estradiol receptor
- **MyoD –** Myoblast determination protein 1
- **MyoG -** Myogenin
- **CEBP/β –** CCAAT enhancer-binding protein beta
- **PPARγ –** Peroxisome proliferator-activated receptor gamma
- **Pax7 –** Paired box protein Pax-7

Abstract

Alterations to the maternal and subsequent *in-ovo* environment during embryonic development, can lead to permanent phenotypic alterations, a phenomenon termed developmental programming. Developmental programming has enormous potential in the chicken meat industry due to meat chickens now spend ~40% of their life *in-ovo*, the stressors encountered by breeder hens throughout production that can affect the *in-ovo* environment during egg formation, and the pedigree breeding structure of the industry. Despite evidence suggesting maternal/*in-ovo* stress can influence progeny body weight **(bwt)**, the physiological mechanisms contributing to performance variation in commercial poultry remain relatively unknown. This thesis therefore aimed to further ascertain performance variations in commercial chicken meat birds in response to alterations to the maternal/*in-ovo* environment, and to identify the contributing physiological mechanisms influencing these phenotypes. Furthermore, nutritional additives to both breeder and progeny diets were investigated as a potential method to alleviate performance variation in response to altered maternal/*in-ovo* environments.

As reductions in offspring bwt have been repeatedly identified in response to both maternal and *in-ovo* stress, the first study aimed to investigate contributing physiological mechanisms. Great grandparent lineage meat chicken embryos were exposed to a corticosterone **(CORT)** or control **(CON)** solution at embryonic day **(ED)** 11, hatched and then fed either a control or arginine supplemented diet until 35 days of age. Exposure to *in-ovo* CORT failed to influence male and female bwt at any stage, whilst dietary arginine supplementation had no effect on bwt or body composition traits. However, *in-ovo* CORT exposure did affect body composition and led to a decrease in total lean mass, and subsequent increase in total fat mass in exposed female birds.

In response to the findings generated in the first study, a second experiment was designed to examine the effects of the maternal environment on offspring body composition traits across both sexes, and whether phenotypes were consistent between genetic lines of chicken meat birds. Breeder hens from two genetic lines, a high performing line, and an underperforming line, were fed either a control or *saccharomyces cerevisiae (SC)* supplemented diet from 23 weeks of age. Eggs were collected from 32 week old birds, with the subsequent progeny hatched and reared under identical conditions until 42 days of age. The results indicated breed differences in growth traits, as well as sex by breed interactions for body composition traits. The provision of *SC* appeared to increase plasma CORT concentrations in breeder hens, and subsequently reduced yolk testosterone concentrations, however *SC* failed to influence any performance measure in both breeder hens and progeny.

A third study was developed to identify whether *in-ovo* exposure to CORT influenced muscle fibre development at hatch through the metabolic actions of the sex steroid hormones within the yolk. This study utilised the *in-ovo* method from Chapter 3 to expose commercial chicken meat birds to CORT at ED 11. Exposure to *In-ovo* CORT did not influence steroid hormone abundance levels in the yolk, except for the conjugate etiocholanolone glucuronide, nor did it affect muscle fibre number and muscle fibre cross sectional area at hatch. However, muscle fascicle area occupied by muscle fibres was reduced in CORT treated birds at hatch, potentially indicating increased intramuscular fat content in CORT treated birds. Furthermore the relative mRNA expression of the CCAAT/enhancer-binding protein beta (CEBP/β) was increased in CORT treated birds. Additionally, total yolk lipid content was decreased in CORT treated birds at hatch.

In summary, this thesis examined the effects of the maternal environment and exposure to *in-ovo* CORT on progeny performance traits, and explored potential physiological mechanisms contributing to noted

differences in body composition measures in chicken meat birds. The results indicate that *in-ovo* exposure to CORT appears to alter body composition, including higher fat mass (% bwt), with both sex and line differences evident in adult birds while no influence on muscle fibre development was detected in embryonic birds. Furthermore, our results suggest progeny phenotypes in response to alterations to the maternal/*in-ovo* environment are a result of a complex interaction between breeder age, bird strain/line and the subsequent *in-ovo* environment created. However, further analysis of the metabolic fate of yolk hormones and the mechanisms in which progeny are exposed to these compounds is required to gain a better understanding as to how the maternal environment can influence performance traits in chicken meat birds. We however do believe that targeting the maternal/*in-ovo* environment provides a novel and innovative method to improve performance outputs as well as flock uniformity in chicken meat birds.

Declaration

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.

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Chapter One:

General introduction to the chicken meat industry and developmental programming.

General introduction to the chicken meat industry and developmental programming

Global poultry production has seen unparalleled expansion over the past five decades, so much so, that the global poultry population has increased to 23 billion individuals birds, five times the number of poultry present 50 years ago (OECD/FAO 2019). The Australian chicken meat industry is no different, where poultry products have become the preferred source of protein in Australian diets, with the average person consuming ~48kg of poultry meat annually (Australian Government 2021). The demand for poultry products has primarily been driven by global population increase, rising per capita income and the continual growth in urbanisation (Haley 2001). However, predictions suggest that global meat consumption will continue to grow, with the demand for animal derived products to increase by up to 70% by the year 2028 (Mottet and Tempio 2017). The poultry industry has so far adapted best to the increasing demand for animal products, primarily driven through the genetic advancements generated through the pedigree structure of the chicken meat industry.

As reviewed by Tallentire *et al.* (2016), the major breeding companies (Aviagen and Cobb) control the pure lines of chicken meat birds, otherwise termed the 'nucleus stock' **(Figure 1)**. These birds are subjected to intense selection parameters for a vast array of performance measures, including growth, breast muscle yield, feed efficiency and integrity of skeletal structures. The resulting progeny produced from the 'nucleus' stock are termed great grandparent birds. Pure lines of great grandparent birds are than cross bred to generate several lines of grandparent birds, which are subsequently cross bred further to generate parent flocks, which are then distributed to integrated producers. From these parent stocks, a final stage of cross breeding between hybrid lines is implemented to produce commercial broiler chickens, those of which are slaughtered for human consumption. This integrated structure has allowed the chicken meat industry to meet consumer

demand for chicken meat products through genetic and nutritional advancements. However, this integrated structure has also positioned the chicken meat industry to be at the forefront of animal production in regards to the applicability of developmental programming mechanisms to current production strategies (Hynd *et al.* 2016).

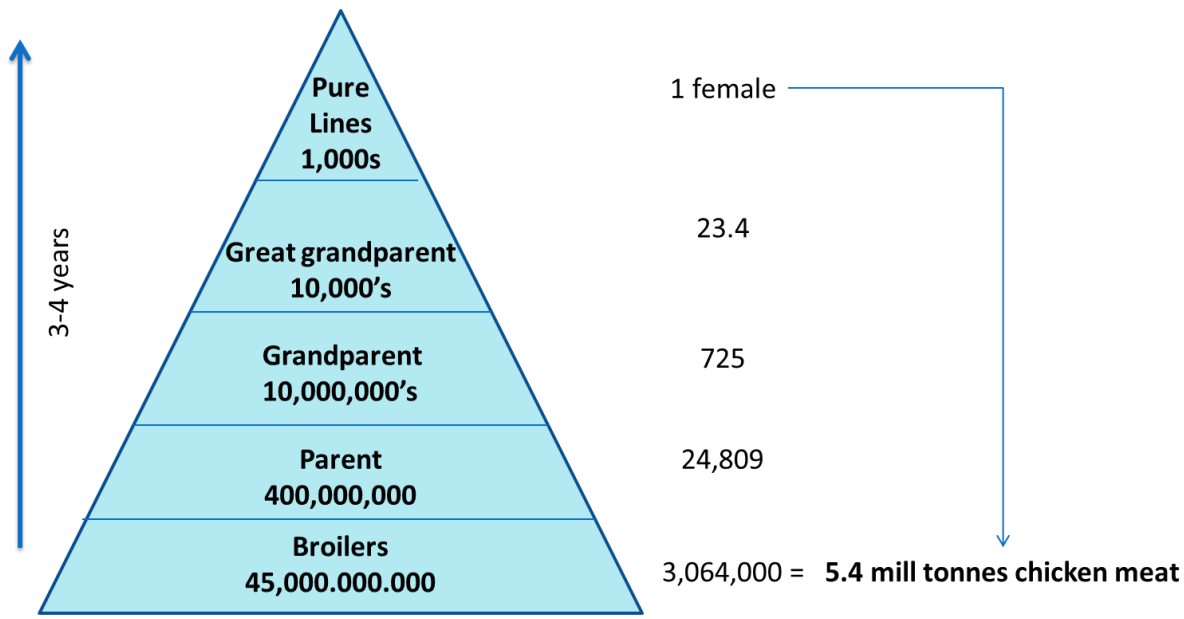

Figure 1: Pedigree breeding structure of the chicken meat industry (Eenennaam *et al.* 2014), highlighting the genetic influence of one pedigree female breeder hen, alongside time line estimates and global bird population estimations of the various pedigree lines of chicken meat birds.

Developmental programming is the idea that the environment an individual encounters during times of reproduction can alter the developmental trajectory of the subsequent progeny, with permanent phenotypic implications through adult life (Sinclair *et al.* 2016). In the chicken, these maternal effects are almost certainly mediated through alterations to the *in-ovo* environment, most likely through nutritional or hormonal alterations to the contents deposited at the time of egg production (Henriksen *et al.* 2011b). Considering

commercial chickens spend almost one third of their life (~40%) within the *in-ovo* environment, alterations to the hormonal/nutritional contents within the egg are likely to influence phenotypic traits displayed in progeny post-hatch (Ho *et al.* 2011). Thus, several strategies currently used in the management of broiler breeder hens may be influencing progeny performance.

Previous work in wild avian species has clearly identified that the maternal environment can influence phenotypic traits in subsequent progeny (Sheriff *et al.* 2017), however, little work as assessed the implications of such effects in commercial poultry production. As performance gains through genetic selection and nutritional advancement are near optimal in commercial poultry, new, innovative methods to enhance production are required to advance the industry forward. Furthermore, as one pure line breeder hen has the potential to influence the genetic composition of up to three million commercial broiler birds (Eenennaam *et al.* 2014), targeting the maternal environment may provide an economically viable method to enhance production in commercial broiler flocks. This is possible through the potential for developmental programming mechanisms to have transgenerational effects on subsequent progeny performance. Therefore, our aim was to investigate the effects of alterations to the maternal/*in-ovo* environment on progeny performance measures in commercial chicken meat birds in relation to current management strategies broiler breeders are exposed to. Additionally, we aimed to investigate the physiological mechanisms that contribute to the noted phenotypic differences identified between progeny as a result of alterations to the maternal/*in-ovo* environment.

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Chapter Two:

The avian maternal environment: the physiological mechanisms driving progeny performance

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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.

Name of Co-Author	Dr. Rebecca Forder		
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Chapter Introduction

The following manuscript was formatted to the standards associated with the Worlds Poultry Science Journal, and was accepted for publication in September 2019 and published in March 2020.

This chapter explores the effects of the avian maternal environment on offspring performance characteristics in both commercial and wild avian species along with relevant connections to mammals. This review identified the potential interaction between chronic maternal stress in broiler breeder hens, and subsequent reductions in progeny performance as a result of severe feed restriction. However, the contributing physiological mechanisms influencing progeny growth rates and body composition remain elusive. The involvement of key hormones, such as GH, IGF-I, T_3, and CORT, in avian growth and metabolism suggests the development of the HPA, GH/IGF-I and HPT axes may either directly or indirectly be influenced by maternal stress. Additionally, the communication both within and between axes may subsequently act to influence progeny growth and body composition in several ways dependent on stressor type, exposure duration and time of exposure.

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The avian maternal environment: exploring the physiological mechanisms driving progeny performance

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2.1 Summary

Environmental factors, both positive and negative, experienced by breeder hens during their reproductive life, can have significant influence on the productive efficiency and health of their progeny. This is particularly important considering that commercial chicken meat birds spend a significant proportion of their life in ovo, and alteration to the in ovo environment can permanently 'program' progeny endocrine pathways. The maternal environment is greatly influenced by factors such as nutrition and stress, both of which play a significant role in the chicken meat breeder industry due to feed restriction practices, ranging from 25% to 80% ad libitum intake. The effects of nutrition and stress and their influence on the maternal environment have been extensively investigated in mammalian literature, primarily focusing on the development and function of the hypothalamic-pituitary-adrenal axis (HPA) in offspring including the exposure of the stress hormone cortisol. Disruption of the HPA axis can inadvertently disrupt other important endocrine pathways, involved in growth and metabolism, including the growth hormone-insulin-like growth factor I axis (GH/IGF-I) and the hypothalamic-pituitary-thyroid axis (HPT). The disruption or 'reprogramming' of metabolic endocrine axes through maternal influences has been linked to variations in progeny performance, including growth rate and body composition; however, the underlying physiological mechanisms responsible for these phenotypic differences still remain unclear, especially in poultry. The aim of this review is to assess current industry practices that may influence the maternal (breeder hen) environment, whilst reviewing the concept of developmental programming, and its application to chicken meat production.

KEYWORDS

Developmental programming; feed restriction; stress; body composition; growth; endocrine axes

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2.2 Introduction

Poultry meat is predicted to be the most consumed animal protein globally by 2022 (Henchion et al., 2014). Estimations suggest approximately 23 billion broilers (three per person) exist globally, producing in excess of 100 million tons of meat annually (Mottet and Tempio 2017). Desirability of chicken meat resides from the consistency of product, low cost, and healthy image portrayed by consumers (Haley 2001). The industry has met increasing consumer demands, utilising advances in genetic gain primarily through selective breeding programs, where superior growth rates and feed conversion ratios (FCRs) are highly sort after (Tallentire, Leinonen, and Kyriazakis 2016) to produce cheap, high-quality meat. Nutritional advancements have enhanced the productive efficiency of commercial poultry, with current industry FCR goals being around 1.5 (Cowieson and Selle 2011).

Through a combination of genetic and nutritional advancements the chicken meat industry has produced an efficient, low-cost animal that produces a high-quality product. Therefore, nutritional manipulation and genetic gains are considered near optimal, leading the industry to investigate alternate strategies to improve production outcomes, whilst maintaining bird health and welfare.

Developmental programming encompasses the idea that alterations to foetal nutrition or hormonal status during embryonic development induce permanent alterations in offspring, which can affect growth mechanisms, metabolic status and structural physiology during its adult life (Reynolds 2010). Developmental programming itself has enormous potential within the chicken meat industry, as commercial birds now spend up to 40% of their life in ovo, a consequence of enhanced post-natal growth (Hynd et al., 2016). Poor nutritional status of the breeder hen, as well as maternal stress, have both been linked to disrupted brain function (Welberg and Seckl 2011) as well as reduced performance (Sinclair et al., 2016). However, the mechanisms which govern the maternal environment that influences production traits remain elusive.

Hormones involved in stress responses, such as corticosterone (CORT), as well as epigenetic modifications during embryonic development, are known to effect various endocrine pathways involved in growth and metabolism (Seckl 2004). The disruption of such pathways through exposure to maternal stressors during development may influence long-term progeny performance. If these physiological mechanisms can be understood, then innovative measures can be implemented in breeder flocks to optimise progeny performance.

This review assessed current industry practices that may influence the maternal (breeder hen) environment, whilst reviewing the impact of the maternal environment on progeny production traits. Discussions centred on the development/function of endocrine axes, primarily involving the HPA, GH/IGF-I and HPT axes, and how disrupted development influences several performance parameters of chicken meat birds.

2.3 Broiler breeders

Advancements in broiler growth and efficiency traits through genetic selection and nutrition have yielded major benefits for producers of commercial birds (Siegel 2014; Zuidhof et al., 2014). A single, pure line, broiler breeder has the potential to influence the genetic makeup of over one million commercial birds, generated through the pedigree structure of the breeder industry (Tallentire et al., 2016). The industry currently utilises feed restriction practices (De Jong et al., 2002) to maintain an optimal body weight (BW) that enhances reproductive efficiency (Renema, Rustad, and Robinson 2007). The use of feed restriction does not result in weight loss in breeder hens, instead growth rate throughout adult life is reduced, promoting reproductive efficiency. Overall breeder flock health improves in relation to feed restriction through reducing susceptibility to cardiac failure, skeletal abnormalities, ascites and obesity (Mench 2002; De Jong and Guemene 2011).

2.4 Feed restriction-induced chronic stress in broiler breeder hens

Industry standards utilise feed restriction ranging between 25% and 50% of a bird's daily ad libitum feed intake during the rearing phase. Restrictions remain in place upon the onset of lay, although the severity can be increased to between 60% and 90% of a bird's daily *ad libitum* intake (Mench 2002; Van Krimpen and De Jong 2014). Such practices can induce chronic stress in breeder hens due to prolonged hunger (De Jong et al., 2002), a likely result of CORT mobilising blood glucose (Li et al., 2009). However, exposure for extended periods to elevated stress hormones, such as CORT, can have negative implications for breeder hen performance and behaviour (Bruggeman et al., 1999; De Jong, Fillerup, and Blokhuis 2005).

Periods of prolonged hunger in breeder hens has been strongly correlated with several stressor indicators, including increased plasma CORT and heterophil/lymphocyte ratio (H/L ratio) (Najafi et al., 2015). Hynd et al. (2016) analysed plasma CORT concentrations in three groups of hens subjected to varying levels of feed restriction based on maternal BW. Low BW breeder hens, exposed to greater feed restriction severity, corresponded with significantly elevated plasma CORT in comparison to the two groups subjected to reduced feed restriction severity (Hynd et al., 2016). Such findings were supported by De Jong et al. (2002) and Najafi et al. (2015) who identified elevated plasma CORT levels in hens subjected to severe feed restriction. Both Savory et al. (1996) and Hocking et al. (2001) used H/L ratio as an indicator of stress, and noted elevated H/L ratios in birds subjected to greater severities of feed restriction, indicating chronic stress. Additionally, behavioural differences have been observed in severely feed-restricted birds, including increased aggression, stereotypic behaviours (i.e. pecking at inanimate objects) and consistent pacing (Van Krimpen and De Jong 2014), all of which were deemed indicative of frustration or boredom (Mench 2002; Sandilands, Tolkamp, and Kyriazakis 2005). Thus, the evidence suggesting that feed restricted breeder hens are suffering from chronic stress is strong. However, exposure to maternal stress, either chronic or acute exposure may ultimately influence performance characteristics in the offspring.

2.5 Maternal nutrition and stress: effects on progeny development

Maternally derived stress, through malnourishment or poor nutrition, is known to interrupt 'normal' progeny development. Exposure to stressors results in glucocorticoid release CORT in avian species (Moisiadis and Matthews 2014; Romero and Reed 2005). Gestational stress in mammalian species is linked with reductions in birthweight, permanent hypertension, hyperglycaemia/hyperinsulinemia (O'Regan et al., 2001), behavioural alterations and poor immunocompetence (Hyatt et al., 2007; Weinstock 2008). Studies conducted in cattle identified reductions in weaning weight, attributed to poor maternal nutritional status, with little to no evidence of compensatory growth beyond 30, months of age (Robinson, Cafe, and Greenwood 2013). Poor maternal nutrition has been shown to impair musculoskeletal growth and increased adiposity in both sheep and pigs, with piglet growth efficiency reduced in those with lower birth weights (Daniel et al., 2007; Gatford et al., 2018). Other mammalian studies have shown substantial cognitive impairment in offspring from maternally stressed dams, including learning and memory incompetence as well as increased anxiety, aggression and fearfulness (Weinstock 2008).

Similar physiological and behavioural impacts have been noted in avian species in response to maternal feeding regimes (Janczak et al., 2007; Gholami et al., 2017) and environmental conditions (Lickliter 2005). Progeny growth rate, hatchling weight and hatchability were decreased in response to maternal stress, in trials with hens and in ovo models (Hayward and Wingfield 2004; Love et al., 2005; Ahmed, Musa, and Sifaldin, 2016). Furthermore, progeny testosterone, an anabolic hormone essential for muscle and skeletal development, is elevated in birds where there has been maternal stress exposure (Henriksen, Rettenbacher, and Groothuis 2013; Singh et al., 2003).

As observed in mammalian progeny, birds can display cognitive disruption, anxiety, aggressive behaviour and delayed sexual maturation (Henriksen, Rettenbacher, and Groothuis 2011b) as well as compromised immunity (Love et al., 2005) in response to maternal stress. Such behavioural alterations may be induced by

functional disruption of the limbic and prefrontal cortex, both of which play important roles in regulating emotion and anxiety. The progeny HPA axis activity can increase upon exposure to maternal stress (Weinstock 2008; Welberg and Seckl 2011), and, like the limbic/prefrontal cortex, has been linked to anxiety disorders. However, these phenotypic alterations appear to be inconsistent within species, and seem to depend on timing, duration and intensity of maternal glucocorticoid elevation (Henriksen et al., 2011b). One factor that must be considered is the sex of the offspring, as, in both birds and mammals, phenotypic outcomes generated by the maternal environment differ depending on gender (Laguna-Barraza et al., 2013).

2.6 The maternal environment: sex-dependent developmental variation in progeny

Studies altering the maternal environment have highlighted significant phenotypic variations between male and female progeny. Low maternal protein intake during the first trimester in cattle resulted in leaner, and heavier male progeny during the post-weaning period. This corresponded to an upregulation in insulin-like growth factor I (IGF-I) mRNA expression (Micke et al., 2015), where IGF-I is a primary endocrine regulator of growth and metabolism (Xiao et al., 2017). In contrast, female progeny were lighter and smaller in stature compared to high protein counterparts, accompanied by decreased IGF-I mRNA expression. Similar findings have been observed in poultry, whereby male chicks from hens fed low crude protein diets had enhanced breast muscle yield, whilst female progeny breast muscle yield did not differ (Van Emous et al., 2015).

Stressors encountered during early-life development can act in a sex-specific manner in relation to offspring growth and behaviour. Breeder hens exposed to stressful environments reduced male offspring growth rate, whilst no effects were identified in females, although the latter did show a stronger immune response (Hynd et al., 2016; Bowling et al., 2018). Similar findings have been observed in European starlings, whereby maternal stress increased male embryonic mortality, whilst female chick quality was significantly enhanced (Love et al., 2005). In other species, CORT administration to sows tended to decrease male piglet

survivability (Mack et al., 2014), whilst disruption of HPA axis regulation was seen in male rats whose dams were exposed to chronic variable stress, accompanied by down-regulation of the glucocorticoid receptor (GR) (Chung et al., 2005; Weinstock 2008). Sex-specific, phenotypic variation, particularly as a result of chronic maternal stress is strongly linked to the development of the HPA axis and its neighbouring endocrine pathways, which are responsible for a range of physiological functions (De Groef, Grommen, and Darras 2008; Lu et al., 2008). Thus, identification of critical time points during HPA axis developmental remains a fundamental requirement in order to understand the influence of the maternal environment.

2.7 Hypothalamo-pituitary adrenocortical axis in chickens

The HPA axis regulates the secretion of glucocorticoids from the adrenal gland, dependent on the level of stress encountered by an individual animal at any one time (McEwen 2007). Briefly, corticotropin-releasing hormone (CRH) and arginine vasotocin (AVT) are released from the hypothalamus in response to a stressor and are transported to the anterior pituitary gland (Blas 2015). In the pituitary, both CRH and AVT stimulate the release of adrenocorticotropic hormone (ACTH) into the blood stream, which promotes the release of glucocorticoids from the adrenal gland (Herman et al., 2016). Regulation of glucocorticoid secretion is mediated via negative feedback mechanisms exerted on the HPA axis. Here glucocorticoids bind to GRs located within the anterior pituitary gland and the hypothalamus (Figure 1), prompting the inhibition of axis activity (Keller-Wood 2011).

The HPA axis plays a significant role in a variety of physiological processes, including glucocorticoid-mediated glycogen breakdown, gluconeogenesis and in allocating resources to enhance survival (Exton et al., 1972). The hormones involved via the HPA axis have the capacity to influence other endocrine pathways, most notably the GH/ IGF-I and HPT axes (Kuhn et al., 2005; De Groef et al., 2008). Therefore, complete

embryonic development of a functional HPA axis is essential to enable a vast variety of biological functions associated with various endocrine pathways.

2.8 Ontogeny of HPA axis development in the chicken

Ontogeny of the HPA axis begins early during embryonic development, and is believed to be fully functional by the time of hatch (Table 1; Jenkins and Porter 2004). The enzymes required for steroid hormone synthesis (i.e. CORT/androgens), along with substrate availability (lipid and cholesterol), appear to be present early on during embryonic development (Ericson and Domm 1969). In vivo administration of ACTH stimulated glucocorticoid synthesis at embryonic day (ED) five, suggesting the adrenal gland is functioning at this time (Pedernera 1971), although the exact point of maturation remains unknown. Additionally, 3β-hydroxysteroid dehydrogenase activity (which converts pregnonelone to progesterone) in the adrenal gland is stimulated by ACTH at ED4 (Ericson and Domm 1969). ACTH synthesis appears to be independent of the CRF-neurons until ED10 (Jenkins and Porter 2004), where ACTH synthesis by the adenohypophysis appears to be regulated by CRF-neurons upon completion of development (Ellestad, Saliba, and Porter 2011). ACTH synthesis may be influenced by the pituitary hormone AVT which acts to release ACTH either directly or through corticotropic releasing factor (CRF). However, the timeframes in which AVT exerts it effects during chick embryonic development are unclear (Grossmann et al., 1995). Additionally, corticotrophs are said to be present at ED10, which coincides with the identification of pro-opiomelanocortin expression (Allaerts et al., 1999), the prohormone involved in ACTH synthesis.

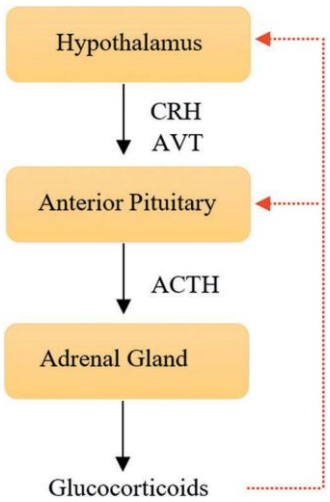

Figure 1. Hypothalamus-pituitary-adrenal axis schematic. Primary endocrine axis involved in the regulation of a stress response and glucocorticoid secretion in the chicken. Development of the HPA axis can be influenced by maternal stress and nutritional factors.

Table 1. Significant time points during the development of the HPA axis in the domesticated chicken (Jenkins and Porter 2004).

Embryonic age	Biological event
4	3β-Hydroxysteroid dehydrogenase activity first appears in adrenal gland
5	Lipids and cholesterol appear in adrenal gland Adrenocortical cells are able to secrete steroids when stimulated by ACTH
6	Vascular connection between the hypothalamus and pituitary is present
7	Corticotrophs in the anterior pituitary first appear, and ACTH is secreted
8	Unstimulated adrenocortical cells can secrete steroids
11	Negative feedback of glucocorticoids can be demonstrated
12	The complete hypothalamo-hypophyseal portal plexus is established
14	CRF neurons become detectable in the hypothalamus
15	Adrenal corticosterone secretion becomes regulated by pituitary ACTH and hypothalamic CRF

The hypothalamic immunoreactive CRF-neuronal system has been reported to be functional at ED14, where CRF-immunoreactive fibres and terminals have been detected (Jozsa et al., 1986). Thus, functioning CRF

neurons within the hypothalamus appear to be fully developed by ED15, where neurons have the ability to secrete CRF. CRF is a known promoter of ACTH from the pituitary gland (Jenkins and Porter 2004), with ACTH acting on the adrenal gland to promote the synthesis of glucocorticoids (Herman et al., 2016).

Two distinct glucocorticoid periods are present during chick embryo development, with a noticeable peak in concentration between ED14 and ED16, potentially stimulated by elevated pituitary ACTH synthesis (Wise and Frye 1973). The second peak occurs just before hatch at ED20 (Scott et al., 1981). Noticeably, the dominating glucocorticoids differ depending on peak timing, with the ED14–16 peak consisting of equal proportions of cortisol and CORT, whilst the ED20 peak consists of primarily CORT (Kalliecharan and Hall 1974). Such variation is said to be induced through increased activity of the adrenal 21-hydroxylase enzyme, which promotes the conversion from progesterone to 11-deoxycorticosterone (Nakamura, Tanabe, and Hirano 1978). Notably glucocorticoid secretion continuously increases up until ED14, which may reflect the growth in adrenocortical cell number, and by ED15 the adrenal gland is responsive and dependent on ACTH stimulation (Jenkins and Porter 2004). Subsequently, due to the complex and extended developmental timeframe that is involved in HPA axis development, disruptions to substrate availability or hormonal signalling can permanently alter HPA axis maturation. This includes maternally derived disruptions, which can arise upon changes to the maternal environment.

2.9 The effects of maternal stress on offspring HPA development

Specific time frames of HPA development are highly species-specific and differ significantly, making any maternal environmental effects complex to understand on a broad scale (Dobbing and Sands 1979). Significant mammalian work analysing HPA programming has involved many species including, pigs, guinea pigs and rats (Gatford et al., 2018). Exposure to external stressors or nutrient restriction during

gestation has shown to reduce the HPA axis response in progeny (Kranendonk et al., 2006b), with males more adversely affected than females (Kranendonk et al., 2006a).

The effects of maternal stress may be mediated indirectly through testosterone, which has an inhibitory effect on HPA axis function through the suppression of AVT (Viau and Meaney 1996). Low plasma testosterone concentrations in rats, upon exposure to pre-natal stress, was reported in male offspring, possibly enhancing basal adrenocortical activity (Kapoor and Matthews 2005). Notably, basal ACTH and pituitary-adrenal response were elevated in female offspring exposed to pre-natal stress, but was reduced, or remained unaffected, in male offspring (Mccormick et al., 1995). Furthermore, ACTH administered to sows during mid-gestation resulted in HPA axis hyperactivity in their offspring. This hyperactivity was correlated to increased plasma cortisol concentrations and clearance time, after progeny were exposed to handling restraint stress (Haussmann et al., 2000). Maternal stress (social stress) during late gestation in sows had no significant implications on progeny HPA axis development, nor plasma cortisol concentrations (Otten et al., 2010), signifying the influence that time of exposure and stressor type has on disruptions to HPA axis development.

Avian models provide an excellent means of studying maternal effects, due to the fact that offspring develop externally from the hen. Avian offspring exposed to maternal stress have reduced negative feedback mechanisms (Wilsterman et al., 2015), disrupting the regulation and subsequent function of the HPA axis. Embryonic exposure to CORT down-regulates mRNA gene expression of AVT and CRH, both of which are significant contributors to negative feedback mechanisms governing the HPA axis. Additionally, GR mRNA expression remains unaffected, whilst GR protein content decreases (Ahmed et al., 2014). Zimmer and Spencer (2014) identified potential enhancement in the negative feedback regulation of the HPA axis, whereby maternal stress significantly reduced GR and mineralocorticoid receptor (MR) mRNA expression. Both receptors play pivotal roles in the regulation and maintenance of baseline glucocorticoids, impacting feedback efficiency. Hyper-activated HPA axis responsiveness was reported in quail progeny exposed to

maternal stress upon exposure to a human handling (Marasco et al., 2012). Henriksen et al. (2013) reported no variation in HPA axis response time or activity after physical restraint stress in offspring produced from hens with chronically elevated CORT levels. Thus significant variability exists regarding the effects of maternal stress on the HPA axis in offspring and its ability to regulate glucocorticoid secretion upon exposure to a stressor (Schoech, Rensel, and Heiss 2011). It is evident that several maternal factors can permanently disrupt HPA axis development in progeny, with further implications for post-hatch phenotypes. However, the physiological mechanisms influencing post-hatch phenotypes remain unclear.

2.10 The HPA-axis and its effects on progeny growth and metabolism

Disruption to growth and metabolic processes initiated via the HPA axis can be mediated directly through glucocorticoid secretion (Mazziotti and Giustina 2013), likely encompassing disrupted feedback mechanisms. At baseline levels, glucocorticoids are necessary in the stimulation of neurogenesis, muscle development and inducing immune system activation (Mcewen et al., 1997; Belanto et al., 2010; Anacker et al., 2013). Baseline CORT concentrations in chicken meat birds vary between 0.75 ng/ml during the dark cycle and ~1.5 ng/ml at the peak of the light cycle (De Jong et al., 2001). Once glucocorticoid concentrations exceed baseline levels, their physiological effects tend to inhibit somatic growth, either directly through binding to GR, or indirectly, through signal disruption of key endocrine pathways associated with performance traits (e.g. GH/IGF-I, HPT) (Scanes 2009; Mazziotti and Giustina 2013). Furthermore, maternal stress, either chronic or as daily doses of acute stress, have been shown to reduce both plasma and yolk concentrations of testosterone, progesterone, whilst increasing concentrations of oestrogen (Henriksen, Groothuis, and Rettenbacher 2011a; Bertin et al., 2019). Such findings signify the potential 'flow on effect' disruption to HPA axis that can have on the functionality of various other endocrine pathways. Additionally, this disruption may occur through dysregulation of negative feedback mechanisms. In chronically stressed animals this may result from adrenal exhaustion, resulting in reduced baseline and peak CORT levels (Rich

and Romero 2005). Additionally, gene expression alteration, and subsequent synthesis of proteins involved in the HPA axis, including CRH, AVT and GR, may disrupt glucocorticoid secretion through negative feedback mechanisms (Kapoor et al., 2006). Thus, prolonged stress response coincides with the disruption of HPA axis functionality and elevated glucocorticoid levels, but whether this correlates to poor growth and metabolism remains unclear.

2.11 Growth hormone – insulin-like growth factor I axis

The GH/IGF-I axis (Figure 2) is a major neuroendocrine pathway involved in avian growth and metabolism (Scanes 2009), and is the major regulatory pathway controlling IGF-I synthesis (Mcmurtry, Francis, and Upton 1997). Growth hormone releasing hormone (GHRH) secreted from the hypothalamus acts upon the anterior pituitary triggering GH secretion (Stanfield and Germann 2009). The liver significantly contributes to plasma IGF-I concentrations (75%), and its secretion is triggered by GH. The remaining 25% is produced from various tissues, with and without initiation from GH (Mcmurtry et al., 1997; Stanfield and Germann 2009). The endocrine activity of both GH and IGF-I involve exerting negative feedback on the hypothalamus, regulating secretion (Yakar and Isaksson 2016). Further components, including thyroid hormones, glucagon and insulin, have been identified as IGF-I secretory regulators (Mcmurtry et al., 1997; Tsukada et al., 1998). Published data have suggested several endocrine factors that, either independently or coherently, regulate IGF-I secretion.

The thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), significantly influence secretion of IGF-I in birds, which has been demonstrated by administration of goitrogens, which reduce IGF-I hepatic mRNA expression, which was later restored following administration of (T_4) (Tsukada et al., 1998). CORT is known to influence the functionality of the GH/IGF-I axis, through its inhibitory effects on GHRH, which reduces growth through suppression of IGF-I synthesis (Fernandez-Vazquez et al., 1995). Consequently, a

dysfunctional or disrupted HPA axis has the potential to directly influence the function of GH/IGF-I and the HPT axes.

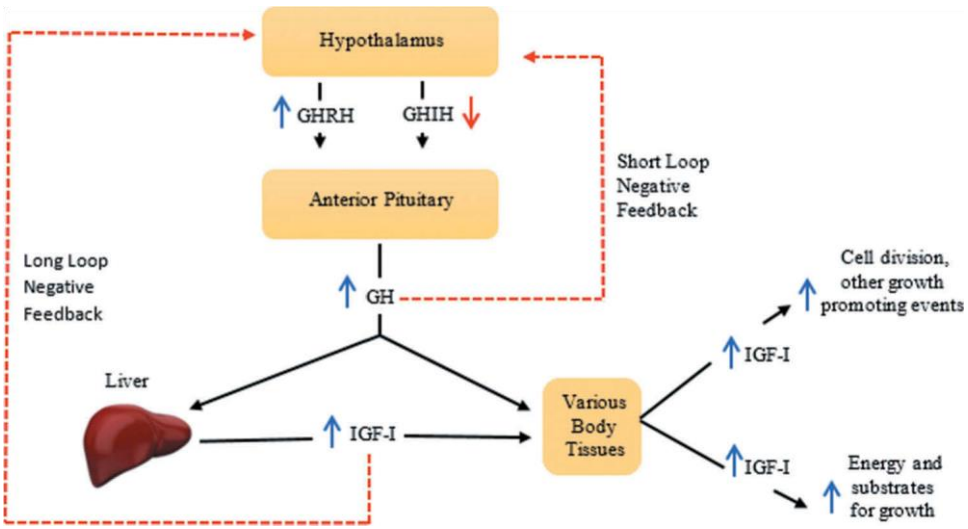

Figure 2. Growth hormone/insulin-like growth factor I axis (GH/IGF-I) schematic. Primary endocrine axis involved in the promotion and regulation of growth events in the chicken. Functionality of the GH/IGF-I axis can be influenced by stress and nutritional factors, reducing the occurrence of growth-promoting events.

2.12 Hypothalamic pituitary thyroid axis

The avian HPT axis is a primary contributor to avian growth and metabolism through its ability to regulate the synthesis of the thyroid hormones, T_3 and T_4. Synthesis initiation begins from the hypothalamus, which releases thyroid-releasing hormone (TRH), which proceeds to act upon the pituitary gland, promoting the release of thyroid-stimulating hormone (TSH). TSH acts directly on the thyroid gland, resulting in the synthesis and secretion of both T_3 and T_4 (Kim 2010). T_4 is the primary form secreted in avian species, comprising approximately 99% of all thyroid hormones secreted (Lam, Harvey, and Hall 1986). T_3 is considered the active form, and, although T_4 is the primary hormone secreted, it is converted to its active form via the iodine-dependant enzymes, iodothyronine deiodinases, in various tissues (Darras et al., 2006).

Regulation of the HPT axis is governed via negative feedback mechanisms, whereby both T_3 and T_4 act on both the pituitary and the hypothalamus to regulate TSH and TRH secretion, which, in turn, regulates thyroid hormone synthesis (Muchow, Bossis, and Porter 2005). Additionally, CRH and CORT both influence HPT axis regulation via inhibiting TSH and TRH synthesis, respectively (Geris et al., 1996).

The HPT axis is functional from ED11-13 (Thommes et al., 1992), and is a pivotal contributor to early growth and development events both in ovo and posthatch (De Groef et al., 2008). The presence of thyroid hormones, primarily T_3, are required to promote differentiation in various body tissues, including sensory function, the nervous system, musculoskeletal development and feathering (integumentary development; McNabb 2006). Considering the similarities in growth regulation and promotion between the HPT axis and GH/IGF-I axis, it was not surprising that communication between the two axes has been shown (Kuhn et al., 2005; De Groef et al., 2008). In fact, TRH is a potent stimulator of GH, to the extent where GHRH is believed to be less of a stimulant then TRH (Kuhn et al., 2005). Considering that communication between endocrine axes exists, the improved or disrupted function of one axis, may indirectly affect the functionality of several others, ultimately influencing performance.

2.13 Maternal stress, endocrine axes and offspring body composition

Although many studies have linked maternal stress and nutritional status on phenotypic alterations on progeny BW and growth, investigations into specific body composition parameters, such as fat, muscle and bone percentage, are somewhat limited. Maternal stress could be associated with the development of undesirable carcase characteristics, such as increased fat deposition and reductions in lean muscle mass (Reynolds 2010).

Published data suggested that elevated glucocorticoids, particularly in stressed individuals, were linked with enhanced fat deposition, primarily around the abdominal, cervical and thigh adipose tissue, as well as the

liver and skeletal muscle (i.e. breast muscle; Cai et al., 2009; Wang et al., 2012). Prenatal glucocorticoid exposure in rats resulted in increased abdominal adiposity, accompanied with increased circulating levels of the appetite regulating hormone leptin (Dahlgren et al., 2001). Similarly ovine offspring, exposed to either dexamethasone or intra-uterine growth restriction, showed adiposity, increasing the risk of obesity (Cardoso and Padmanabhan 2019). Exposure to in utero heat stress in pigs resulted in offspring exhibiting increased adiposity and reduced lean muscle mass, similar to that seen in individual animals exposed to a stressor (Johnson et al., 2015a, 2015b).

CORT functions to increase fatty acid synthesis through increased expression of acetyl-CoA and fatty acid synthase, both key regulators mediating fat deposition (Cai et al., 2009, 2011). Additionally, CORT promotes the expression of fatty acid transport protein 1 (FATP1) and insulin receptors, ultimately increasing fatty acid uptake within skeletal muscle (Wang et al., 2012). Considering elevated CORT appears to promote adiposity, its inhibitory effects on protein synthesis and subsequent promotion of muscle degradation is not unexpected. This may result from disruptions to mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase signalling pathways. These signalling pathways appear to influence growth retardation upon exposure to glucocorticoids, as exposure decreases phosphorylation of both the MTOR and ribosomal protein S6 protein kinase, both regulators of protein synthesis (Wang et al., 2015). Additionally, circulating concentrations of uric acid reportedly increase in response to CORT elevation, with uric acid a primary bi-product of protein catabolism (Song et al., 2011). Gluconeogenesis within the liver was reportedly increased in response to CORT, further reflecting the degenerative effects of stress on protein synthesis (Gao et al., 2008). Significant evidence exists suggesting exposure to various forms of stressor promotes adiposity whilst simultaneously degenerating lean muscle mass; however, conclusive links connecting maternal stress to these phenotypic traits remain elusive.

Reductions in protein synthesising capabilities or a failure of skeletal muscle to uptake glucose have been proposed as potential causes (Wang et al., 2015). Additionally, the metabolic actions of GH are reportedly suppressed in mammalian progeny exposed to maternal stress, a potential side-effect of reduced proportions and densities of somatotropic cells (Lutz, Dufourny, and Skinner 2006). Furthermore increased adiposity may be a consequence of expanded adipocytes, a potential consequence of maternal stress, allowing for the accommodation of greater lipid quantities (O'Connell et al., 2010). Moreover, reduced activity of the adipose-specific glucose transporter (GLUT4) has been reported in sheep, which was seen in offspring from malnourished dams exhibiting increased adipose tissue deposition in adult life (Gardner et al., 2005).

In humans, high levels of maternal T_4 were associated with reductions in offspring body mass index and total fat mass (Godoy et al., 2014). Exposure to heat stress in chickens significantly reduced T_3 serum concentrations, and T_3 to T_4 ratios (He et al., 2019), suggesting that glucocorticoids act in a suppressive manner on thyroid hormone synthesis. Furthermore, the synthesising capacity of the HPT axis may be directly influenced by the HPA axis, where glucocorticoids act to inhibit the synthesis of both TSH and TRH. It is therefore possible that, due the interaction between the HPA and HPT axes, suppression of thyroid hormone secretion, primarily the active form (T_3), may ultimately influence body composition due to its known influence on metabolic rate (Scanes 2011).

The effects of maternal stress on progeny body composition have primarily been investigated in mammalian species. Little to no work has analysed the effects of maternal stress on progeny body composition in broilers. Thus, the utilisation of feed restriction measures in breeder hens may, unintentionally, promote undesirable carcase characteristics in commercial birds. A detailed understanding of how the maternal environment, primarily maternal stress, influences offspring body composition would provide the poultry industry a unique and economically viable method to improve production outputs. However, this requires a more fundamental understanding of the physiological mechanisms at play, which currently remain unclear.

2.14 Conclusion

This review identified the potential interaction between chronic maternal stress in broiler breeder hens, and subsequent reduction in progeny performance as a result of severe feed restriction, which is currently implemented in the broiler breeder industry. Although feed restriction is necessary to ensure optimal reproductive output and bird health, the negative impacts on progeny growth and production efficiency are of concern. However, the contributing physiological mechanisms influencing progeny growth rates and body composition remain elusive.

The involvement of key hormones, such as GH, IGF-I, T_3 and CORT, in avian growth and metabolism suggests the development of the HPA, GH/IGF-I and HPT axes may be either directly or indirectly be influenced by maternal stress. Additionally, communication within and between axes may subsequently act to influence progeny growth and body composition in several ways dependent on stressor type, exposure duration and time of exposure. As broilers are under constant metabolic stress (De Jong and Guemene 2011), the detrimental physiological effect of a prolonged stress response is likely to be increasingly prominent. Further studies are required to better understand the effects of the maternal environment on offspring development and the role of maternal nutrition. Although significant research is required, maintenance of the maternal environment has the potential to provide a cost-effective method of enhancing chicken meat production, in an industry that has almost optimised its production standards.

Disclosure statement

No potential conflict of interest was reported by the authors.

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J. L. Angove is a PhD student currently working at The University of Adelaide, where his work aims to investigate breeder bird management and its influence of progeny performance. Joshua completed a bachelor of science (animal science) with first class honours in 2017, and was awarded the prestigious Gary Sansom memorial scholarship in 2019 by Agrifutures Australia.

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Chapter Three:

In-ovo corticosterone administration alters body composition irrespective of arginine supplementation in 35 day old chicken meat birds

Statement of Authorship

Statement of Authorship

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Contribution to the Paper	Designed experiment, collected experimental data, performed laboratory work, data analysis, data interpretation, manuscript author
Overall percentage (%)	85 %
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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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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

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Previous work conducted by our research group (2014-2018) identified that alterations to feed restriction practices in broiler breeder hens led to growth differences in subsequent progeny hatched from these hens, despite progeny being reared together under identical conditions. The differences in progeny growth rates identified by (Bowling *et.al.* Unpublished) were therefore hypothesised to be a result of alterations to the level of maternal stress encountered in these hens as a result of the level of feed restriction the hens were exposed to during their production phase.

In addition, further work by (Bowling *et.al.* Unpublished) identified that embryonic exposure to the stress hormone corticosterone at embryonic day 11 in chicken meat birds led to similar differences in progeny growth to those identified in the original study in breeder hens. In order to investigate the effects of maternal stress on progeny growth and performance, an *in-ovo* model was chosen to simplify the experimental procedure by removing the potential confounding factors associated with assessing the breeder hen environment, including housing environment, temperature variations and human handling. Furthermore, dietary supplementation with the amino acid arginine was utilised in the present chapter as a method of alleviating the expected growth differences between control and corticosterone exposed progeny. Arginine was chosen due to its well documented ability to improve growth rates in commercial chickens, whilst being involved in protein synthesis mechanisms, therefore potentially increasing bodyweights and total lean mass at market weight.

***In ovo* corticosterone administration alters body composition irrespective of arginine supplementation in 35-day-old female chicken meat birds**

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3.1 Abstract

Context. Exposure to maternal hormones can permanently alter an embryo's developmental trajectory. Maternal mediated effects have significant potential in the chicken meat industry, as breeder hens are feed restricted in a bid to improve performance. Evidence suggests breeder hens are chronically stressed, resulting from periods of prolonged hunger. However, evidence linking embryonic exposure to early-life stress and altered offspring phenotype in meat chickens is lacking. Additionally, methods to alleviate the phenotypic consequences of early life stress have not been comprehensively explored. Nutritional supplementation with amino acids, such as arginine (Arg), may provide one such option, as Arg reportedly enhances performance characteristics in chicken meat birds.

Aims. An *in ovo* study was conducted to investigate whether exposure to *in ovo* stress altered offspring performance in meat chickens. Additionally, Arg was supplemented post-hatch to alleviate reductions in performance, hypothesised to occur as a result of exposure to corticosterone.

Method. A total of 400 eggs were divided into two groups and administered a corticosterone (CORT) or control (CON) solution at embryonic Day 11. At hatch, birds were separated into four groups based on *in ovo* and dietary treatments: CORT-Control, CORT-Arg, CON-Arg and CON-Control. Birds fed supplementary Arg diets received an Arg: lysine inclusion of 125%.

Bodyweight (bwt) and feed conversion were recorded weekly. Birds were euthanised at embryonic Day 15, Day 0, 7, 21 (n = 40 birds/time point), 28 and 35 (n = 48 birds/time point) for organ collection. A total of 12 additional female birds were euthanised and subjected to a dual-energy X-ray absorptiometry scan for body composition at Day 35.

Results. Neither *in ovo* nor diet treatments influenced bwt, bwt gain, feed conversion or plasma corticosterone at any time point, nor did any *in ovo* by diet interaction exist. Female birds exposed to CORT exhibited significantly greater fat mass (%bwt; P = 0.007) and reduced lean mass (%bwt; P = 0.026) compared with CON females at Day 35. Supplementary Arg did not influence bird body composition.

Conclusions. These findings suggest *in ovo* exposure to CORT may negatively influence body composition of female birds.

Implications. Understanding the effects of the maternal/*in ovo* environment may provide a novel approach to further improve carcass quality and flock uniformity.

Keywords: amino acids, animal stress, broilers, fat deposition, poultry.

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3.2 Introduction

Human consumption of chicken meat products has risen exponentially over the past five decades, and continues to grow on a global scale (Salter 2017; Seidavi *et al.* 2019). The desirability of chicken meat arises from the ability to produce a relatively cheap, healthy and versatile product that is widely consumed by a range of cultures (Haley 2001). Thus, chicken meat production has seen unparalleled expansion to meet the continually increasing consumer demand. Such expansion has resulted in the chicken meat industry being at the forefront of animal production, where advances in animal nutrition and genetics are near optimal (Zuidhof *et al*. 2014). Therefore, the industry requires new and innovative approaches to advance chicken meat production, with breeder hens and their management strategies providing a viable avenue to do so.

The maternal environment can be described as the overall environment a female encounters at the time of reproduction (i.e. gestation/egg production; Reynolds *et al*. 2010). Several known factors can influence the maternal environment, including stress, nutrition, environmental chemicals and individual health. Such factors have the potential to alter offspring developmental trajectory, with permanent phenotypic effects (Sinclair *et al.* 2016). A significant body of work exists in mammals, primarily humans (Welberg and Seckl 2011), rodents (Weinstock 2008; He *et al*. 2020) and large production species (Mack *et al*. 2014; Sinclair *et al*. 2016; Gatford *et al*. 2018), investigating the phenotypic effects displayed by progeny as a result of exposure to maternal stress. In the majority of cases, maternal stress tends to reduce birthweights (Sinclair *et al.* 2016), although some offspring would encounter 'catch-up' growth periods and exhibit increased growth rates or bodyweight (bwt) in adult life (Berghänel *et al*. 2017). Similar findings have been reported in wild birds, where maternal stressors, such as malnourishment, increased predation risk and seasonal induced stress all reduced offspring growth, hatching success and survival (Love *et al*. 2013; Sheriff *et al*. 2017). More recently, the effects of varied maternal environmental conditions have been demonstrated in commercial poultry, whereby the progeny of severely feed restricted broiler breeder hens exhibited reduced hatch weight, growth rate and feed conversion ratio (FCR; Gholami *et al*. 2017; Bowling *et al*. 2018).

Feed restriction in breeder flocks is a common management practice throughout the chicken meat industry (De Jong and Guemene 2011). Such protocols are utilised to enhance reproductive capacity, while improving bird health by reducing the occurrence of ailments associated with metabolic disorders (Mench 2002). Birds are feed restricted from 1 week of age and throughout their adult life, with restriction levels varying between 25 and 80% of a bird's normal daily *ad libitum* intake, depending on age and reproductive status (Mench 2002). Several studies have shown that hens subjected to feed restriction exhibit elevated stress biomarkers, including plasma corticosterone and heterophil :lymphocyte ratios (De Jong *et al*. 2002; Najafi *et al*. 2015). However, the ramifications of such stressors, and their ability to influence the hormonal and nutritional composition of the egg appear complex (Groothuis *et al*. 2019). Additionally, the physiological mechanisms contributing to variations in offspring phenotypes in response to maternal stress remain elusive, but appear more multifaceted than a direct transfer of corticosterone from hen to egg (Groothuis *et al*. 2019; Angove and Forder 2020). However, given that commercial meat chickens now spend ~40% of their life within the egg, maternally derived alterations to the *in ovo* environment may have significant implications on the phenotypes displayed by offspring posthatch (Ho *et al*. 2011).

Despite evidence suggesting the maternal environment does influence the concentration of hormones, such as corticosterone and testosterone, within the egg (Henriksen *et al*. 2011*a*; Almasi *et al*. 2012), and subsequent offspring performance (Henriksen *et al*. 2013; Bowling *et al*. 2018), the physiological consequences of alterations to the *in ovo* environment still remain unclear. Additionally, methods to alleviate the physiological consequences of exposure to early life stress have not been extensively investigated. Providing exposed offspring with nutritional supplementation, including amino acids, such are arginine (Arg), is one approach to negate the effects of early-life stress. Arg is reported to improve production efficiency and bwt in chicken meat birds (Xu *et al*. 2018), while supressing plasma corticosterone in porcine (Ma *et al*. 2010) and aquaculture (Costas *et al*. 2013) species subjected to chronic stress. Additionally, increased secretion of metabolic and growth-related endocrine factors, such as growth hormone, insulin-like growth factor I (Oh *et al*. 2017) and the thyroid hormones (Ebrahimi *et al*. 2014), has been reported in Arg supplemented birds.

Therefore, a study was conducted to identify the effects of *in ovo* exposure to corticosterone on growth and body composition in chicken meat birds. Additionally, dietary Arg supplementation was provided post-hatch to determine if Arg would alleviate any consequences of *in ovo* exposure to corticosterone. It was hypothesised that birds exposed to *in ovo* corticosterone would exhibit a reduction in bwt and bwt gain. Additionally, the provision of supplementary Arg was hypothesised to alleviate the growth consequences induced via *in ovo* corticosterone exposure, by increasing total bwt in commercial chicken meat birds.

3.3 Materials and methods

All animal use and experimental protocols were approved by the University of Adelaide Animal Ethics Committee (S-2018058), and the Primary Industries and Regions South Australia Animal Ethics Executive Committee (# 08/18).

3.3.1 In-ovo treatment

Fertile eggs (Cobb 500 broiler breeder, n = 400) were provided by a commercial breeder (HiChick Breeding Company, Bethel, SA, Australia), and set in an incubator as per standard incubation conditions (see below). At embryonic Day (ED) 11, eggs were divided into two treatment groups: corticosterone (CORT) and control (CON). ED 11 was selected as the injection age in accordance with (Ahmed *et al*. 2014*b*), in a bid to use an extreme model of *in ovo* stress to understand the phenotypic consequences that could arise in subsequently hatched progeny. Prior to injection, all eggs were candled, and infertile eggs removed from the study. Eggs were randomly allocated to CORT (n = 200) or CON (n = 200) treatments. CORT eggs were injected with 100 µL of a 10 mM PBS solution (Sigma-Aldrich, Castle Hill, NSW, Australia, #1002596158; pH 7.4) containing 1 µg corticosterone dissolved in ethanol (Sigma-Aldrich, #1001988418), with the administered dose rate determined in accordance with previous publications (Janczak *et al*. 2006; Ahmed *et al*. 2014*b*). The CON treatment group was injected with 100 µL of the 10 mM PBS solution. All injections were administered into the chorioallantoic membrane of the egg, through the air sac (Fig. 1), using

a 1-mL insulin syringe. Selleys glass silicone was used to re-seal the injection site, after which the eggs were returned to the incubator.

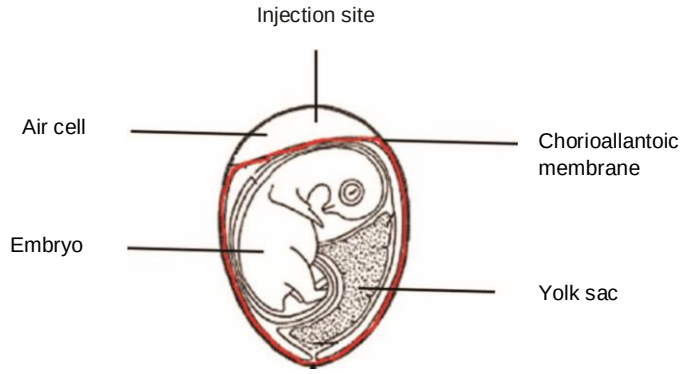

Fig. 1. Schematic diagram demonstrating the site of injection into the chorioallantoic membrane, via the air sac. Figure obtained from Vargas *et.al*.2007.

Table 1. Diet composition for control and arginine supplemented feed utilised throughout the trial.
+Arg, control diet supplemented with arginine: lysine inclusion of 125%; SBM, soybean meal; DCP, digestible crude protein; Lys, lysine.

Feed ingredient	Starter		Grower	
(% diet)	Control	+Arg	Control	+Arg
Wheat	54.255	67.819	59.119	73.899
SBM	35.029	43.786	30.469	38.086
Vegetable oil	4.408	5.51	5.265	6.581
Lysine	0.265	0.331	0.217	0.271
Methionine	0.317	0.396	0.256	0.32
Threonine	0.136	0.17	0.111	0.139
Valine	0.062	0.078	0.03	0.038
Salt	0.038	0.048	0.04	0.05
Na bicarb	0.556	0.695	0.517	0.646
Limestone	0.815	1.019	0.596	0.745
DCP	2.848	3.56	2.31	2.888
Xylanase	0.02	0.025	0.02	0.025
Choline Cl	0.9	1.125	0.8	1

Maxiban	0.05	0.063	0.05	0.063
Premix	0.3	0.375	0.2	0.25
	99.999	124.9988	100	125
+Arg (%)		0.32		0.25
Arg:Lys ratio (%/%)		125.3188		125.25

3.3.2 Animals and tissue collection

Eggs were acclimatised at room temperature (26C) for 15 h, then set in an incubator at 38C and 55% humidity between ED 0 and ED 18, and at 36.7C and 60% humidity from ED 18 until hatching (ED 21). Eggs were rotated 90 hourly throughout the incubation period until ED 18, when all eggs were placed in hatching trays. At ED 15 and hatch (Day 0), 20 birds per treatment (n = 40) were humanely killed. Total weights were recorded for bwt, yolk sac (ED 15 only), heart, liver and brain, and a blood sample collected via cardiac puncture (Day 0 only).

At hatch, 212 birds were individually weighed, tagged and randomly allocated to one of four treatment groups (*in ovo* · diet) as follows: (1) CON-Control (n = 55), (2) CON-Arginine (n = 57), (3) CORT-Control (n = 50), and (4) CORT-Arginine (n = 50). Birds were allocated across five rearing pens/ treatment lined with wood shavings, and provided with *ad libitum* access to feed and water. The control diet was a standard poultry diet, whereas the Arg supplemented diet was a standard poultry diet + 25% Arg, which was included as an Arg :lysine ratio of 125% (Table 1). Birds were fed starter diets from Day 0 to 21, and finisher diets from Day 21 to 35. The lighting schedule was 23 h light and 1 h dark from Day 0 to 5, and 16 h light and 8 h dark from Day 5 to 35. Shed temperature and ventilation were maintained to standard management procedures throughout the trial.

Individual bird bwt was recorded weekly, along with pen total feed intake. A total of 10 birds per treatment (n = 40) were euthanised at Day 7 and 21, and 12 birds per treatment (n = 48)

were euthanised at Day 28 and 35. Organ weights were collected for the heart, liver, spleen and brain, and a blood sample was obtained via cardiac puncture. Whole left and right breast muscle tissue was removed and weighed at Day 35.

Body composition, including total lean mass, total fat mass, bone mineral content and bone mineral density was quantified using a dual-energy X-ray absorptiometry scan. A subsample of female birds, three per treatment (n = 12), were humanely killed by cervical dislocation at Day 35 and individually frozen to ensure correct body orientation was achieved for scanning. Scanning was conducted at the South Australian Research and Development Institute (Gilles Plains, SA, Australia).

3.3.3 Plasma corticosterone

Blood samples collected at Day 0 (n = 40) and Day 35 (n = 48) were centrifuged at 1200*g*. The plasma was removed and analysed for corticosterone concentrations at the University of Western Australia, Animal Biology Department, using a validated radioimmunoassay corticosterone 125I RIA KIT (MP Biomedical, Orangeburg, NY, USA).

3.4 Statistical analyses

A Chi-square test was used to analyse the sex ratio and hatchability data. Continuous data were checked for normality by the Shapiro–Wilk test. Normally distributed data, including ED 15 to Day 28 bwt and Day 7–21 bwt gain, FCR, body composition, and Day 0 plasma corticosterone were subjected to a full factorial mixed model analysis for *in ovo*, diet and sex factors. Non-normalised data were analysed using non-parametric tests, including the Mann–Whitney *U*-test and Kruskal–Wallis test, which included Day 28 bwt gain, Day 35 bwt and bwt gain, and Day

35 plasma corticosterone. Correlation analyses were performed using the Pearson's coefficient for normalised data, and Spearman's coefficient for non-normalised data. All statistical analyses were conducted in IBM SPSS Statistics 25 (Armonk, NY, USA). A probability level of <5% ($P < 0.05$) was deemed as significant.

3.5 Results

Total hatchability was 76.8%, with no significant differences between *in ovo* treatments observed (CON 80%, CORT 73.7%; $P = 0.144$). The 4.2% mortality recorded throughout the trial was acceptable by industry standards and deemed unrelated to the treatments allocated. The offspring sex ratio was not significantly different between treatments ($P = 0.341$), with 47.4% females and 52.6% males hatching for CORT treated eggs, compared with 53.5% female and 46.5% males hatching for CON-treated eggs.

3.5.1 Growth and performance

ED 15 bwt did not differ between *in ovo* treatments; however, male birds were significantly heavier compared with females ($P = 0.011$). Total weekly bwt between treatment groups did not differ at any time point (Table 2). Significant interactions between *in ovo* · sex and diet · sex for Day 35 bwt were identified following non-parametric analysis; however, further analysis showed this to be a difference between sexes only, as is presented in Table 2. Additionally, bwt did not differ in response to the main effects of *in ovo* treatment or dietary Arg supplementation at any time point.

Bwt gain did not differ between treatment groups at any stage, nor were any interactions (*in ovo* · sex or diet · sex) identified. Additionally, no difference was present for the main effects of *in ovo* and diet. As expected, male birds exhibited significantly greater bwt and bwt gain

from Day 14 onwards ($P < 0.05$). FCR did not significantly differ between treatments ($P = 0.962$), nor between *in ovo* ($P = 0.802$) and diet ($P = 0.637$) independently.

3.5.2 Body composition

Fat %bwt was significantly higher for CORT-treated birds compared with CON-treated birds ($P = 0.007$; Table 3). Conversely, lean %bwt was significantly lower in

CORT-treated birds compared with CON-treated birds ($P = 0.026$). No significant differences in bone mineral content ($P = 0.902$) nor bone mineral density ($P = 0.481$) were detected. Additionally, no difference was identified in relation to diet, with no diet by *in ovo* interaction observed.

In addition to the birds sent for dual-energy X-ray absorptiometry scanning, breast muscle yield (%bwt) was compared from birds dissected at Day 35 to further assess body composition. No differences nor interactions were detected for sex, diet and *in ovo* factors (Table 2).

3.5.3 Plasma corticosterone

Plasma corticosterone concentrations did not significantly differ between male and females exposed to either CORT or CON solutions, nor did dietary Arg supplementation influence these measures. No interactions were observed between any factors at Day 0 or Day 35 (Table 4). However, male birds exposed to a CON solution tended to display higher plasma corticosterone concentrations compared with CORT treated birds.

A significant positive correlation (r = 0.681, P = 0.021) was identified between plasma corticosterone and bwt in 35-dayold CORT-treated female birds, which was not replicated in CON-treated female birds. Additionally, plasma corticosterone was negatively correlated (r = –0.664, P = 0.026) to bwt in CON-treated male birds at Day 35, with no such correlation identified in CORT-treated males.

3.5.4 Organ weights

CORT treatment did not significantly influence ED 15 organ weights in either males or females. Furthermore, organ weights between treatment groups did not differ significantly at any sampled time point, and no significant diet, sex or *in ovo* interactions were identified. No correlations were identified between plasma corticosterone concentration and both spleen and liver weights (%bwt) at Day 0 and 35.

Table 2. Weekly bodyweights of both male (M) and female (F) chicken meat birds exposed to either a corticosterone (CORT) or control (CON) injection at embryonic Day (ED) 11.

Birds were fed either a control or arginine (Arg) supplemented diet. Values are mean ± s.e.m. Statistical significance was determined at the 95% confidence interval ($P < 0.05$). Means with different (a, b) lowercase letters within a column are different dependent on sex. Means with different (y, z) lowercase letters within a column are different dependent on diet and sex. Means with different (1, 2) numerals within a column are different dependent on *in ovo* and sex. BM, breast muscle; FCR, feed conversion ratio; The statistical test could not be run for the corresponding factors; n.s., not significant at $P < 0.05$.

In ovo	Diet	Sex	*n*	ED 15 (g)	Day 0 (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)	Day 28 (g)	Day 35 (g)	BM yield (%bwt)	Total FCR
CON	Con	M	7	11.9 ± 0.3a	55.3 ± 1.7	156.1 ± 3.8	438.9 ± 11.7a	937.7 ± 24.8a	1667.3 ± 45.3a	2602.3 ± 101.1a,y,1	20.21 ± 1.11	1.31 ±.014
CON	Con	F	12	10.5 ± 0.4b	55.4 ± 1.1	155.2 ± 3.0	427.5 ± 8.9b	871.7 ± 16.5b	1444.4 ± 29.7b	2036.2 ± 43.7b,z,2	21.22 ± 0.48	
CORT	Con	M	8	10.9 ± 0.2a	54.4 ± 1.4	154.7 ± 2.9	450.1 ± 8.2a	963.1 ± 14.4a	1728.6 ± 34.7a	2644.0 ± 51.9a,y,1	20.64 ± 0.58	1.31 ±.013
CORT	Con	F	12	10.4 ± 0.3b	54.1 ± 1.4	153.7 ± 3.2	419.1 ± 9.0b	861.9 ± 15.2b	1427.4 ± 27.9b	1999.8 ± 47.6b,z,2	20.72 ± 0.56	
CON	Arg	M	–	–	54.1 ± 1.0	148.6 ± 3.5	420.8 ± 10.2a	947.5 ± 19.5a	1690.7 ± 36.3a	2524.7 ± 69.4a,y,1	20.17 ± 0.96	1.31 ±.014
CON	Arg	F	–	–	55.7 ± 1.1	154.0 ± 3.2	424.4 ± 7.6b	881.9 ± 12.9b	1460.1 ± 18.2b	2099.4 ± 39.6b,z,2	19.53 ± 0.79	
CORT	Arg	M	–	–	54.8 ± 1.0	157.9 ± 3.1	442.3 ± 9.8a	959.2 ± 19.9a	1693.9 ± 47.2a	2616.6 ± 82.5a,y,1	21.70 ± 0.47	1.30 ±.013
CORT	Arg	F	–	–	55.8 ± 1.3	158.3 ± 2.8	432.8 ± 7.3b	880.9 ± 14.3b	1469.9 ± 23.3b	2018.6 ± 38.7b,z,2	19.98 ± 0.71	
P-value												
In ovo				n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	–	n.s.	n.s.
Diet				–	n.s.	n.s.	n.s.	n.s.	n.s.	–	n.s.	n.s.
Sex				0.011	n.s.	n.s.	0.027	<0.001	<0.001	<0.001	n.s.	–
In ovo · sex				n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.	–
In ovo · diet				–	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Diet · sex				–	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.	–
In ovo · diet · sex				–	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	–

Table 3. Day 35 body composition of female chicken meat birds exposed to either a corticosterone (CORT) or control (CON) injection at embryonic Day 11.

Birds were fed either a control or arginine (Arg) supplemented diet. Values are mean ± s.e.m. Means with different (a, b) lowercase letters within a column are significant for *in ovo* treatment. %bwt, variable normalised as a percentage of bodyweight; BMC, bone mineral content; n.s., not significant at $P < 0.05$.

In ovo	Diet	*n*	BMC (%bwt)	Fat (%bwt)	Lean (%bwt)
CORT	Con	2	1.13 ± 0.04	9.86 ± 1.44a	87.82 ± 1.31a
CORT	Arg	3	1.16 ± 0.04	8.36 ± 0.86a	89.53 ± 0.66a
CON	Con	2	1.13 ± 0.11	4.76 ± 0.52b	91.94 ± 1.31b
CON	Arg	3	1.17 ± 0.03	6.66 ± 0.40b	91.59 ± 0.59b
P-value					
In ovo			n.s.	0.007	0.026
Diet			n.s.	n.s.	n.s.
In ovo · diet			n.s.	n.s.	n.s.

Table 4. Plasma corticosterone concentrations (ng/mL) at hatch (Day 0) and Day 35 post-hatch of both male (M) and female (F) chicken meat birds exposed to either a corticosterone (CORT) or control (CON) injection at embryonic Day 11.

Birds were fed either a control or arginine (Arg) supplemented diet. Values are mean ± s.e.m. n.s., not significant at $P < 0.05$.

In ovo	Diet	Sex	*n*	Corticosterone Day 0 (ng/mL)	*n*	Corticosterone Day 35 (ng/mL)
CORT	Con	Male	4	15.01 ± 7.44	5	8.88 ± 2.77
CORT	Con	Female	6	15.40 ± 4.25	6	12.03 ± 3.08
CORT	Arg	Male	3	11.13 ± 4.53	6	19.39 ± 5.51
CORT	Arg	Female	3	9.17 ± 2.76	6	14.94 ± 4.29
CON	Con	Male	4	29.10 ± 10.33	5	11.52 ± 4.07
CON	Con	Female	6	13.23 ± 5.22	8	14.51 ± 3.11
CON	Arg	Male	9	27.23 ± 8.96	7	17.15 ± 6.90
CON	Arg	Female	1	6.53 ± 0.00	5	11.74 ± 4.75
P-value						
In ovo				n.s.		n.s.
Diet				n.s.		n.s.
Sex				n.s.		n.s.
In ovo · diet				n.s.		n.s.
In ovo · sex				0.088		n.s.
Diet · sex				n.s.		n.s.
In ovo · diet · sex				n.s.		n.s.

3.6 Discussion

The effects of early-life stress have to date been explored primarily in mammalian species (Sinclair *et al*. 2016; Gatford *et al*. 2018) and wild birds (Sheriff *et al*. 2017), the majority of which have investigated growth traits and survival rates in offspring, as well as susceptibility to various diseases. Studies investigating the effects of *in ovo* exposure to corticosterone on production traits in chicken meat birds are limited, but are pertinent, given that breeder birds

are routinely feed restricted (De Jong and Guemene 2011), exposing them to the associated stressors induced through prolonged periods of hunger (De Jong and Guemene 2011). Studies have revealed that maternal corticosterone is transferred to the egg (Almasi *et al*. 2012), and elevated maternal corticosterone may correlate to increased hormone levels deposited into the egg (Hayward and Wingfield 2004). Furthermore, elevations in corticosterone within the egg have been linked to a reduction in growth rate (Ahmed *et al*. 2014*b*) and immunological traits (Saino *et al*. 2003; Bowling *et al*. 2018), as well as alterations in bird behaviour (Janczak *et al*. 2007; Ahmed *et al*. 2014*b*). The current study explored whether exposure to *in ovo* corticosterone influenced performance traits, while investigating whether dietary supplementation with Arg would negate any consequences of *in ovo* exposure to corticosterone.

The primary finding of this study was the influence of CORT on female Day 35 body composition, where total fat content was increased in CORT-treated female birds, accompanied with a decrease in total lean mass. These results should, however, be taken with caution due to the limited sample size available (n = 5/per treatment), but do warrant further consideration. Additionally, body composition was not measured in male birds, as an insufficient number of animals remained at the conclusion of the trial. Although specific regions of increased adiposity were not quantified in the current study, increased exposure to glucocorticoids is reported to increase the fat content around the abdominal, cervical and thigh regions of a bird (Cai *et al*. 2009; Wang *et al*. 2012). Glucocorticoids themselves have been documented to directly influence body composition in both pigs and ruminants, increasing adiposity, while simultaneously decreasing lean mass (Greenwood *et al*. 2009), coinciding with the findings presented in this paper. In the chicken, glucocorticoid exposure has been documented to promote fatty acid synthesis, through the actions of acetyl-CoA carboxylase and fatty acid

synthase (Cai *et al*. 2011), both of which mediate the biochemical pathway regulating fat deposition.

The mechanisms governing offspring adiposity and the relationship with *in ovo* corticosterone, as documented in our findings, remain unclear. The increase in female bird adiposity may have arisen as a consequence of hyperphagic behaviour, which has been reported in mammalian offspring exposed to early-life stress, and may arise through disrupted appetite regulating mechanisms (Greenwood *et al*. 2009). Total pen FCR did not differ between CORT- and CON exposed birds in the current study. However, this does not eliminate the possibility of hyperphagic behaviour, as it was not possible to record individual bird feed intake, which should be incorporated in future studies. Quantification of the appetite-regulating hormone, leptin, would also be beneficial, as leptin concentrations reportedly increase in mammalian offspring exposed to early-life stress (Dahlgren *et al*. 2001). Whether exposure to early-life stress disrupts the appetite-regulating functions of leptin is unclear, although studies in humans have suggested that early-life stress has the potential to 'reorganise' neural pathways regulating energy expenditure and intake (Entringer *et al*. 2012). Another possibility is that the increased total fat content of female CORT-treated birds may result from an increase in the number of adipocytes that are derived from mesenchymal stem cells. This is because glucocorticoids, such as corticosterone, reportedly promote adipogenic commitment of mesenchymal stem cells at the point of differentiation (Feldman 2009), which, like muscle fibre number (Smith 1963; Sporer *et al*. 2011), occurs embryonically in the chicken (Smith 1963), providing an additional avenue to investigate the contributing mechanisms.

The differences in body composition between female CORT- and CON-treated birds may alternatively be a result of protein degradation. Glucocorticoids are reported to disrupt the signalling capacity of neural pathways associated with the regulation of protein metabolism and cell proliferation processes (Wang *et al*. 2015). Thus, if *in ovo* exposure to CORT brought

about such neural disruptions during embryonic development, a subsequent reduction in total lean mass is likely to be expected. Additionally, the conversion of proteins into glucose is upregulated upon exposure to glucocorticoids (Gao *et al.* 2008), ensuring homeostatic blood glucose levels to maintain energy requirements under stress. Therefore, if total lean mass decreased in CORT-exposed females, this may have resulted as a consequence of protein catabolism (Song *et al.* 2011). However, this appears unlikely, as plasma corticosterone concentrations did not vary between CORT and CON treated birds.

There are conflicting results between studies assessing plasma corticosterone concentrations in response to maternal stress. The present study did not identify differences in plasma corticosterone concentrations at Day 0 and 35 in response to *in ovo* and/or dietary treatments. Separate studies in egg and meat chickens also found no differences in plasma corticosterone concentrations in progeny produced from maternally stressed hens (Henriksen *et al.* 2013; Bowling *et al.* 2018). Conversely, the *in ovo* administration of 1 µg corticosterone increased Day 42 plasma corticosterone concentrations in laying hens (Ahmed *et al.* 2014*a*). The variations in these results could be attributed to stressor type, duration and/or severity, all of which can manipulate offspring phenotypes (Henriksen *et al.* 2011*b*). Yolk glucocorticoids and embryonic *in ovo* metabolism are documented to interact, regulating embryonic exposure (Vassallo *et al.* 2014; Vassallo *et al.* 2019). Therefore, the initial concentration of glucocorticoids within the *in ovo* environment is likely to influence embryonic exposure to free corticosterone (Vassallo *et al.* 2019). The ramifications of exposure to increased levels of free corticosterone is likely to influence several embryonic developmental processes, including that of endocrine pathways, such as the hypothalamic–pituitary–adrenal axis (Ahmed *et al.* 2014*b*). However, phenotypic consequences of *in ovo* corticosterone exposure are generally not apparent until after hatch. For instance, hypothalamic–pituitary–adrenal axis feedback is reportedly disrupted (Wilsterman *et al.* 2015) in birds exposed to *in ovo* stress, accompanied

with suppressed mRNA expression of hypothalamic–pituitary–adrenal regulating genes (Ahmed *et al*. 2014*b*). Such endocrine disruption is likely to induce a variety of different phenotypes in adult birds, making it difficult to develop interventions that can alleviate such phenotypic variation.

Evidence linking the provision of supplementary Arg and alterations to an individual's stress response is limited at best. Studies in chronically stressed fish (Costas *et al*. 2013) and pigs (Ma *et al*. 2010) have reported reduced plasma cortisol in animals supplemented with Arg. Such findings indicate that Arg may attenuate a stress response, where it is documented to inhibit adrenocorticotropic hormone-induced corticosterone synthesis through the actions of nitric oxide (Cymeryng *et al*. 1999). Additionally, chicks provided L-arginine before exposure to social isolation demonstrated sedative behaviours, a potential outcome from the interaction between creatine (metabolite of Arg) and the central nervous system (Suenaga *et al*. 2008). As plasma corticosterone concentrations did not differ between CORT and CON-treated birds fed a control diet, whether supplementary Arg in chicken diets can reduce corticosterone synthesis under periods of stress could not be determined. Further work is required to understand whether Arg can influence corticosterone secretion in chickens during periods of stress, both acute and chronic, and what phenotypic outcomes may arise from such an interaction.

The addition of Arg to the diets of both CORT- and CON treated birds failed to influence bodyweight and body composition measures in 35-day-old birds. This is somewhat unexpected, as Arg reportedly promotes protein synthesis (Wu *et al*. 2009), and increases total lean mass (Castro *et al*. 2019). Evidence suggests Arg can promote the synthesis of hormones, such as the thyroid hormones, growth hormone and insulin-like growth factor I, all of which are readily involved in regulating and promoting growth and developmental processes (Ebrahimi *et al*. 2014; Oh *et al*. 2017). Arg is also reported to enhance the signalling capacity of neural pathways controlling cellular proliferation/ differentiation (Oh *et al*. 2017). Although

the effects of additional Arg on body composition measures is not yet clear in chicken meat birds, several studies have reported significant bwt increases in Arg-supplemented birds (Gao *et al*. 2017; Xu *et al*. 2018; Castro *et al*. 2019). However, the present study failed to replicate these results in chicken meat birds fed a diet containing +25% Arg, with genetic interference providing a potential explanation to the variation in results across studies.

The current study collected eggs from a great grandparent broiler breeder line, with the offspring 'broilerised', mimicking a commercial production strategy. Genetic variation between/within breeds utilised must be considered as a factor influencing the phenotypes identified. As broiler breeders exhibit greater genetic/phenotypic variability (Siegel 2014), 'broilersing' pure-line progeny of great grandparent descent may have masked the effects of supplementary Arg on performance traits, those of which have been observed in commercial birds (Castro *et al*. 2019). The influence of great grandparent genetics also provides a possible explanation for the lack of growth differences between birds receiving CON or CORT *in ovo* treatments. Studies reporting significant reductions in offspring growth rates in response to maternal or *in ovo* corticosterone have primarily used laying hens (Henriksen *et al*. 2013; Ahmed *et al*. 2014*a*; Ahmed *et al*. 2016). For instance, Ahmed *et al*. (2014*a*) recorded significantly reduced growth rates in layer birds exposed to corticosterone during embryonic development; however, as previously noted, plasma corticosterone concentrations in these birds also differed. Separate studies that manipulated maternal stress on the hen rather than using *in ovo* models also reported reductions in offspring growth rates (Henriksen *et al*. 2013; Peixoto *et al*. 2020). Considering laying hens are bred specifically for egg production, they differ – both physiologically and genetically – to meat chickens (Willson *et al*. 2018), which may contribute to the variations in results. Separate studies have used other avian species, including starlings (*Sturnus vulgaris*; Love *et al*. 2005) and Japanese quail (*Coturnix japonica*; Hayward and Wingfield 2004; Langen *et al*. 2018), where phenotypic variations in growth have

also been identified. Thus, further work is required to understand the effects of supplementary Arg and *in ovo* corticosterone on production traits in commercial grower birds, as breed variation in phenotypic responses may no longer be negligible.

3.7 Conclusions

This study has identified the potential effects of elevated corticosterone during embryonic development on body composition in chicken meat birds. The *in ovo* administration of CORT increased total fat mass, while simultaneously reducing lean mass in Day 35 female broiler chickens. Unfortunately, male body composition was not measured in this study, as an insufficient number of male birds remained at the conclusion of the trial. Therefore, the findings from this study warrant further investigation to determine the effects of alterations to the *in ovo* environment and its influence on subsequent body composition in adult life. Furthermore, the mechanisms contributing to variations in body composition remain unclear. If these mechanisms can be understood, the utilisation of developmental programming provides a novel approach to enhance carcass quality and flock uniformity.

Conflicts of interest

The authors declare no conflicts of interest.

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Chapter Four:

Growth, feed efficiency and body composition differ between progeny from two lines of chicken meat bird, irrespective of breeder supplementation with *Saccharomyces cerevisiae*

Statement of Authorship

Statement of Authorship

Title of Paper	Progeny performance measures do not differ in response to maternal supplementation with a *Saccharomyces cerevisiae* metabolite in separate lines of broiler breeders
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Principal Author

Name of Principal Author (Candidate)	Mr. Joshua Angove
Contribution to the Paper	Experimental design, experimental work, data collection, statistical analysis, data interpretation, development of manuscript (primary author)
Overall percentage (%)	80%
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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Co-Author Contributions

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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Chapter Introduction

The following manuscript was formatted for the Animal Nutrition Journal with the intention to submit for publication.

In Chapter Three, the growth and bwt results generated failed to replicate those results previously identified in both breeder hen and *in-ovo* trials conducted by our research group. However, a major finding was the apparent influence of CORT on body composition parameters at 35 days post hatch, which appears to be a relatively unexplored area of research. Unfortunately, the body composition data was opportunistic and could only be collected from female birds in the previous chapter, as an insufficient number of males remained at the trials conclusion.

Therefore, the work presented in Chapter Four aimed to better understand whether alterations to the maternal environment encountered by breeder hens would influence body composition measures in the subsequent progeny produced, in either a sex or line dependant manner. Two lines of broiler breeder hens were utilised in the present study to assess whether phenotypic differences in progeny as a result of alterations to the maternal environment were consistent between lines. The two lines of broiler breeder hens differed in performance, where one line produced severely wet excreta, accompanied with reduced performance, whilst the second line was deemed a high performing line of breeder hen. Previous evidence from our research group indicated that the poorer performing line may be under greater physiological stress, and therefore would provide a good model to explore the maternal line influence on progeny development. In addition, the yeast metabolite Diamond V XPC® was included in the diets of both breeder hens. The addition of Diamond V XPC® was included firstly as a potential method to alleviate the gut dysbiosis experienced by the underperforming line of breeder hen, and has been conducted as a separate piece of work. The effects of maternal Diamond V XPC®

supplementation has previously however been documented to influence progeny performance in chicken meat birds, and thus we aimed to assess whether such effects were obtainable under Australian rearing conditions, and whether any effects were consistent between genetic lines.

Progeny performance measures do not differ in response to maternal supplementation with a *Saccharomyces cerevisiae* metabolite in separate lines of broiler breeders

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4.1 Abstract

New, innovative methods are required to advance chicken meat production, with optimisation of breeder hen rearing practices providing a novel approach to do so. Studies from the US and Brazil have identified that breeder supplementation with *Saccharomyces cerevisiae* ***(SC)*** metabolites improves progeny feed conversion **(FCR)** and breast muscle **(BM)** yield. Therefore a study was designed to investigate the effects of *SC* supplementation in broiler breeders reared under Australian management conditions, and its impacts on progeny performance.

Breeders (n = 240) from two genetic lines, (Line A) and (Line B) were reared until 23 weeks, and then separated by line and diet (control vs *SC* (1000ppm) into four treatments, Line A: Control, Line A: *SC*, Line B: Control and Line B: *SC*. At 32 weeks, 82 eggs/treatment were collected (n = 328), 300 were incubated, with remaining eggs analysed for yolk corticosterone **(CORT)** and testosterone concentration. Chicks (n = 160) were hatched and separated based on maternal treatment. Chicks had *ab libitum* access to feed and water, whilst bodyweight **(bwt)** and pen FCR were measured weekly. At d 42, eight birds per treatment (n = 32) were sacrificed and dual-energy x-ray absorptiometry scanned for body composition **(BC)**. Progeny blood samples were collected at d 21, 37 and 42, and analysed for CORT concentration.

Supplementing breeder hens with *SC* had minimal influence on yolk hormone concentrations, progeny BC, bwt, plasma CORT or FCR. Plasma CORT concentrations were higher in Line A progeny at d 21 ($P = 0.036$), d 37 ($P = 0.001$) and d 42 ($P = 0.003$). Bwt and bwt gain increased from d 7–35 and d 0-28 respectively in Line B progeny ($P < 0.05$). Line A birds yielded more BM compared to Line B ($P < 0.001$) at d 42. Diet x sex interactions were present for lean mass ($P = 0.032$) and fat mass ($P = 0.038$), where Line A male progeny had increased lean mass and decreased fat mass compared to Line A females, as well as Line B males and females, the three of which did not differ.

These findings suggest supplementing breeders with *SC* does not influence progeny performance in chicken meat birds. However, additional research is required to understand how breeder flock age contributes to progeny phenotypes as a result of maternal supplementation with nutritional additives.

Key words: feed restriction, breeder nutrition, chicken meat birds, developmental programming

4.2 Introduction

The production of chicken meat has undergone rapid expansion over the past several decades (Seidavi *et al.* 2019), resulting in an efficient animal that yields a range of desirable products at an affordable price (Haley 2001). Such advancements have primarily come about through decades of intense selective breeding programs, with particular emphasis on growth, breast muscle **(BM**) yield and feed efficiency traits.

Broiler breeders share the same genetic potential for fast growth and high feed intake as commercial grower birds (De Jong and Guemene 2011). Due to the emphasis on genetic selection for growth and feed efficiency in commercial birds, broiler breeders are routinely exposed to varying degrees of feed restriction throughout their life in a bid to reduce metabolic disorders and improve reproductive output (Van Krimpen and De Jong 2014). Although current production strategies are in place to maintain both breeder hen and commercial bird performance, advancements in breeder management, as well as breeder nutrition, may provide a novel approach to improve performance characteristics in commercial meat birds.

The breeder or maternal environment can encompass a range of factors that a hen encounters throughout her reproductive life, including, environmental chemicals, stress and nutritional status (Sinclair *et al.* 2016). Maternal nutrition has been documented in several species, including porcine (Mack *et al.* 2014), bovine (Robinson *et al.* 2013) and ovine (Penagaricano *et al.* 2014), as well as commercial chickens (Dixon *et al.* 2016; Li *et al.* 2019), to alter the developmental trajectory of subsequent progeny. In the chicken, the ability for environmental conditions encountered by the hen to alter progeny development and performance is likely driven though alterations to the nutritional and hormonal composition of the egg (Henriksen *et al.* 2011b). Furthermore, as commercial chicken meat birds spend approximately 40% of their

life *in-ovo*, the concept that breeder hen environmental conditions can initiate progeny phenotypic variation in meat birds is an area worth investigating (Dixon *et al.* 2016).

The applicability of the breeder hen environment to chicken meat production is primarily associated with the feed restriction measures implemented into broiler breeder flocks. Current feed restriction measures are implemented to maintain hen reproductive performance, with restriction severities ranging from 25-80% of a birds daily *ab libitum* intake (Van Krimpen and De Jong 2014). Studies have identified that feed restricted birds' exhibit a form of chronic stress (De Jong *et al.* 2002; Zulkifli *et al.* 2015), likely resulting from exposure to periods of prolonged hunger. Furthermore, studies have shown significant reductions in progeny growth rates and immunity (Bowling *et al.* 2018), as well alterations in behavioural traits (Janczak *et al.* 2007) in progeny produced from severely feed restricted hens. The physiological mechanisms driving such performance alterations in progeny remain elusive, but may involve variations in yolk hormone concentrations (Ho *et al.* 2011). These include corticosterone (Hayward and Wingfield 2004) and testosterone (Henriksen *et al.* 2011a), both of which are documented to influence embryonic (Giraudeau *et al.* 2017; Peixoto *et al.* 2020b), and post hatch development (Ahmed *et al.* 2016; Angove *et al.* 2021).

For the chicken meat industry, the optimisation of breeder hen management may provide a novel approach to improve performance parameters in subsequent progeny. Previous work has utilised alternative feeding mechanisms (De Jong *et al.* 2005; Moradi *et al.* 2013) and feed bulking agents (Sandilands *et al.* 2006) as a means to alleviate feed restriction induced stress in broiler breeder hens. However, the use of such methods to alleviate stress associated with feed restriction has failed to gain widespread acceptance, likely a result of increased production cost and/or minimal effectiveness (Mench 2002). Thus, further methods to alleviate the physiological consequences of such maternal stressors in both broiler breeders and their

progeny are required, with maternal supplementation with nutritional additives providing one such method.

Recent studies have identified yeast metabolites sourced from *Saccharomyces cerevisiae* ***(SC)***, as a dietary additive that can enhance poultry performance. Its addition to poultry diets reportedly reduces feed conversion ratio **(FCR)**, enhances immune function and increases growth rates (Gao *et al.* 2008; Roto *et al.* 2017). Additionally, functioning of the gastrointestinal tract **(GIT)** and intestinal absorptive capacity is documented to improve in *SC* supplemented birds (Lee *et al.* 2005). The provision of *SC* metabolites has also been linked to reductions in stress biomarkers such as plasma CORT and heterophil/lymphocyte **(H/L)** ratios in commercial broilers (Nelson *et al.* 2018; Price *et al.* 2018). Additionally, feeding *SC* to 39 week old broiler breeders reduced progeny FCR and improved total BM yield (% bwt) (Kidd *et al.* 2013). However, whether the reported benefits of maternal *SC* supplementation are transferable to the Australian poultry industry has not been investigated. Therefore, a study was developed to investigate whether supplementing breeder diets with *SC* influenced progeny performance post-hatch under Australian rearing conditions. Considering *SC* reportedly reduces both plasma CORT and H/L ratios, the addition of *SC* to breeder diets was hypothesised to reduce feed restriction induced-stress. Two lines of breeder hens were used to assess whether the physiological effects in both breeders and subsequent progeny, in response to maternal *SC* supplementation, are consistent between lines, as recent work has highlighted genetic differences in response to maternal stress (Peixoto *et al.* 2020a; Peixoto *et al.* 2020b). Furthermore, the inclusion of *SC* in breeder diets was hypothesised to improve progeny performance metrics through alterations in yolk hormone concentrations, specifically corticosterone and testosterone.

4.3 Materials & Methods

All animal use and experimental protocol were approved by the University of Adelaide Animal Ethics Committee (S-2018-068) and the Primary Industries and Regions South Australia Animal Ethics Executive Committee (#14/18).

4.3.1 Breeder hens

Two hundred and forty Cobb 500 great grandparent **(GGP)** broiler breeders from two genetic lines that differ in performance, Line A (n = 120) and Line B (n = 120), were imported and reared on a commercial farm until 23 weeks of age. At 23 weeks, birds were then separated into four treatment groups (n = 60 per treatment) dependent on breeder line and diet (Control vs *SC* treated). Thus, the four treatment groups were: (Line A: Control), (Line A: *SC*), (Line B: Control) and (Line B: *SC*). Birds were housed on a commercial farm, in pens consisting of 10 hens and one rooster, with six replicates per treatment. Animals in the control group received the farm's formulated broiler breeder diet (commercial in confidence), while *SC* supplemented birds were fed the same diet with *SC* supplemented at 1000 ppm as an addition. Birds had restricted access to feed and water and were fed once daily, with total quantities determined the day before, dependent on average bird body weight **(bwt)** and reproductive status. Ventilation, lighting and shed temperature were all maintained to industry specification and in accordance with the industry partner's commercial conditions. At peak lay (32 weeks of age), 10 birds per treatment (n = 40), were randomly selected and a blood sample collected via the left brachial vein. Blood samples were centrifuged at 2000 g for 10 minutes, after which plasma was collected and stored at -20^{o}C.

4.3.2 Plasma corticosterone

A commercially available enzyme-linked immunosorbent assay (ELISA; corticosterone ELISA kit ADI-900–097, Enzo Life Sciences, Farmingdale, NY) was used to measure corticosterone concentration in plasma samples collected at 32 weeks, following the manufacturer's protocol for small plasma samples. Steroid displacement reagent (10 µL at 1:100) was added to 10 µL of each sample. Samples were vortexed and left to stand for five minutes before 380 µL of ELISA buffer was added to make a final 1:40 dilution. All samples, standards (32, 160, 800, 4000 and 20,000 pg/mL), blanks, as well as positive and negative controls were assayed in duplicate on a 96 well plate. The absorbance of each assay was read at a wavelength of 405 nm with a plate reader (BIO-RAD, Benchmark Plus Microplate Reader, South Australia).

4.3.3 Heterophil: lymphocyte counts

Whole blood (10 µl) from each bird sampled at 32 weeks of age was placed onto a glass slide, using the two slide (wedge) technique to produce a blood smear (Ward 2013). Smears were air-dried and fixed in methanol for 20 minutes following the protocol outlined by (Ballard and Cheek 2016). Slides were stained with Wright-Giemsa stain in an automated slide stainer (Siemens Hematek, Siemens, South Australia) and cover slipped. A total of 100 cells including heterophils, eosinophils, basophils, lymphocytes and monocytes were counted at ×100 magnification (oil immersion lens). Subsequent H/L ratios were determined.

4.3.4 Progeny

At 32 weeks of age, 82 eggs per treatment were collected from the farm (n = 328) and transported to the University of Adelaide, Roseworthy Campus where they were incubated

(288 AEH, IM Incubators, Rockingham, Western Australia). Incubator settings were as follows: d 0-18, temperature: 37.7ºC, humidity: 55% and d 18-21, temperature: 36.7ºC, humidity: 60%. At embryonic day **(ED)** 15, 10 eggs per treatment (*n* = 40) were randomly selected and sampled for embryonic tissue which included liver, muscle, jejunum, yolk and brain. Liver, yolk sac and brain samples were weighed along with bwt. All samples were snap frozen in liquid nitrogen and stored at -80ºC.

At hatch (d 0), 160 viable chicks were placed in rearing pens which housed ~10 birds, with 4 replicates per treatment (Line A: Control, *n* = 38, Line A: *SC*, *n* = 43, Line B: Control, *n* = 37, Line B: *SC*, *n* = 42). Birds were subjected to a lighting schedule of 23:1 light: dark from d 0 - 4, after which a 16:8 light: dark schedule was implemented. Birds were fed *ab libitum* a standard commercial starter diet (2250320, Laucke Mills, South Australia) from d 0-21, and from d 21-42 birds were fed a commercial finisher diet (2252120, Laucke Mills, South Australia). Birds had unlimited access to water and were housed in a temperature and ventilation-controlled environment, which was set to industry standard guidelines. Individual bird bwt and total pen feed intake were recorded weekly.

At d 21 and 42, 10 birds per treatment (*n* = 40), were humanely euthanised via cervical dislocation for tissue sampling. Samples collected included liver, brain, muscle and jejunum, which were weighed before being snap frozen in liquid nitrogen and stored at -80ºC. Breast muscles, both left and right, were removed and weighed from d 42 birds. Primary feathers four and six from the left wing were plucked after euthanasia and stored at room temperature for CORT analysis.

Additionally, at d 42 a sub-sample of birds, 8 per treatment (*n* = 32), split evenly between sex (*n* = 4), were humanely killed and frozen in a specific orientation for dual-energy x-ray absorptiometry (DEXA) scanning. Birds were scanned to determine overall body composition,

with the procedure performed in conjunction with the South Australian Health and Medical Research Institute (Gilles Plains, SA, AUS).

4.3.5 Yolk, blood & feather hormonal analysis

Seven yolk samples per treatment (n = 28), collected from 32 week old hens were homogenised in 1 mL of phosphate buffer saline solution (#P5493, Sigma Aldrich, North Ryde, Australia) (1x **PBS**, pH 7.4) per/g of egg yolk. Samples were divided into 1 mL aliquots and frozen at -80°C.

Blood samples were taken from all euthanised birds at d 21 and 42, via cardiac puncture and from live birds at d 37 via the jugular vein. Blood samples were centrifuged at 2000 g for 10 minutes before plasma was collected and stored at -20^{o}C.

Yolk and plasma samples were sent to the University of Western Australia to determine CORT (plasma and yolk) and testosterone (yolk only) concentrations utilising a validated radioimmunoassay method with a detection limit of 0.05 ng/mL.

Feather CORT was extracted following the methods of Bortolotti *et al.* (2009). Briefly, primary feathers four and six were ground to a powder utilising a bead homogeniser (#85220, Qiagen, Haan, Germany), and placed in a sterile vial with the addition of 10 mL of molecular grade methanol (#34860, Sigma Aldrich, North Ryde, Australia). Vials were shaken vigorously at room temperature for 30 minutes, before being placed in an orbital shaker (#OM11, Ratek, Victoria, Australia) over night at 50^{o}C. Incubated samples were then washed twice in 2.5 mL of methanol and the vial contents filtered using a vacuum pump (#2511, Welch®, Germany) to ensure all feather remnants were removed. Washed samples were then placed in a 50^{o}C water bath and methanol evaporated under a nitrogen stream. Samples were then resuspended in 500

uL of PBS (pH 7.6) and stored at -20^{o}C. CORT concentration was determined using an enzyme linked immunoassay kit (ADI-9000-097, Enzo Life Sciences, New York) in accordance with the manufacturer's instructions.

4.3.6 Statistical analyses

All data were analysed using IBM®, SPSS® Statistics 25 (Armonk, NY, USA). Hatchability and progeny sex ratio were analysed using a chi-squared test. Continuous data were checked for normality by the Shapiro–Wilk test. Normally distributed data were analysed by a full factorial linear mixed model for the factors diet, line and sex. The means of significant interactions were separated using a generalised linear model pair-wise comparison. Non-normalised data, which included d 42 progeny plasma CORT and d 42 bwt, progeny bwt gain from d 35-42 and d 42 progeny feather CORT, were analysed using the nonparametric Mann-Whitney U and Kruskal-Wallis tests. When significant interactions were identified in non-normalised data sets, a non-parametric pairwise comparison was conducted to separate the means. A probability level of less than 5% ($P < 0.05$) was deemed as statistically significant.

4.4 Results

4.4.1 Breeder hen

4.4.2 Stress biomarkers

H/L ratios did not differ between the two breeder lines at 32 weeks of age; even though H/L ratios were higher in Line A hens. However, substantial variability in the H/L counts was present for both lines, as evidenced by the standard error of the mean **(Table 1)**. *SC* supplementation did not influence H/L ratio in either line of breeder hen, although variations

within counts appeared to reduce in *SC* supplemented birds. *SC* supplemented birds recorded higher plasma CORT concentrations, with the effect of diet approaching statistical significance (P = 0.056). No line x diet interactions were observed for H/L ratio or plasma CORT concentration.

4.4.3 Yolk hormonal composition

Yolk CORT concentration did not differ in response to maternal *SC* supplementation, or between lines **(Table 1)**, although CORT concentrations were higher in the yolk of Line B birds (P = 0.069). No differences in yolk testosterone concentrations were observed between both line and diet factors, although testosterone concentrations were lower in the yolk of *SC* supplemented birds. No line x diet interaction was observed.

Table 1: Heterophil lymphocyte (H/L) ratio and plasma corticosterone (CORT) concentrations, as well as yolk CORT and yolk testosterone (T) concentrations in fertile day 0 eggs from two genetic lines of broiler breeders (Line A and Line B), fed a control (Con) or *SC* supplemented diet. All samples were collected at 32 weeks of age. Values are mean ± SEM.

Breeder Line	Diet	H/L ratio	Plasma CORT (ng/mL)	Yolk CORT (ng/g)	Yolk T (ng/g)
Line A	Con	0.96 ± 0.32	4.56 ± 1.29	141.6 ± 14.05	10.25 ± 1.88
Line A	*SC*	0.95 ± 0.14	7.08 ± 2.55	119.9 ± 6.56	9.99 ± 1.35
Line B	Con	0.66 ± 0.24	3.84 ± 1.27	144.8 ± 6.93	11.87 ± 1.46
Line B	*SC*	0.63 ± 0.10	8.71 ± 2.56	152.6 ± 7.56	9.45 ± 0.91
P-value					
Line	-	*0.130*	*0.887*	*0.069*	*0.709*
Diet	-	*0.325*	*0.056*	*0.475*	*0.349*
Line x Diet	-	*0.901*	*0.357*	*0.124*	*0.460*

4.4.4 Hatchability & sex ratio

Total hatchability was increased in Line A birds fed *SC* (70.6%) compared to control fed hens (64%), whilst hatchability was increased in Line B control fed hens (69%) compared to *SC* supplemented hens (62.6%), however no statistically significant differences were identified. Breeder line did not influence hatchability ($P = 0.991$). In addition, neither line ($P = 0.568$) or diet ($P = 0.572$) influenced progeny sex ratio separately.

4.4.5 Progeny

4.4.6 Growth & performance

At ED 15, total bwt was significantly reduced in female embryos from Line A hens compared to Line A males ($P = 0.034$), with no such difference observed in embryos from Line B hens. A line x diet interaction existed at ED 15, where total bwt was increased in embryos from *SC* supplemented Line B hens, compared to *SC* supplemented Line A hens ($P = 0.024$) **(Fig. 1)**. However, this difference was lost at hatch (Line A Control: 41.53g, Line A *SC*: 42.42g, Line B Control: 42.64g, Line B *SC*: 41.53g). A line x diet interaction was recorded for d 7 bwt ($P = 0.005$), where bwt was increased in control fed Line B progeny (144.21g ± 2.76) compared to both control (121.32g ± 2.20) and *SC* fed (127.51g ± 2.75) Line A progeny, whilst *SC* supplemented Line B progeny (135.60g ± 2.60) recorded increased bwt to that of control fed Line A progeny only. Between d 14 and d 35, total bwt was increased in Line B progeny compared to that of Line A **(Fig. 2.A)**. No difference in bwt was observed between lines at d 42 ($P = 0.724$). No further line x diet interactions were observed for progeny bwt throughout the trial. Supplementing 32 week old breeder hens with *SC* appeared to have no influence on progeny bwt from d 14 – 42.

Weekly bwt gain followed a similar trend to total weekly bwt, with reduced bwt gain recorded for Line A progeny at d 7 ($P = 0.001$), d 14 ($P = 0.003$), d 21 ($P < 0.001$) and d 28 ($P = 0.021$) compared to Line B progeny **(Fig. 2.B)**. Maternal supplementation with *SC* did not influence bwt gain at any stage independent of all other factors. No difference in bwt gain was observed between lines from d 28-35, nor were any two-way or three-way interactions present. From d 35 - 42, Line A hatched progeny exhibited greater bwt gain than progeny produced from Line B hens ($P = 0.006$), with no two or three way interactions identified.

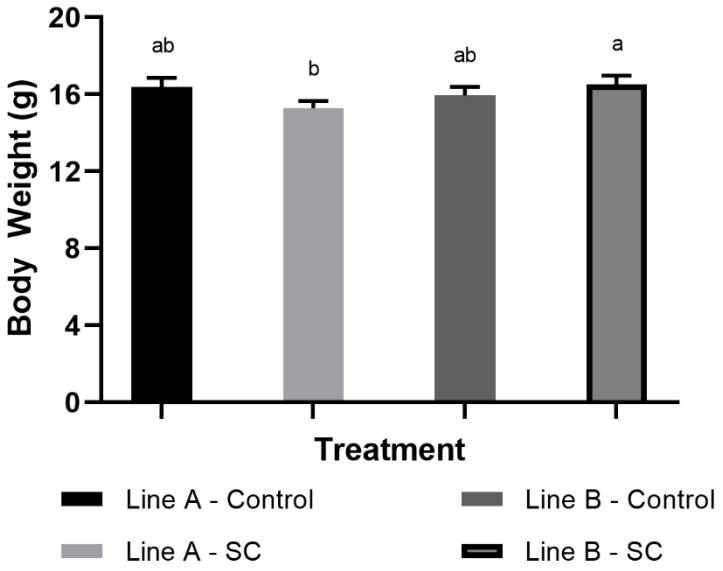

Fig. 1. Embryonic Day 15 bwt of chicken meat birds hatched from two genetic lines of breeder hens (Line A and Line B), fed a control or *SC* supplemented diet. Values are mean ± SEM. Different superscripts indicate significance at $P < 0.05$.

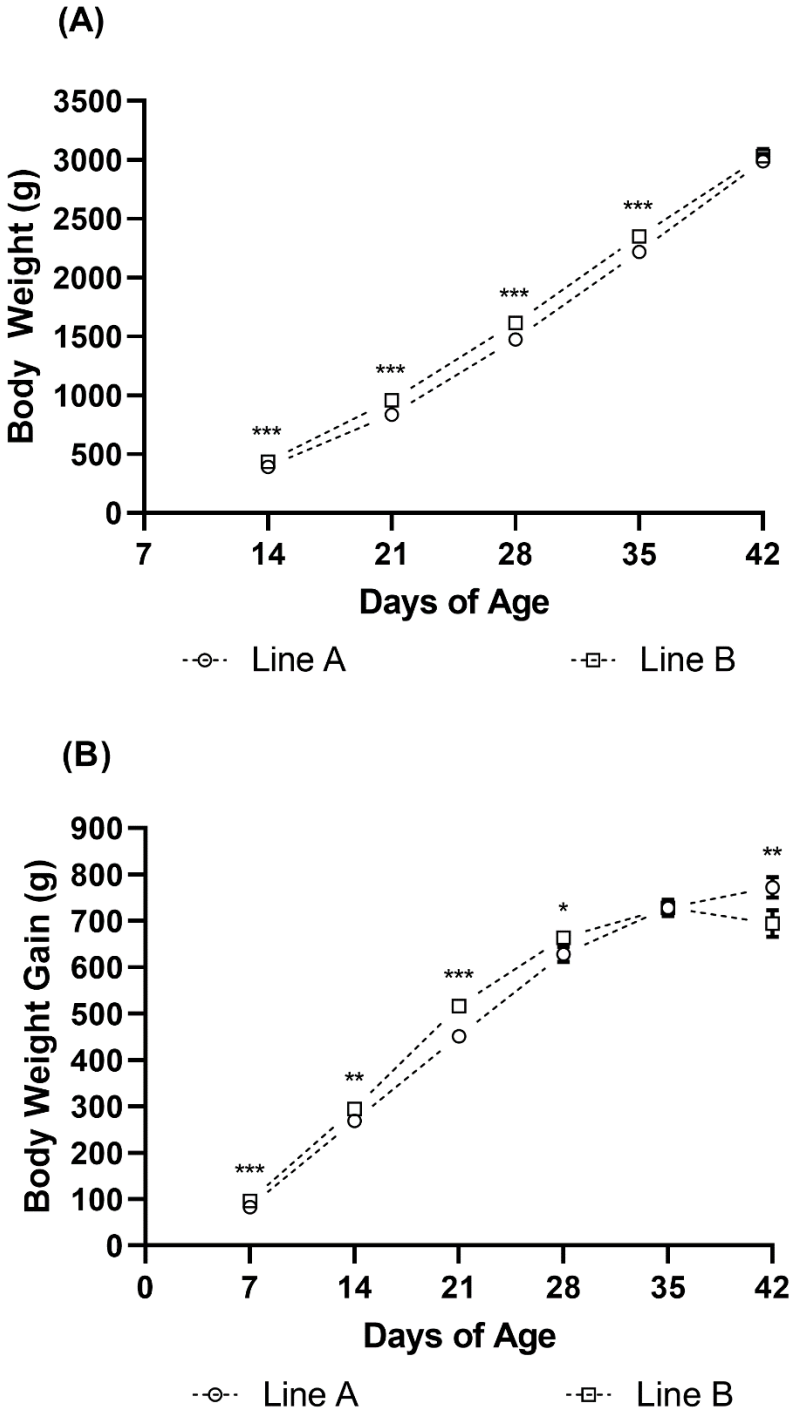

Fig. 2. Weekly bwt **(A)** from d 14 – 42 and bwt gain **(B)** from d 7 – 42 of chicken meat birds hatched from two genetic lines of breeder hens (Line A and Line B). Progeny were reared under identical conditions and fed a standard poultry starter and finisher diet. Values are mean ± SEM. Significant differences indicated by * ($P < 0.05$), ** ($P < 0.01$) and, *** ($P < 0.001$).

No line x diet interaction was observed in relation to progeny FCR at any age following full factorial analysis. Progeny hatched from Line B hens exhibited reductions in FCR from d 7-14, (Line A – 1.25 ± 0.01, Line B – 1.19 ± 0.02, $P = 0.009$) and d 14-21, (Line A – 1.31 ± 0.02, Line B – 1.24 ± 0.02, $P = 0.041$), however no difference was observed from d 21 days onwards. Total FCR throughout the whole trial period did not differ between line (Line A - 1.43 ± 0.02, Line B – 1.40 ± 0.03, $P = 0.467$) or diet (Control – 1.41 ± 0.03, *SC* – 1.43 ± 0.02, $P = 0.597$). Additionally, no line by diet interaction was identified in relation to total FCR ($P = 0.705$).

4.4.7 Body composition

A sex x line interaction was present for total bone mineral content **(BMC) (Fig. 3.A)** ($P = 0.007$), where BMC was higher in Line B male progeny. BMC did not differ between sexes in Line A hatched progeny. A further sex x line interaction was present in relation to total fat mass **(Fig. 3.B)** ($P = 0.036$), where Line A hatched male progeny exhibited reduced total fat mass, whilst an increase in total lean mass was observed **(Fig. 3.C)** ($P = 0.032$). No such differences were identified between progeny hatched from Line B hens. Line A hatched progeny exhibited greater BM yield to that of Line B ($P < 0.001$) **(Fig. 3.D)**, whilst BM yield was increased in female progeny of both lines ($P = 0.005$). No 3-way interaction was identified in relation to any BC measure following full factorial analysis. Maternal *SC* supplementation from 23-32 weeks failed to influence any progeny BC measure.

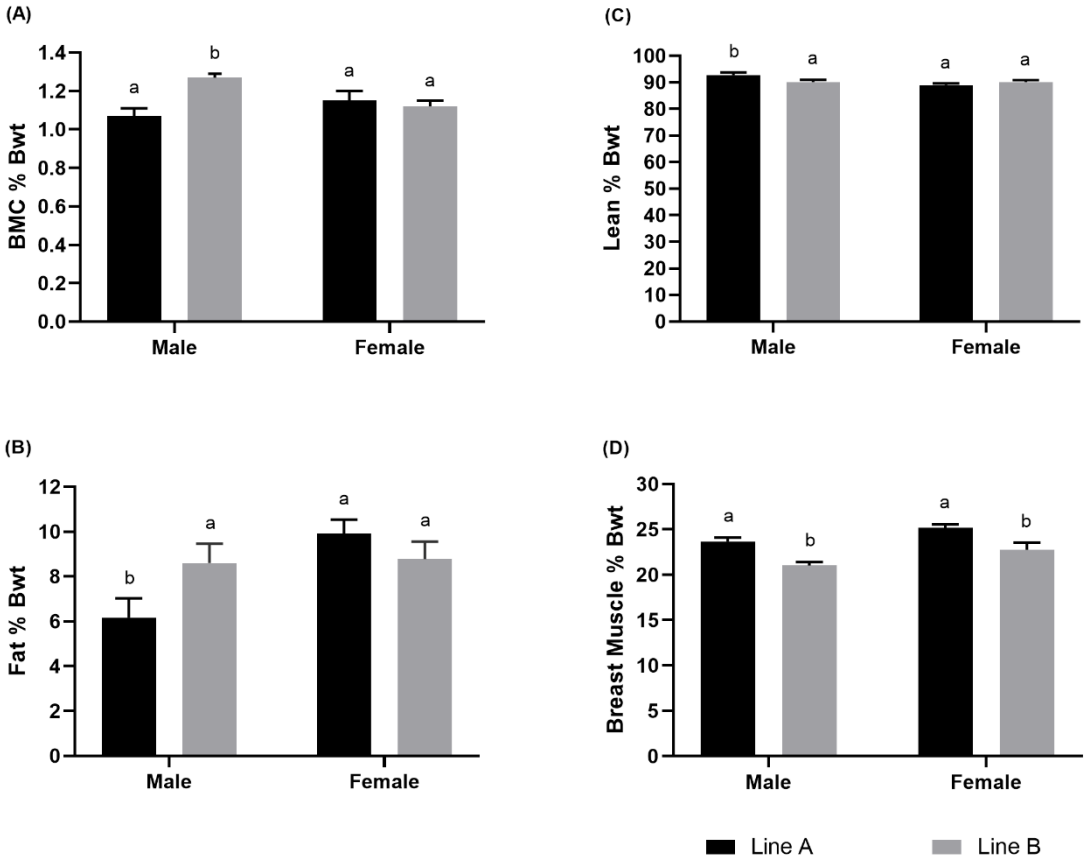

Fig. 3. Day 42 body composition of both male and female chicken meat birds hatched from two lines of breeder birds (Line A and Line B). Progeny were reared under identical conditions and fed a standard poultry starter and finisher diet. Body composition was analysed via DEXA scan, measurements recorded were for **(A)** bone mineral content (BMC), **(B)** total fat mass and **(C)** total lean mass. BM yield **(D)** was measured from a subset of birds humanely culled at d 42. [a-b] Different superscripts indicate significant (< 0.05) differences. Values are mean (% bwt) ± SEM.

4.4.8 Plasma & feather corticosterone

Maternal *SC* did not influence progeny plasma corticosterone concentrations, independent of line, at d 21 or d 37. At d 42, a line x sex effect was identified (P = 0.025), with plasma CORT concentrations higher in Line A male progeny, compared to Line B male progeny **(Fig. 4.A)**. No difference in d 42 plasma CORT concentration was observed between female progeny

hatched from Line A or B hens, although concentrations in Line A hatched birds were higher. A line x diet interaction was also identified at d 42 (P = 0.023), whereby progeny from Line B *SC* supplemented hens had reduced plasma CORT to that of progeny hatched from Line A control fed birds **(Fig. 4.B)**. Progeny plasma CORT concentrations were higher in Line A birds at d 21 (Line A – 17.48 ± 1.76 ng/mL, Line B – 13.48 ± 1.53 ng/mL, P = 0.036), and d 37 (Line A – 9.67 ± 1.65 ng/mL, Line B – 3.56 ± 0.76 ng/mL, P < 0.001). Maternal diet did not influence feather CORT concentrations, although CORT concentrations were higher in *SC* supplemented birds (Control – 15.79 ± 1.73 pg/mL, *SC* – 24.30 ± 4.28 pg/mL, P = 0.612). Feather CORT concentration did not differ between Line A and Line B hatched progeny (P = 0.909). No 2-way, nor 3-way interactions were identified following non-parametric analysis.

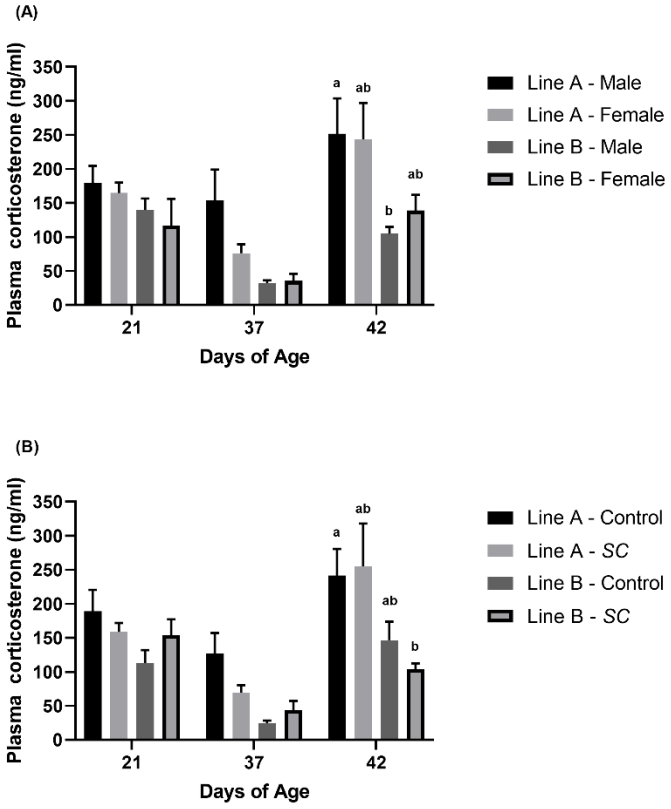

Fig. 4. Plasma corticosterone concentration at 21, 37 and 42 days of age of both male and female **(A)** chicken meat birds hatched from two genetic lines of breeder hens (Line A and Line B) fed a control or *SC* supplemented

diet **(B)**. Progeny were reared under identical conditions and fed a standard poultry starter and finisher diet. Values are mean ± SEM. [a-b] mean values with different superscripts are significantly different ($P < 0.05$).

4.4.9 Yolk & organ weights

Yolk mass as a percentage of egg weight at ED 15 differed between line and diet factors independently. Yolk mass was increased in Line B progeny (25.49 g ± 0.57) compared to Line A progeny (22.06 g ± 0.67, $P = < 0.001$), whilst eggs from control fed hens recorded higher yolk mass (24.84 g ± 0.67) compared to those from *SC* fed hens (22.89 g ± 0.73, $P = 0.023$). Total egg weight did not differ between lines, nor diet, with no interaction identified. Liver weight (% bwt) was significantly increased in progeny from control fed hens at ED 15, whilst liver weights only differed in a line dependent manner at d 22 ($P = 0.011$) and d 42 ($P = 0.007$), with increased liver weights in Line B progeny. Both heart weight (% bwt) and spleen weight (% bwt) did not differ between line and diet at any time point, nor were any interactions observed following full factorial analysis. At d 22, brain weight (% bwt) was increased in Line A progeny ($P = 0.006$). At d 42, a 3-way interaction was observed for brain weight (% bwt) for the factors of line, diet and sex ($P = 0.010$), where female progeny from Line A hens fed *SC* recorded higher brain weights to all other treatments.

4.5 Discussion

In the present study, maternal supplementation from 23-32 weeks with an *SC* metabolite did not significantly influence progeny growth, body composition or feed efficiency. However maternal *SC* provision appeared to increase feather CORT concentrations in progeny, and influenced progeny plasma CORT through a line by diet interaction at d 42. Additionally,

performance measures, including growth rate and body composition, as well as plasma CORT concentrations, did differ between progeny hatched from separate broiler breeder lines.

This study, to the best of our knowledge, is the first to investigate the effects of *SC* metabolite supplementation on baseline stress biomarkers in broiler breeders raised in a commercial setting. The provision of *SC* to breeder diets appeared to increase plasma concentrations of CORT in supplemented hens. In fact, the addition of *SC* elevated plasma CORT concentrations to a level previously documented in feed restricted breeder hens (Hynd *et al.* 2016), whilst control bird plasma CORT concentrations were within recorded baseline levels (Najafi *et al.* 2015; Arrazola *et al.* 2019). Therefore, the provision of *SC* to breeder hens may directly inflate some form of stress response in supplemented birds leading to an increase in plasma CORT concentration. Alternatively, *SC* could trigger an alteration to the regulation of metabolic events within the hen, which in turn can lead to elevated CORT concentrations (de Jong *et al.* 2003). Additionally, *SC* is documented to influence serum lysozyme concentrations when fed to poultry, as well as the humoral immune response upon exposure to several pathogens (El-Husseiny *et al.* 2008; Gao *et al.* 2008). This suggests a potential immunomodulatory effect on the intestine, which could inflate/subdue any corresponding stress response (Nelson *et al.* 2018; Price *et al.* 2018).

However, these findings differ from previous work, where plasma CORT concentration and H/L ratios were reduced in *SC* supplemented commercial birds exposed to standard production conditions, as well as stressors including heat stress, the reuse of old litter and live coccidiosis vaccination (Nelson *et al.* 2018; Price *et al.* 2018). The studies by Price *et.al.* (2018) and Nelson *et al.* (2018) supplemented *SC* to commercial broiler chickens, whereas our study provided *SC* to feed restricted GGP breeder hens. Therefore the effectiveness of *SC* metabolites to reduce stress in chicken meat birds may be influenced by stressor type, severity and duration (i.e. chronic feed restriction vs acute heat stress) (Henriksen *et al.* 2011b). The inability of *SC*

to reduce both plasma CORT and H/L ratio in the current study may also arise through age or genetic related differences in the activation/regulation of a stress response between GGP and commercial birds (Tallentire *et al.* 2016). Additionally, differences in mRNA expression of key genes involved in the avian stress response have been identified between broilers and layers (Khan *et al.* 2015). Whether such differences are present between GGP and commercial chicken meat birds is unclear, but provides a potential explanation to the differing effects of *SC* on stress biomarkers between genetic lines of chicken meat birds. Future studies may investigate the effects of feed restriction severity on the effectiveness of *SC* to mediate a stress response in broiler breeders. Additionally, future studies may also incorporate feeding *SC* metabolites to both breeder birds and their progeny to investigate whether the stress response in subsequent generations differs in response to *SC* supplementation, and whether differences (if any) are consistent between lines.

Although the addition of *SC* to breeder diets appeared to influence stress biomarkers, yolk concentrations of both CORT and testosterone did not significantly differ in a diet dependent manner. Additionally, no significant differences in yolk hormonal contents were observed between eggs produced from either Line A or B hens. However eggs produced from Line B hens did contain increased yolk concentrations of CORT, suggesting either a difference in breeder hen stress response or basal plasma CORT concentrations between genetic lines. However, these results should be taken with caution, as the yolk concentrations of CORT are substantially higher than those previously recorded. The reason for such a dramatic increase in yolk CORT concentration is not clear, although breed, strain (Peixoto *et al.* 2020a) and breeder management factors (Rao *et al.* 2009) can all influence maternal hormone transfer. However, the radioimmunoassay utilised to measure yolk CORT and testosterone concentrations in this study should be considered as another influencing factor, as yolk hormone concentrations can differ significantly dependent on the methodology used to measure such analytes (Groothuis *et*

al. 2019). Nonetheless, the ability to influence the contents deposited into the egg may differ between lines, providing a potential explanation as to why yolk hormone contents differ between lines of breeder hens relative to one another.

The *in-ovo* environment encountered during development is an integral component in generating the phenotypes displayed during adult life in avian species (Ho *et al.* 2011). Although *SC* did not influence yolk CORT concentrations in this study, further work is required to investigate its usefulness in response to breeder flock age, feed restriction severity and other factors influencing the breeder environment. Such work is of importance, as exposure to maternal stressors has previously been shown to increase yolk CORT concentrations (Hayward and Wingfield 2004; Almasi *et al.* 2012). Even though the provision of *SC* appeared to increase plasma CORT concentrations in both lines of breeders, this did not coincide with an increase in yolk CORT concentrations. This suggests that although *SC* supplementation appears to influence baseline CORT concentrations in broiler breeders, such effects are not readily transferred to the egg in the form of CORT. Instead, the effects of elevated CORT in breeder hens may induce phenotypic variation in progeny via other hormones, such as testosterone, an androgen in which CORT is well documented to interact with (Brown and Spencer 2013).

The concentrations of yolk testosterone in the present study, similar to yolk CORT concentrations, were substantially higher than those previously reported in broiler chickens (Ho *et al.* 2011). This again is likely a result of the methodology used to measure testosterone concentrations within the yolk, although like CORT, yolk testosterone concentrations can differ dependent on breed, strain (Peixoto *et al.* 2020a), and management factors (Rao *et al.* 2009). Nonetheless, the addition of *SC* to breeder diets was hypothesised to increase yolk testosterone concentration (Henriksen *et al.* 2011a), as plasma CORT concentrations in birds supplemented with *SC* are reported to reduce. Increased plasma CORT concentration reportedly reduces yolk testosterone concentrations (Henriksen *et al.* 2011a), which, although

not significant, was observed in *SC* supplemented birds. The reduction in yolk testosterone concentrations may result from the reported interaction between the HPA axis and the hypothalamic pituitary gonadal axis, where glucocorticoids inhibit gonadotropin releasing hormone and androgen hormone synthesis (Brown and Spencer 2013). Thus, a decrease in yolk testosterone, in response to elevated maternal CORT, would likely contribute to reductions in performance measures in subsequent progeny, as testosterone is essential for musculoskeletal development (Akmal *et al.* 2019). Such improvements in performance characteristics were not identified in progeny produced from control fed hens, where yolk testosterone concentrations were increased at ED 0. Therefore, it remains unclear as to whether maternal *SC* supplementation influenced progeny production parameters through changes in yolk hormone concentrations in the present study.

Although concentrations of yolk testosterone within the egg appeared to be influenced by diet, and agreed with breeder hen plasma CORT concentrations, no variations in progeny performance was identified. However, the apparent differences in yolk testosterone concentrations in eggs produced from control and *SC* supplemented hens may not have been biologically significant enough to induce a phenotypic response in the subsequently hatched progeny. Maternal *SC* provision has previously been documented to improve production characteristics in broiler progeny produced from supplemented hens (Kidd *et al.* 2013; Araujo *et al.* 2018). These findings however appear to be age dependent, with no or minimal effects identified in birds hatched from 32-35 week old hens. However, progeny hatched from 39 to 45 week old hens supplemented with an *SC* metabolite displayed improvements in both FCR and BM yield (Kidd *et al.* 2013; Araujo *et al.* 2018), suggesting a breeder age effect. Therefore the lack of variation in performance measures in progeny hatched from *SC* supplemented hens in the present study coincides with those from Kidd *et al.* (2013); Araujo *et al.* (2018), as progeny were hatched from 32 week old hens. Additionally, although progeny bwt (d 7-35)

and bwt gain (d 7-28) differed in a line dependent manner, these differences are likely of genetic origin (Jia *et al.* 2018), or result from an interaction between the embryonic genome and the *in-ovo* environment encountered (Peixoto *et al.* 2020a; Peixoto *et al.* 2020b). Alterations to the *in-ovo* environment, particularly the hormonal composition of the egg are of particular intrigue, as previous work has identified an age effect in relation to egg hormone and nutrient composition (Sudo *et al.* 2004; Veiga-Fernandes and Pachnis 2017).

Breeder flock age may also explain why FCR was not reduced in progeny hatched from *SC* supplemented hens in our study, as were observed in the studies by Kidd *et al.* (2013); Araujo *et al.* (2018). Instead, birds hatched from Line B hens recorded reduced FCR from d 7-21 to that of Line A birds, which coincided with increased bwt gain and total bwt, and again is likely a result of metabolic differences between lines of chicken meat birds. Additionally, the studies by Kidd *et al.* (2013); Araujo *et al.* (2018) utilised single sex pens, whereas the present study utilised mixed sex pens as an indicator of performance. Nonetheless, the physiological mechanisms influenced by the provision of maternal *SC* which account for the differences in progeny FCR between studies still remains unclear. Yeast cultured products, including *SC*, are documented to improve gastrointestinal maturation (de los Santos *et al.* 2007) when provided to chickens, allowing for improvements in gastrointestinal health, absorption capacity and nutrient utilisation (Sun *et al.* 2020). Therefore, further studies are required, which should incorporate larger scale trials where single sex pens are used, allowing for robust FCR data to be produced to better understand the effects of maternal *SC* supplementation in broiler breeders.

Compared to growth and feed efficiency traits, little work has investigated the interactions between progeny BC and the breeder environment, particularly in avian species. In the present study, providing *SC* to broiler breeders did not influence any BC measure analysed in 42 day old birds. Instead BC appeared to be influenced by line and sex, whereby

Line A hatched male progeny exhibited greater total lean mass and reduced total fat mass compared to Line A and B females, as well as Line B males. Female chicken meat birds do exhibit increased fat content compared to males (Zhao *et al.* 2015), and thus, the lack of variation between sexes of Line B hatched progeny is unexpected. Furthermore, although plasma CORT concentrations were higher in Line A male progeny, this is unlikely to be a contributing factor to the noted differences in BC between breeds. This is primarily due to CORT acting in an inhibitory manner in regards protein anabolism, where increased exposure instead promotes fat lipogenesis and subsequent fat accumulation (Dong *et al.* 2007; Cai *et al.* 2009). Instead, the variations in BC could be attributed to breed differences in metabolic rate, as has previously been identified between breeds of chickens (Buzała *et al.* 2015), or alterations to the *in-ovo* environment encountered, which also differs between breeds (Ho *et al.* 2011), and has recently been shown to influence BC measures in chicken meat birds (Angove *et al.* 2021). Even so, the lack of variation in BC measures between progeny produced from control or *SC* supplemented hens is somewhat surprising considering the studies by Kidd *et al.* (2013); Araujo *et al.* (2018) identified significant improvements in BM yield. The current study identified no such differences in BM yield, nor were any differences in total lean mass, nor fat mass identified between control and *SC* fed birds. The differences in BM yield between studies may again be in relation to breeder flock age, although further studies are required to understand how supplementing *SC* to breeder hens can alter progeny body composition measures in chicken meat birds, and how breeder flock age influences such parameters.

Studies investigating the effects of the breeder hen environment have also reported alterations in plasma CORT concentrations and modified expression of HPA regulating genes in subsequent progeny (Hayward *et al.* 2006; Janczak *et al.* 2007; Ahmed *et al.* 2014; Wilsterman *et al.* 2015). The present study identified a diet x line interaction in plasma CORT concentrations at d 42 between progeny hatched from Line A control and Line B *SC*

supplemented hens. The reasons for the increase in plasma CORT concentrations in progeny hatched from Line A controls hens, at d 42 only, is not clear. However the noted differences may be a result of increased variability in the plasma CORT concentrations of Line A control and Line A *SC* progeny, as CORT concentrations overall appeared to still be increased in Line A progeny, compared to that of Line B. Furthermore, no diet x line interactions were identified at both d 21 and d 37, where plasma CORT concentrations were instead significantly increased in Line A progeny compared to that of Line B. Previous studies have reported differences in plasma hormone concentrations between lines of broiler chickens, including the thyroid hormones (Tona *et al.* 2004). However, to the best of our knowledge, this study is the first to highlight such differences between progeny hatched from two lines of meat chicken regarding plasma CORT concentrations. Studies do suggest that plasma CORT concentrations differ between breeds (Ericsson and Jensen 2016). For instance, CORT levels tend to be higher in commercial layers in response to a stressor compared to commercial meat birds (Saito *et al.* 2005), however this may be influenced by separate factors, including metabolic stress (de Jong *et al.* 2003), bird age and management protocols (Scanes 2016). Additionally, plasma CORT provides an instantaneous measure of stress at a single point in time, and can be influenced by a range of factors including time of day, collection time and collection technique (Fairhurst *et al.* 2013). Feather CORT has been touted to provide a better representation of CORT exposure over a prolonged period of time (Freeman and Newman 2018). Although feather CORT concentrations did not differ significantly in this study, concentrations were elevated in progeny from both lines of *SC* supplemented hens. Such an increase agrees with the elevation in plasma CORT identified in *SC* supplemented breeder hens, suggesting that maternal supplementation with *SC* may influence baseline CORT exposure in the same manner in both hen and progeny. However, a more consistent approach is required to better understand the influence of maternal *SC* on breeder hen CORT concentrations, and whether such alterations

are transferred between generations. Nonetheless, the provision of *SC* to both Line A and B hens failed to influence plasma and feather CORT concentrations in subsequent progeny in a manner that was biologically relevant to growth and performance.

4.6 Conclusion

The findings from this study suggest supplementing breeder hens with *SC* metabolites did not influence progeny growth rates and BC when hatched from 32 week old GGP hens. Future work analysing the effects of hen age on progeny performance, and the effects *SC* supplementation has on such characteristics may provide a novel approach to improve progeny performance in birds hatched from aging flocks. Additionally, the variations in growth rate and BC, as well as plasma CORT in birds hatched from separate lines of breeder hen are likely of genetic origin, but may also be attributed to interactions between embryonic genotype and the *in-ovo* environment encountered. Therefore, breeder hen nutrition may provide a novel approach to improve production output and flock uniformity in the chicken meat industry if an effective and accessible additive can be identified. This is largely due to the potential transgenerational transfer of performance improvements between breeder lines within the pedigree breeding structure. Thus further work is required to understand whether performance outcomes induced through altered breeder nutrition are consistent in progeny produced at various stages of the production cycle (i.e. great grandparent vs grandparent vs parent lines).

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Chapter 5:

In-ovo corticosterone exposure does not influence yolk steroid hormone relative abundance or skeletal muscle development in the embryonic chicken.

Statement of Authorship

Statement of Authorship

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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 to 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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Chapter Introduction

Considering carcass quality is an important performance measure in chicken meat production, it is surprising that little attention has focused on the effects of the maternal environment on such measures. The findings from both chapters three and four highlighted significant differences in body composition measures in response to alterations to the *in-ovo* environment, as well as between progeny hatched from separate lines of broiler breeder hens. Although these findings suggest the maternal/*in-ovo* environment encountered does influence progeny body composition, the contributing physiological mechanisms are not clear.

As previously discussed, maternally derived alterations in progeny performance across avian species is likely mediated through endocrine or nutritional alterations to the *in-ovo* environment. Significant work has assessed the effects of yolk steroid hormones on post-hatch phenotypes in birds, primarily due to the wide range of phenotypes displayed in response to such alterations. Sex steroids, particularly androgens, are an essential component in both embryonic and post-hatch musculoskeletal development, as they regulate muscle fibre development, as well as adult myoblast development, an essential component for post-hatch muscle hypertrophy. In addition, early exposure to glucocorticoids hormones, such as corticosterone, has the potential to directly influence body composition phenotypes, as well as mediate such alterations through well documented interactions with the sex steroids. In fact, a doctorate of veterinary medicine project conducted by Mr. Mitchell Crago in 2019 (See Appendix 2) measured yolk testosterone concentrations at embryonic day 15 in both corticosterone and control treated birds sourced from the work presented in Chapter 3. In this project, embryonic exposure to corticosterone appeared to influence yolk testosterone concentrations in a sex dependent manner. Therefore, the aim of Chapter 5 was to investigate whether *in-ovo* exposure to corticosterone influenced early musculoskeletal development in the chicken, as well as the relative abundance of various yolk steroids throughout embryonic

development. Additionally, this study utilised eggs collected from a parent line of breeder hens, where the subsequent progeny represent commercial broiler chickens raised for human consumption, instead of the previously utilised great grandparent lineages.

***In-ovo* corticosterone exposure does not influence yolk steroid hormone relative abundance or skeletal muscle development in the embryonic chicken.**

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5.1 Abstract

The *in-ovo* environment encountered by developing embryos can directly affect post-hatch phenotypes, and is likely influenced by maternal factors, including stress. *In-ovo* exposure to corticosterone (CORT) reportedly reduces growth rates in wild birds and production poultry, whilst altering body composition in meat chickens. However, the mechanisms governing alterations in growth rate and body composition are not clear, but could involve myogenic commitment of mesenchymal stem cells, and/or potentially through the actions of yolk steroid hormones. Therefore, this study aimed to investigate whether *in-ovo* CORT exposure influenced yolk steroid hormone content, as well as early myogenic development in meat chickens.

Fertile eggs (n = 550) were divided between two groups and administered a CORT or control (CON) solution at embryonic day (ED) 11. At ED 0 and ED 5, baseline yolk samples were collected from 40 eggs (n = 20/time point). At ED 15 and hatch, 84 embryos (n = 42/treatment) were humanely killed, where yolk, breast muscle (BM), and liver samples were collected. Yolk

lipid content and the relative abundance of 15 steroid hormones within the yolk were measured at ED 0 (n =10), ED 5 (n = 10), ED 15 (n = 40) and ED 21 (n = 40). Additionally, muscle fibre number (MFN), cross sectional area (CSA) and fascicle area occupied by muscle fibres were measured in left (n = 40) and right (n = 40) BM sections obtained at hatch. Furthermore, relative mRNA expression of the *MyoD, MyoG, Pax7, PPARγ* and *CEBP/β* genes, along with the androgen, progesterone and estrogen receptors, was measured in hatch BM samples (n = 40).

Exposure to *in-ovo* CORT reduced fascicle area occupied by muscle fibres at hatch ($P < 0.001$), but failed to influence MFN ($P = 0.774$), CSA ($P = 0.826$) or hatch weight ($P = 0.406$). Relative mRNA expression of *CEBP/β* was increased in CORT treated birds at hatch ($P = 0.040$). CORT exposure increased the relative abundance of etiocholanolone glucuronide in CON females compared to CORT males at hatch ($P = 0.035$). Yolk lipid content was reduced in CORT exposed birds at hatch ($P = 0.062$).

These findings suggest *in-ovo* CORT exposure does not influence early muscle development through the actions of yolk steroid hormones, but may instead promote adipogenesis in meat chickens. A further understanding of such pathways will allow manipulations to be implemented to current production strategies to further improve progeny performance.

Keywords: poultry, androgens, yolk fat, developmental programming, stress, body composition

5.2 Introduction

The phenotypic traits displayed by offspring as they transition into adulthood largely results from a complex interaction between genetic and maternal factors encountered during embryonic development (Groothuis et al., 2019;Peixoto et al., 2020a;Peixoto et al., 2020b). These maternal factors are a result of the environment encountered by a mother at the time of

gestation, or in oviparous species, egg formation. In avian species, maternally derived differences in offspring phenotypes are likely mediated through hormonal or nutritional alterations to the composition of the egg at the time it is produced (Henriksen et al., 2011b;Angove and Forder, 2020). A range of factors can influence the composition of a subsequently produced egg, including but not limited to, environmental chemicals, nutritional status and stress (Sheriff et al., 2017;Gatford et al., 2018;Angove and Forder, 2020).

Maternal stress itself has been the focus of a significant number of studies, both in wild birds (Love et al., 2013;Berghänel et al., 2017;Sheriff et al., 2017) and production poultry (Henriksen et al., 2013;Bowling et al., 2018;Peixoto et al., 2020a;Peixoto et al., 2020b). However, the ramifications of maternal stress on subsequent progeny performance has not been investigated to the same extent, especially in production species. More recent studies have highlighted the impact of both maternal and embryonic stress on growth and bodyweight **(bwt)** measures in commercial poultry. Increasing feed restriction severity in breeder hens elevated maternal plasma corticosterone **(CORT)** concentrations and led to reductions in male progeny bwt (Bowling et al., 2018), whilst *in-ovo* exposure to CORT reduced bwt in commercial layers from three weeks of age (Ahmed et al., 2014b). Although these studies highlight the ability of maternal/*in-ovo* stress to influence growth in commercial poultry, whether or not such alterations in growth were in response to altered fat mass, lean mass or originate from another biological source, remains unclear. Recent findings have shown that *in-ovo* exposure to CORT at embryonic day **(ED)** 11 led to an increase in total fat mass, accompanied with a decrease in total lean mass, in 35 day old female commercial meat chickens (Angove et al., 2020). These findings suggest alterations to adult body composition may be achieved through manipulations of the *in-ovo* environment during critical developmental time points. In fact essential components for post-hatch musculoskeletal growth form during the period of embryonic

development (Halevy et al., 2006), and thus alterations to the *in-ovo* environment has the potential to influence post-hatch body composition.

Muscle fibre development occurs embryonically in the chicken, where muscle fibre number is set by the time of hatching (Smith, 1963;Halevy, 2020). Muscle fibres are derived from the commitment of multipotent progenitor stem cells to embryonic myoblasts which then proliferate and differentiate to form multinucleated myofibres (Herbst and Bhasin, 2004;Velleman, 2007). Thus, the period in which progenitor cells commit, and embryonic myoblasts differentiate may provide a critical time frame in which alterations to the *in-ovo* environment can influence post-hatch body composition by altering the total number of muscle fibres. Post hatch muscle growth is due to hypertrophy, however myonuclei do not undergo cellular division, and thus external nuclei are required to facilitate hypertrophy mechanisms (Moss and Leblond, 1971). The predominant source of external nuclei are from a quiescent population of adult myoblast cells, termed satellite cells **(SC)**, which are detectable from ED 13 in the chicken (Halevy et al., 2006). SC numbers peak around 2-3 days post hatch, after which SC's become quiescent and remain so until required to initiate muscle growth or repair (Halevy et al., 2006). However, the period in which SC's proliferate and differentiate, thought to be up until d 7 post hatch (Halevy et al., 2006), is likely to influence post-hatch muscle growth, as this period is essential in determining the quiescent SC population available for muscle hypertrophy.

The state (i.e. proliferation, differentiation, quiescent) of SC's is under tight myogenic regulation, primarily from the muscle-specific basic helix-loop-helix family of transcription factors, including the myoblast determination protein 1 **(*MyoD*)** and myogenin **(*MyoG*)** genes (Olson, 1990). *MyoD* is primarily expressed in proliferating SC's with *MyoD* expression peaking at hatch in the chicken (Halevy et al., 2004). *MyoG* expression peaks at 3 days post hatch, coinciding with SC differentiation (Halevy et al., 2004). Furthermore, the paired box

protein Pax-7 **(Pax7)** gene is documented to be involved in SC mediated skeletal muscle development, with expression levels coinciding with the presence of quiescent SC's (Halevy et al., 2004). Thus alterations to the expression of myogenic regulator genes as a result of exposure to *in-ovo* stress throughout development may influence post-hatch body composition traits.

Glucocorticoid hormones, including CORT, reportedly regulate the differentiation of mesenchymal stem cells to the various cell lineages (Salloum et al., 2013), and the proliferation and differentiation of SC's during their active phase (Salloum et al., 2013). Additionally, glucocorticoid exposure reportedly increases expression of the adipogenic regulator genes peroxisome proliferator-activated receptor gamma **(*PPARγ*)** and CCAAT/enhancer binding protein beta **(*CEBP/β*),** both of which promote adipogenic commitment of mesenchymal stem cells (Otto and Lane, 2005). Thus, alterations to the concentrations of glucocorticoids within the *in-ovo* environment could shift embryonic myoblast proliferation and differentiation, potentially altering the number of muscle fibres that develop. Additionally, *in-ovo* stress may inhibit myogenic commitment of multipotent stem cells, potentially reducing the number of quiescent SC's that develops.

Alternatively, glucocorticoids interact with various other endocrine growth regulators, including gestagen (Henriksen et al., 2011a), estrogen (Henriksen et al., 2011a) and androgen hormones (Brown and Spencer, 2013). For instance, increasing maternal CORT concentrations in 33 week old layer hens decreased yolk concentrations of testosterone and progesterone in freshly laid chicken eggs (Henriksen et al., 2011a), whilst exposure to social isolation stress during the first four weeks post hatch led to a decrease in plasma testosterone in male birds at 16 weeks of age (Natt et al., 2015). Sex steroids also regulate skeletal muscle development (Li et al., 2020), where they promote increased myoblast number and myofibre size (Herbst and Bhasin, 2004;Li et al., 2020) whilst aiding the commitment of mesenchymal stem cells to the

myogenic line (Herbst and Bhasin, 2004). However, for *in-ovo* exposure to steroid hormones to influence embryonic and post-hatch muscle development, embryos must first be exposed to these hormones (Groothuis and Schwabl, 2008;von Engelhardt et al., 2009), which by nature, are highly lipophilic (Moore and Johnston, 2008). How lipophilic hormones are transferred from the lipid rich environment of the yolk, to the water-rich environment of the embryo remains unclear. Recent evidence suggests steroids are metabolised early during embryogenesis into inactive conjugates (Kumar et al., 2019;Vassallo et al., 2019). Whether these conjugates enable the transfer of steroids into the embryo, or act as a form of storage mechanism until the absorption of yolk lipids, which occurs primarily during the later stages embryogenesis (Yadgary et al., 2010), is not known.

The impact of early life stress on yolk hormone abundance and fat content during late embryonic development and the possible relationship with musculoskeletal development is also not well understood, which is surprising given the potential impact on body composition (Gao et al., 2008;Angove et al., 2021). Therefore, a study was designed to investigate the effects of *in-ovo* exposure to CORT on embryonic musculoskeletal development, and steroid yolk hormone abundance in meat chickens. Additionally, yolk lipid content was measured to assess any impact of *in-ovo* exposure to CORT. It was hypothesised that CORT would decrease the relative abundance of steroid hormones and lipid content within the yolk. It was further hypothesised that *in-ovo* exposure to CORT would inhibit myogenic development, reducing MFN at hatch and/or the expression of myogenic regulator genes, whilst increasing expression of adipogenic regulatory genes.

5.3 Materials and Methods

All animal use and experimental protocols were approved by the University of Adelaide Animal Ethics Committee (S-2020-034).

5.3.1 In-ovo Treatment

Fertile eggs (Cobb 500 parent breeder, *n* = 550) were collected from a commercial hatchery (Baiada Hatchery, Willaston), and set in an incubator as per standard incubation conditions (see below). At ED 11, eggs were randomly allocated to either a CORT, or control **(CON)** treatment. Prior to injection, eggs were candled and infertile eggs removed. CORT treated eggs (*n* = 255) were injected with 100 µL of 10mM phosphate buffer saline **(PBS)** solution containing 1 µg of CORT dissolved in absolute ethanol, while CON treated eggs (*n* = 255) were injected with 100 µL of 10mM PBS solution containing the same concentration of absolute ethanol. Immediately before injection, a 23 gauge needle was used to puncture a hole in the egg. Injections were administered into the chorioallontoic membrane, through the air sac, using a 1 mL insulin syringe. Selleys glass silicone was used to re-seal the injection site. Eggs were returned to the incubator following injection.

5.3.2 Animals and Tissue Collection

Prior to incubation, eggs were acclimatised at room temperature (23^{o}C) for 13.5 hours, then set in an incubator at 38^{o}C and 55% humidity between ED 0 and ED 18, and at 36.7^{o}C and 60% humidity from ED 18 until hatching (ED 21). Eggs were rotated 90^{o} hourly until ED 18, where eggs were placed into hatching trays. At ED 0, a sub sample of eggs (*n* = 20) were opened, egg quality measurements were taken and yolk samples obtained. Whole yolks were separated from

egg whites, weighed and homogenised, after which homogenates were aliquoted, snap frozen in liquid nitrogen and stored at -20^{o}C until further analysis.

At ED 5, a further sub-sample of eggs (*n* = 20) were opened and the egg contents emptied into a sterile petri dish. The embryo was removed from the egg contents and weighed, after which the embryo was decapitated, with embryonic head and body samples snap frozen in liquid nitrogen and stored at -80^{o}C. The yolk sac membrane was weighed, snap frozen in liquid nitrogen and stored at -80^{o}C. The remaining egg contents were placed in 50mL falcon tubes and centrifuged at 3000 rpm for 10 minutes. After centrifugation, the yolk supernatant was transferred to a clean tube, snap frozen in liquid nitrogen, and stored at -80^{o}C.

At ED 15, 42 birds per treatment (*n* = 84) were removed from their eggs and humanely killed by decapitation. After euthanasia, total bwt was measured, after which the liver, yolk sac, left and right breast muscles **(BM)** were removed and weighed. Both left and right BM were horizontally sliced in half, where bottom sections were stored in 3 mL of 10% buffered formalin, and top sections placed in cryogenic tubes. A yolk sample was collected from the yolk sac after its removal from the embryo. The gender of each bird was identified to ensure equal sex distribution within treatments (*n* = 21 samples/treatment/sex). All samples, other than those stored in 10% buffered formalin, were snap frozen in liquid nitrogen and stored at -80^{o}C until further analysis.

An additional 42 birds per treatment (*n* = 84) were humanely killed at hatch (Day 0) via cervical dislocation and total bwt recorded. Liver, yolk sac, yolk, as well as left and right BM were removed, weighed and stored as described at ED 15, along with gender identification. Additionally, a blood sample was collected immediately after euthanasia via cardiac puncture.

5.3.3 Yolk Hormone Extraction

Yolk samples collected at ED 0, (*n* = 10), ED 5 (*n* = 10), ED 15 (*n* = 40; 10 per treatment/sex) and hatch (*n* = 40; 10 per treatment/sex) were thawed and homogenised in an MP BIO science Quick homogeniser (FastPrep-24 5G, Santa Ana, CA, USA) using Lysing Matrix C (MP Bio Science, #116912050-CF, Solon, OH, USA). Following homogenisation, 200 mg of yolk was weighed out in a clean 5 mL tube and 2 mL of molecular grade methanol (Sigma Aldrich, #34860, Castle Hill, New South Wales, Australia) was added. All samples were vortexed for 60 seconds, snap frozen in liquid nitrogen and stored at -20^{o}C overnight to precipitate the lipid and protein contents. The next day, frozen samples were centrifuged at 2000 rpm for 20 minutes, at 4^{o}C. The ~2 mL supernatant was transferred to a separate clean vial and stored at -20^{o}C. An additional 2 mL of methanol was added to the original homogenised yolk sample from which the supernatant was removed, snap frozen in liquid nitrogen and stored over night at -20^{o}C. The following morning, samples were centrifuged again under the same conditions as previously described, with the supernatants combined into a single vial and stored at -20^{o}C.

The methanol extracts collected were subjected to solid phase extraction **(SPE)** to separate the free and conjugated hormones. Briefly, 40 mL of MilliQ water was added to a 50 mL falcon tube, after which the 4 mL methanol extract was added. MilliQ water (6 mL) was added to the tube containing the original methanol extract, then transferred to the 50 mL falcon tube, ensuring all methanol sample was transferred. The falcon tubes were vortexed thoroughly to ensure adequate mixing. Samples were extracted using SPE cartridges (waters SEP-PAK plus tC18 environmental SPE cartridge #WAT036800) and a vacuum pump (Rocker 400, SKU 167400-11(22), Kaohsiung City, Taiwan). SPE cartridges were conditioned using 5 mL of methanol, followed by 5 mL of MilliQ water, then samples were gradually loaded into 20 mL reservoirs attached to the SPE cartridge. The eluted solvent was discarded. After the samples

were loaded onto the cartridge, 5 mL of 2% methanol in MilliQ water was added to the reservoir to wash the cartridge and remove possible interfering compounds still linked to the SPE phase. The cartridge was then left to dry for 5 minutes using the vacuum pump (10 mmHg). The free hormones were eluted from the cartridge using 5 mL of diethyl ether (Chem-Supply, #EA012, Gillman, SA, Australia) and collected in a 20 mL glass test tube. Conjugated hormones were eluted from the cartridge using 5 mL of methanol, with the eluent collected in a separate glass test tube. Following eluent collection, all samples were dried down in a nitrogen evaporator (Biotage, TurboVap LV #415000, Uppsala, Sweden), using a nitrogen flow of 3.5 mL/min and a temperature of 30ºC. Samples were reconstituted with methanol (100uL) and dichloromethane (100uL) and vortexed for 4 seconds. The reconstituted samples were transferred into a 4 mL Eppendorf tube and stored at 3ºC for 10 minutes to allow precipitation of possible proteins still present. Each sample was then centrifuged for 10 minutes at 14000 rpm, the supernatant collected and transferred to a HPLC vial fitted with a 200 µl glass insert. Samples were stored at 4ºC until further analysis.

5.3.4 Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS)

LC-MS/MS analyses were performed at the Metabolomics Australia/ Australian Wine Research Institute using an Agilent 1290 infinity II HPLC coupled to AB Sciex 4500 QQQ instrument. Data was acquired using multiple reaction monitoring **(MRM)** in positive ionization mode for free hormones and negative ionisation mode for conjugated hormones. Briefly, the liquid chromatography **(LC)** separation was performed on a Phenomenex Phenyl Hexyl column (2.1 x 150mm, 3µm) with mobile phase A (0.1% formic acid, 0.5% methanol MilliQ water) and mobile phase B (0.1% formic acid in acetonitrile). For both free and conjugated fractions, the flow rate was 0.4 mL/min. The linear gradient was as follows: 0 min:

0% B, 5 min: 20% B, 10 min: 30% B, 15 min: 50% B, 20 min: 70% B, 25 min: 95% B, 27.5 min: 10% B, 28 min: 90% B, 28.5 min: 10% B. For the free hormone fraction, column temperature was set at 40°C, source temperature was 450°C and the injection volume was 10 µL. The curtain gas, ion source gas 1 and ion source gas 2 were 10, 40 and 50 PSI respectively with an ion spray voltage of 5500 V. For the conjugated fraction, column temperature was set at 40°C, source temperature was 500°C and the injection volume was 10 µL. The curtain gas, ion source gas 1 and ion source gas 2 were 35, 50 and 65 PSI respectively with an ion spay voltage of 5500 V. Mass spectrometry parameters were optimised based on 3 standards, testosterone (Sigma-Aldrich, #86500, Castle Hill, New South Wales, Australia) CORT (Sigma-Aldrich, #27840, Castle Hill, New South Wales, Australia) and etiocholanolone glucuronide (Kinesis, D607, Redland Bay, Queensland, Australia).

For the free hormone fraction analysis, 15 metabolite compounds of the sex steroid targets testosterone, progesterone and estradiol, as well as corticosterone were targeted **(Table 1)**. Two MRM transitions "Precursor Ion -> Product Ion" were recorded for each compound, except for estrone where only one MRM transition was monitored. The MRM transition of "precursor ion" to "quantifier ion" was used to record compound intensity. The MRM transition of "precursor ion" to "qualifier ion" was monitored to increase the confidence of the putative identification. For the analytical reference standards testosterone, corticosterone and etiocholanolone glucuronide, the mass spectrometer was optimised to achieve maximum sensitivity for the compounds characteristic MRM transitions. Where pure analytical standards were not available, MRM transitions monitored were determined based on previous studies described in the literature and mass spectroscopy/mass spectroscopy spectra present in online databases (m/z Cloud). Where differing isomers of separate compounds shared a similar formula and molecular structure (11β-hydroxy-progesterone/11-deoxycorticosterone), the same transitions were monitored and could not be separated. The compounds 3α

androstanediol, 3β androstanediol, 5α dihydrotestosterone, 5β dihydrotestosterone and androsterone could not be detected at any time point using the previously described methodology and where therefore excluded from the study.

Table 1. MRM acquisition – scan segments for target compounds.

Compounds	Precursor Ion	Quantifier Ion	Qualifier Ion	Polarity
Estrone	271	253	/	Positive
17β-Estradiol	273	213	109	Positive
Androstenedione	287	97	109	Positive
Testosterone	289	97	107	Positive
5α Dihydrotestosterone	291	255	273	Positive
5β Dihydrotestosterone	291	255	273	Positive
Androsterone	291	255	273	Positive
Etiocholanolone glucuronide	291	255	273	Positive
3α Androstanediol	293	257	275	Positive
3β Androstanediol	293	257	275	Positive
Progesterone	315	109	97	Positive
Pregnonelone	317	109	97	Positive
Corticosterone	347	329	311	Positive
11β-hydroxyprogesterone	331	295	109	Positive
11β-deoxycorticosterone	331	295	109	Positive

5.3.5 Yolk Lipid Extraction

Yolk lipids were extracted following the methods of Yadgary et al. (2010). Briefly, ED 0 (n = 10), ED 5 (n = 10), ED 15 (n = 40 per treatment/sex) and hatch (n = 40 per treatment/sex) yolk

samples were thawed, after which samples were homogenised in an MP BIO science Quick homogeniser using Lysing Matrix C. Approximately 250 mg of homogenised yolk was combined with 5 mL of a 2:1 vol/vol chloroform-methanol solution in a new tube and left at room temperature for 30 minutes. Afterwards, 2 mL of Milli Q water was added to each sample, vortexed, and incubated overnight at 4^{o}C. The next morning, the 2.5 mL bottom layer was transferred into a clean, previously weighed, test tube, then oven dried at 105 ^{o}C. Tubes with remaining lipid content were weighed, after which yolk lipid content was calculated.

5.3.6 Muscle Fibre Development

Histological evaluations were performed on 10 birds per treatment/sex (n = 40 birds) at hatch, with both left and right BM samples evaluated for each individual bird (n = 80 samples in total). Tissue specimens were dehydrated in a graded series of ethanol then orientated to ensure transverse fibre sectioning and embedded in paraffin wax. Samples were sectioned at 5µm using a microtome (Micron, #904030, Walldorf, Germany). Standard haematoxylin and eosin staining was used to visualise the muscle tissue. Sections were examined by light microscopy at x40 magnification with 5 images taken from randomly selected locations within a sample. Images were analysed for MFN, CSA and percentage of fascicle area occupied by muscle fibres using the image analysis software ImageJ, with a minimum of 300 muscle fibres analysed per individual left and right BM sample.

5.3.7 Isolation and Quantification of Total RNA from Chicken Breast Muscle.

Total RNA from n = 32 (n = 8 treatment/sex) chicken BM samples was isolated using an Rneasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturers instruction. Briefly, 50 mg of chicken BM tissue (combined left and right) was

homogenised in 900 µL of Qiazol reagent (Invitrogen, Carlsbad, CA) using the MP BIO QuickPrep tissue homogeniser, CoolPrep Adapter and lysing matrix D (MP Bio Science, #116913050-CF, Solon, OH, USA) under the following conditions, speed: 6.0 m/s, time: 40 seconds, cycles: 2 (150 second interval). Homogenates were stored at -80 ^{o}C until further analysis.

Thawed homogenates were combined with 100 µL of gDNA eliminator solution and 180 µL of chloroform (Sigma Aldrich, #C2432, Castle Hill, NSW, Australia), then vortexed. Samples were left to stand at room temperature for 3 minutes, then centrifuged at 10,000 rpm for 15 minutes at 4^{o}C. The upper aqueous phase (300 µL) was combined with 300 µL of 70% ethanol, mixed, and transferred onto the Rneasy columns. The on column wash steps were performed to manufacturer's specifications. Post wash, Rneasy columns were placed into new sleeves, and centrifuged for one minute at 14,000 rpm to remove residual wash buffer, then placed into new 2 mL collection tubes along with 50 µL of Rnase-free water and centrifuged for one minute at 10,000 rpm. A further 50 µL of Rnase-free water was added, followed by centrifugation at 10,000 rpm for one minute. RNA integrity was analysed using the RNA tape station (Agilent technologies 2200, Santa Clara, California, United States), with all RNA integrity numbers (RIN) $\geq$ 8.0. Sample purity and concentration was determined using UV spectrophotometry (NanoDrop 2000, ThermoScientific). Pure RNA samples were stored at -80^{o}C until further analysis.

5.3.8 cDNA Synthesis

RNA concentrations from chicken BM samples were normalised to 200 ng/µL using a liquid handling robotics system (EpMotion %075; Eppendorf, Hamburg, Germany). A High Capacity RNA-to-cDNA kit (#4387406, Applied Biosystems, Carlsbad, CA, USA) was used to

synthesise complementary DNA **(cDNA)** in accordance with the manufactures specifications. Reactions were incubated for 60 minutes at 37ºC, followed by a 5 minute incubation period at 95ºC to inactivate reverse transcriptase. Incubation steps were carried out using a thermomixer (Thermomixer C; Eppendorf, Hamburg, Germany). Reactions were then cooled to 4ºC and placed on ice. 1:4 dilutions of stock cDNA were created using TE buffer (#12090-015, Applied Biosystems, Carlsbad, CA, USA) and stored at -80ºC until further analysis.

5.3.9 Real-time Quantitative PCR (qPCR)

Primer sequences and references are presented in **Table 2**. A master mix was created before each assay, consisting of SYBR reagent 2x power (Applied Biosystems, #4367659, Warrington, UK) and both forward and reverse oligonucleotides. Stock cDNA (1:4) was diluted five-fold to 1:20 with TE buffer (#12090-015, Carlsbad, CA, USA) using the above mentioned robotics system. Diluted cDNA (6 µL) was combined with 19 µL of master mix, after which the cDNA/SYBR mixture (5 µL) was transferred in triplicate to a 384-well MicroAmp Plate (Applied Biosystems, Carlsbad, CA, USA). A total of n = 32 cDNA preparations were examined along with a 5-point standard curve (1:8, 1:32, 1:128, 1:512, 1:2048 dilutions of pooled cDNA and TE buffer blank), prepared by pooling a portion of 8 random cDNA samples. Quantitative PCR was performed using a real time-PCR Machine (Quant Studio 6 Flex, Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 95ºC/10min for 1 cycle, 95ºC/15s, 60ºC/20s and 72ºC/40s for 40 cycles and 95ºC/15s, 60ºC/15s and 95ºC/15s for melt curve data acquisition. Quant studio™ Real-time PCR software was used to process the data. Tata-binding protein ***(TBP)***, β-actin ***(ACTH)*** and glyceraldehyde-3-phoshate dehydrogenase ***(GAPDH)*** were chosen as reference genes. NormFinder (Andersen et al., 2004) was used to identify the optimal normalisation gene, with *TBP* identified as most stable. The Pflaffl method (Pfaffl, 2001) for relative quantification and

normalisation to reference genes was used to determine the relative expression of *Pax7*, *MyoD*, *MyoG*, androgen receptor **(*AR*)**, estrogen receptor alpha **(*ER-a*)**, progesterone receptor **(*PR*)**, *PPARγ* and *CEBP/β*, see **Table 2** for primers.

Table 2. Primers used in real-time quantitative PCR

Target Gene	Primer sequence (5'-3')	Amplicon length (bp)	Reference	Accession Number
Reference genes				
GAPDH	F: CAACCCCCAATGTCTCTGTT R: TCAGCAGCAGCCTTCACTAC	94	(Gilani et al., 2018)	NM_204305
TBP	F: GTCCACGGTGAATCTTGGTT R: GCGCAGTAGTACGTGGTTCTC	128	(Gilani et al., 2018)	NM_205103
β-actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	152	(Liu et al., 2015)	NM_205518
Steroid receptors				
AR	F: AAGAAGCTGGGCAGTCTGAAR: AGCAGGTTGGAGAAGGAGTC	214	(Li et al., 2020)	NM_001040090
ERα	F: TATTGATGATCGGCTTAGTCTGGCR: CGAGCAGCAGTAGCCAGTAGCA	145	(Liu et al., 2015)	NM_205183.2
PR	F: ATCATCGTTCTATTCACTGT R: CTCGTTCTCATCTCATCAA	168	(Liu et al., 2015)	NM_205262.1
Satellite cell development genes				
Pax7	F: AGTTCGATTAGCCGTGTGCT R: CTCTTCAAAGGCAGGTCTGG	185	(Li et al., 2020)	NM_205065
MyoD	F: ATGTCCCATACTGCCTCCAG R: GTCTTGGAGCTTGGCTGAAC	235	(Li et al., 2020)	NM_204214
MyoG	F: GGCTTTGGAGGAGAAGGACT R: CAGAGTGCTGCGTTTCAGAG	N.P	(Cazzato et al., 2014)	NM_204184
Adipocyte Differentiation genes				
PPARγ	F: CCACTGCAGGAACAGAACAA R: CTCCCGTGTCATGAATCCTT	249	(Piestun et al., 2017)	NM_001001460.1

CEBP/β	F: GCCGCCCGCCTTTAAA R: CCAAACAGTCCGCCTCGTAA	N.P	(Zhang et al., 2015)	NM_205253.2

N.P = Not provided

5.3.10 Statistical Analysis

Continuous data sets were checked for normality using the ShapiroWilk Test. Total MFN, CSA and muscle fibre (% of fascicle area) data sets obtained from both left and right BM sections were combined and analysed as a whole. Yolk hormone relative abundance was analysed for sex x treatment at ED15 and hatch only (sex and treatment were unknown and unapplied at ED0 and ED5). ED 15 17β-Estradiol, ED 15 and hatch 11β-hydroxy-progesterone/11-deoxycorticosterone and hatch etiocholanolone relative abundance data were log transformed to meet the ShapiroWilk Test requirements for normality. Normalised mRNA expression values were used in the analysis of gene expression data. Normally distributed data were subjected to a full factorial univariate analysis for the factors of sex and treatment. Additionally, dissector was added as a random factor to ensure no dissector bias was present. A generalised linear model pair-wise comparison was used to separate means between treatment groups where statistical significance was identified using the Fisher's least significant difference (LSD) adjustment for multiple comparisons. Data sets that did not meet the ShapiroWilk test requirements for normality were subjected to non-parametric Mann-Whitney U and Kruskal-Wallis tests. A non-parametric pairwise comparison was used to separate statistically significant means from non-parametric analyses. Correlation analyses were performed using the Pearson's co-efficient for normalised data, and Spearman's co-efficient for non-normalised data. All data were analysed using IBM®, SPSS® Statistics 25

(Armonk, NY, USA). A probability level of less than 5% ($P < 0.05$) was deemed statistically significant.

5.3 Results

5.4.1 Embryonic Growth

A treatment x sex interaction was identified at ED 15 ($P = 0.040$), whereby bwt was increased in CORT males compared to CORT females **(Figure 1:A),** however this effect was lost at hatch ($P = 0.847$, **Figure 1:B**). There were no differences between sexes, exposed to CORT or CON treatments.

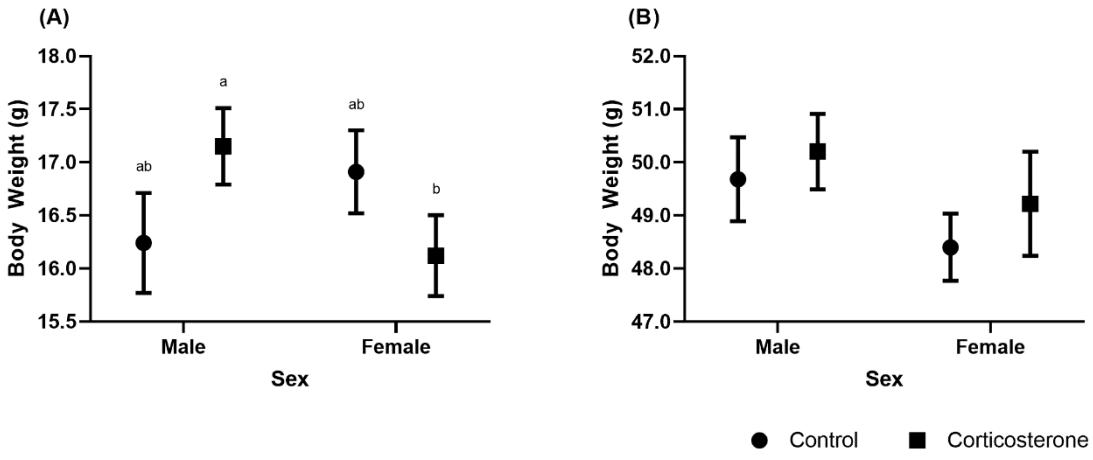

Figure 1. Male and female bwt at embryonic day 15 **(A)** and hatch **(B)** in chicken meat birds exposed to a CON or CORT solution via the chorioallantoic membrane at embryonic day 11. Values are mean ± SEM. Different superscripts indicate significant ($P < 0.05$) differences.

No differences in egg weight were recorded at ED 15 between treatments ($P = 0.0345$) or sex ($P = 0.992$), nor was any interaction identified ($P = 0.086$). Yolk sac weight at ED 15 was significantly greater in CORT treated birds ($P < 0.001$) **(Figure 2)**, irrespective of sex ($P =$

0.149). No sex by treatment interaction was identified for yolk sac weight at hatch ($P = 0.981$), nor did treatment ($P = 0.434$), or sex ($P = 0.495$), influence yolk sac weight at hatch. However, although non-significant, yolk sac weight was higher in CORT treated birds at hatch (CORT = 6.88g ± 0.27, Control = 6.57g ± 0.22). A significant positive correlation was identified between yolk sac weight and hatch bwt ($r = 0.644$, $P < 0.001$).

Liver % bwt did not differ between CORT and CON treated birds at both ED 15 ($P = 0.503$) and hatch ($P = 0.672$), nor between sexes (ED 15: $P = 0.0625$, hatch: $P = 0.309$), whilst no interaction between sex and treatment was observed at either time points (ED 15: $P = 0.237$, hatch: $P = 0.140$).

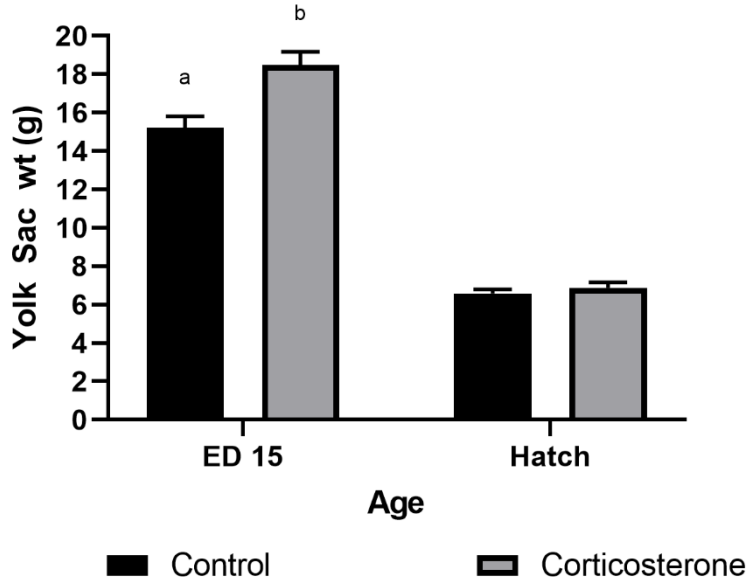

Figure 2. Yolk sac weight at embryonic day 15 and hatch in chicken meat birds exposed to a CON or CORT solution via the chorioallantoic membrane at embryonic day 11. Values are mean ± SEM. Different superscripts indicate significant ($P < 0.05$) differences.

5.4.2 Yolk Hormone Relative Abundance

Testosterone, Androstenedione, Etiocholanolone Glucuronide

The relative abundance of testosterone **(Figure 3:A)** and androstenedione **(Figure 3:B)** within the yolk appeared to peak at ED 0, with similar abundance levels detected at ED 5. At ED 15, the relative abundance of testosterone declined and detection levels remained consistent between ED 15 and hatch. It should be noted that testosterone was detected in n = 28 of a possible 40 samples at ED 15, and n = 27 of a possible 40 samples at hatch, whilst androstenedione was undetected at ED 15, and detected in n = 1 sample at hatch. No sex by treatment interaction was identified for testosterone at ED 15 (P = 0.890), and no differences were identified for treatment (P = 0.467) or sex (P = 0.856). Furthermore, no two-way interaction existed for treatment and sex at hatch (P = 0.833), whilst no differences in testosterone abundance were identified for treatment (P = 0.659) and sex (P = 0.111) when analysed separately at hatch. As androstenedione was undetectable in the yolk at ED 15, and was only detected in one sample at hatch, statistical analyses were not performed.

Etiocholanolone glucuronide was undetected in the yolk at ED 0, and was detected in n = 7 samples at ED 5. Etiocholanolone glucuronide abundance peaked at ED 15, after which abundance levels tended to decline by hatch **(Figure 3:C)**. When analysed separately at ED 15, no sex by treatment interaction was identified (P = 0.931), nor were any differences present for treatment (P = 0.232) and sex separately (P = 0.346). At hatch (ED 21), a sex by treatment interaction was identified (P = 0.021), where yolk etiocholanolone glucuronide abundance was significantly increased in CON females, compared to CORT males, with no further interactions between treatment groups observed.

Progesterone, Pregnonelone and 11β-hydroxy-progesterone/11-deoxycorticosterone

The relative abundance of progesterone **(Figure 3:D)** and pregnonelone **(Figure 3:E)** within the yolk were highest at ED 5 and ED 0 respectively, after which levels declined at both ED

15 and hatch. However it should be noted that progesterone was detected in *n* = 34 samples at ED 15, and *n* = 16 samples at hatch whilst pregnonelone was detected in *n* = 24 of a possible 40 samples at ED 15, and *n* = 3 of 40 samples at hatch. Analyses for ED 15, did not identify any sex by treatment interactions for progesterone (*P* = 0.501) or pregnonelone (*P* = 0.984), nor were there differences identified between treatment (progesterone: *P* = 0.740, pregnonelone: *P* = 0.928), or sex (progesterone: *P* = 0.176, pregnonelone: *P* = 0.925). Statistical analyses could not be performed for the factors of treatment and sex at the hatch time point due to an insufficient sample size.

11β-hydroxy-progesterone/11-deoxycorticosterone relative abundance levels peaked at the ED 15 time point, after which relative abundance levels appeared to decline up until hatch **(Figure 3:F)**. At ED 15, no two-way sex by treatment interaction was identified (*P* = 0.997), nor were there any differences between sex (*P* = 0.979), or treatment (*P* = 0.200). Furthermore, no sex by treatment interaction was identified at hatch (*P* = 0.564), nor were any sex (*P* = 0.783), or treatment (*P* = 0.142) effects present.

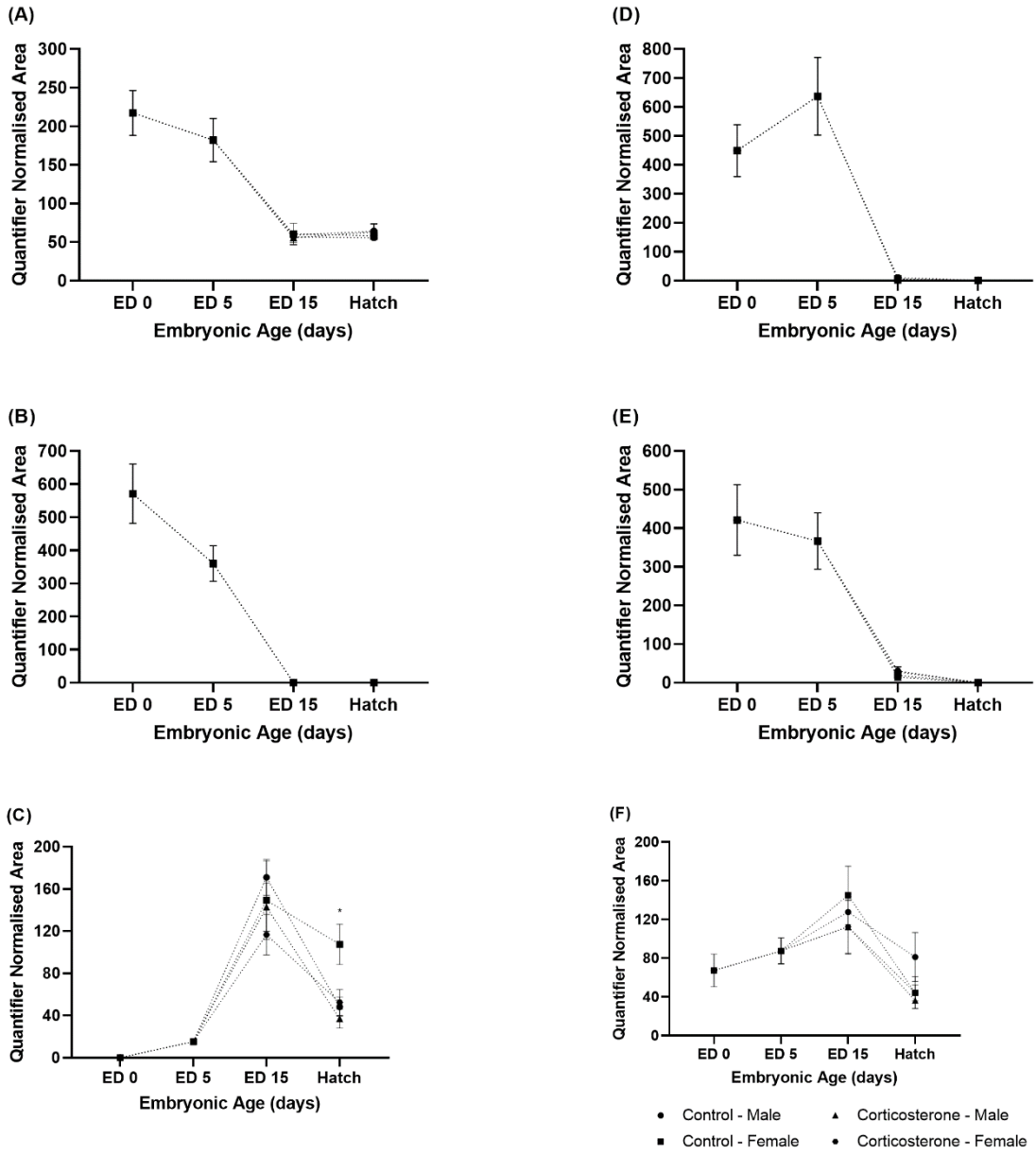

Figure 3. The relative abundance of (A) testosterone, (B) androstenedione, (C) etiocholanolone glucuronide, (D) progesterone, (E) pregnonelone and (F) 11β-hydroxy-progesterone/11-deoxycorticosterone in the yolk of commercial male and female broiler chickens at ED 0 (n = 10), ED 5 (n = 10), ED 15 (n = 40) and hatch (n = 40). Embryos were exposed to a CON or CORT solution via the chorioallantoic membrane at embryonic day 11. Values are expressed as means ± SEM. Quantified normalised area refers to the integrals of the area under the chromatographic peak. * indicates statistical ($P < 0.05$) significance.

Corticosterone

The relative abundance of CORT within the yolk was detected in *n* = 2 samples at ED 0, and *n* = 6 samples at ED 5, whilst the relative abundance of CORT peaked at ED 15, however significant variability existed between treatments **(Figure 4)**. CORT relative abundance appeared to decline between ED 15 and hatch, although significant variability within treatment groups was again observed at hatch. No interaction of sex and treatment were identified at ED 15 ($P = 0.766$) and hatch ($P = 0.941$), nor were treatment (ED 15: $P = 0.612$, hatch: $P = 0.790$), or sex (ED 15: $P = 0.351$, hatch: $P = 0.900$) effects identified at either time points.

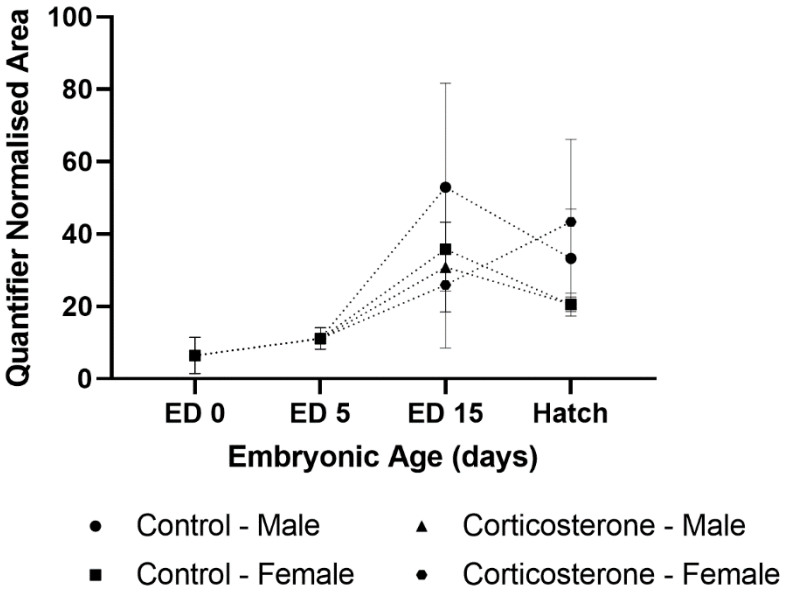

Figure 4. The relative abundance of corticosterone in the yolk of commercial male and female broiler chickens during embryonic development. Embryos were exposed to a CON or CORT solution via the chorioallantoic membrane at embryonic day 11. Values are expressed as means ± SEM. Quantified normalised area refers to the integrals of the area under the chromatographic peak.

17β-Estradiol and Estrone

The relative abundance of estrone in the yolk peaked at ED 0 and declined throughout the course of embryonic develop **(Figure 5:A)**. Estrone was undetectable at ED 15, and detected in n = 8 samples at hatch and therefore could not be statistically analysed for treatment or sex effects.

The relative abundance of 17β-Estradiol in yolk appeared to rise between the period of ED 0 and ED 15, with abundance levels peaking at the ED 15 time point **(Figure 5:B)**. After ED 15, the relative abundance of 17β-Estradiol declined up until the point of hatch, with 17β-Estradiol abundance being lowest at the point of hatch. At ED 15, no sex by treatment interaction was identified (P = 0.411), nor were sex (P = 0.966), or treatment (P = 0.185) effects observed. Similarly at hatch, no sex by treatment interaction was observed (P = 0.494), along with no sex (P = 0.892), or treatment (P = 0.203) effects. Furthermore the relative abundance of 17β-Estradiol in the yolk positively correlated with the relative abundance of 11β-hydroxy-progesterone/11-deoxycorticosterone in the yolk at hatch (r =0.929, P < 0.001).

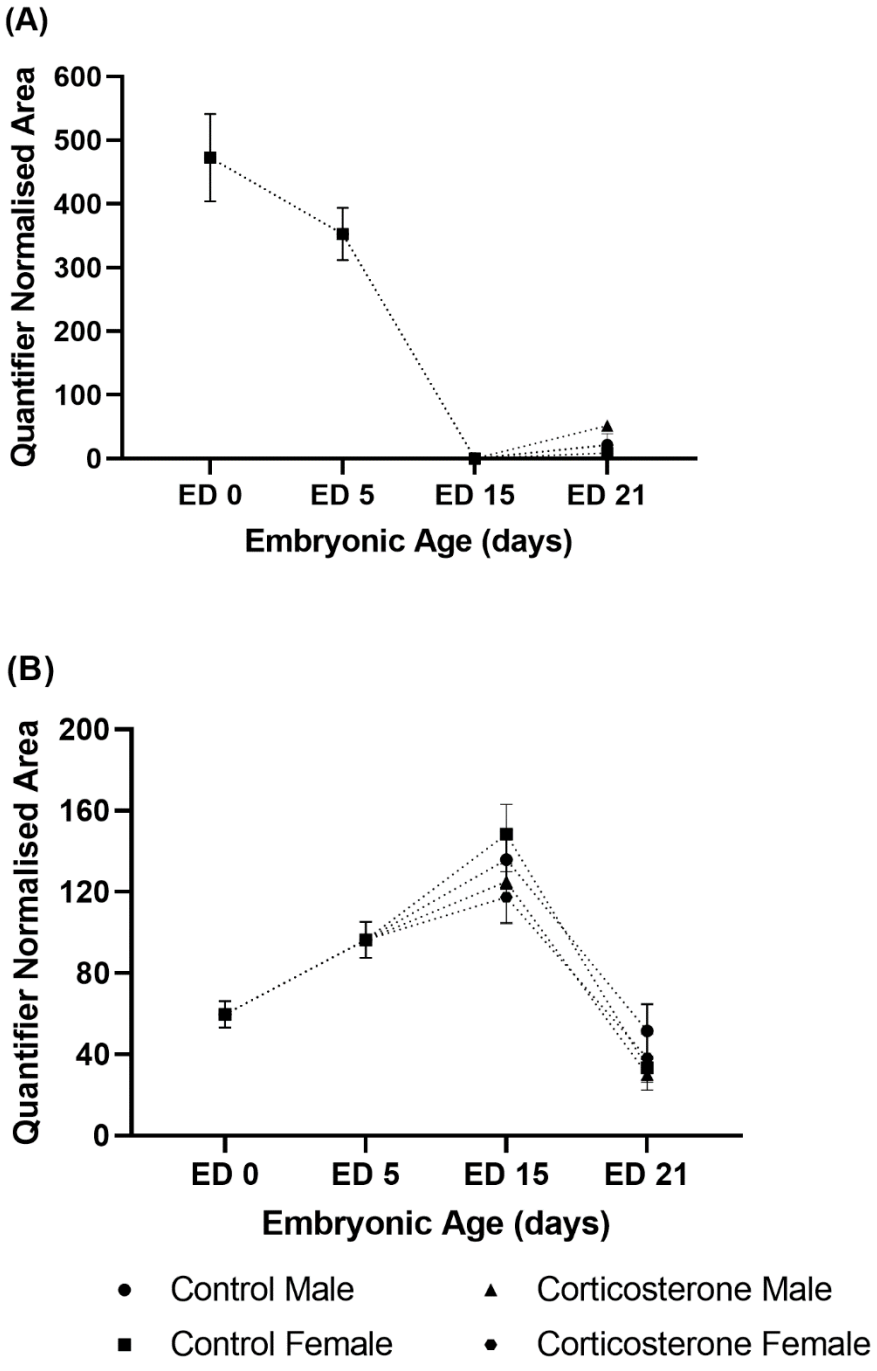

Figure 5. The relative abundance of (A) estrone, and (B) 17β-Estradiol in the yolk of commercial male and female broiler chickens during embryonic development. Embryos were exposed to a CON or CORT solution via the chorioallantoic membrane at embryonic day 11. Values are expressed as means ± SEM. Quantified normalised area refers to the integrals of the area under the chromatographic peak.

5.4.3 Embryonic Musculoskeletal Development

MFN at hatch did not differ between CON (2.95 ± 0.07 x 10^4 fibres per mm^2 fascicle area) and CORT (2.98 ± 0.07 x 10^4 fibres per mm^2 fascicle area) treated birds (P = 0.774), nor between sexes (P = 0.672) (Male: 2.99 ± 0.07 x 10^4 fibres per mm^2 fascicle area, Female: 2.95 ± 0.07 x 10^4 fibres per mm^2 fascicle area). No interaction was identified between the factors of sex and treatment in relation to MFN. Average muscle fibre CSA followed a similar trend, with no significant difference identified between CON (36.81± 0.75 μm^2) and CORT (37.06 ± 0.79 μm^2) treated birds (P = 0.826), nor were any differences identified between male (36.52 ± 0.81 μm^2) and female (37.27 ± 0.74 μm^2) birds (P = 0.459), whilst no two-way interaction was observed for the factors of treatment and sex (P = 0.527). A positive correlation was identified between CSA and yolk sac weight at hatch, which approached statistical significance (r = 0.304, P = 0.051). Additionally, a significant negative correlation was present between MFN and CSA (r = -0.941, P < 0.001). The percentage of fascicle area occupied by muscle fibres was significantly greater in CON treated birds (54.61 ± 0.31 %) compared to CORT treated birds (52.59 ± 0.28 %) (P < 0.001). No sex differences were observed (P = 0.443), whilst no two-way interaction for the factors of sex and treatment was identified (P = 0.269). A negative correlation was identified between MFN and the percentage of total fascicle area occupied by muscle fibres (r = -0.318, P = 0.040).

5.4.4 Yolk Fat

Baseline yolk fat percentage was 25.23 ± 0.92 at ED 0, and 11.96 ± 1.31 at ED 5 **(Figure 6)**. At ED 15, yolk fat percentage did not differ between treatments (P = 0.479). The difference in yolk fat percentage between sexes approached significance at ED 15 (P = 0.058), with fat

percentage increased in male birds, with no two-way interaction identified at ED 15 (P = 0.566). At hatch, yolk fat percentage appeared to increase in CON treated birds compared to CORT treated birds (P = 0.062). No difference in yolk fat percentage was identified between sexes at hatch (P = 0.138), nor was a sex x treatment interaction present (P = 0.642). A negative correlation was identified between yolk fat content at hatch, and hatch bwt (r = -0.364, P = 0.021).

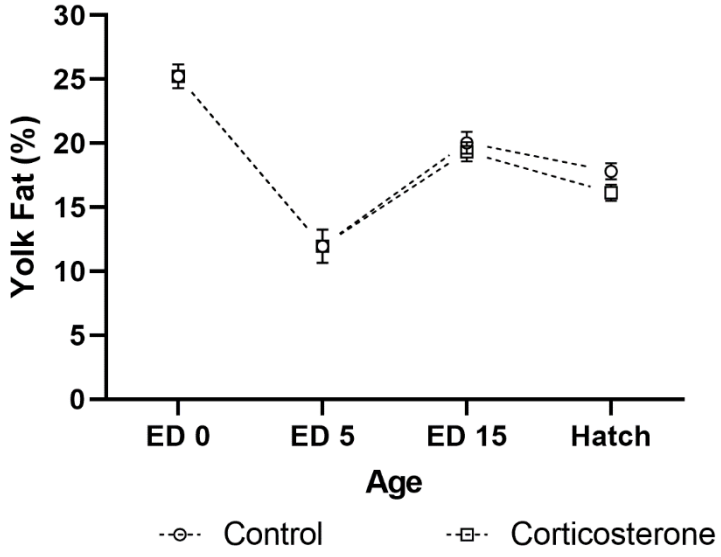

Figure 6. Percentage of fat content within the yolk of chicken meat birds at embryonic day 0, 5, 15 and hatch. Embryos were exposed to a CON or CORT solution at embryonic day 11 via the chorioallantoic membrane. Values are mean ± SEM.

5.4.5 Relative mRNA Expression

The relative expression of *CEBP/β* was significantly increased in CORT birds compared to CON treated birds (P = 0.040; **Figure 7**), whilst no sex effect was identified when analysed separately (P = 0.551), and no significant interaction was identified (P = 0.186). No interaction for the factors of sex and *in-ovo* treatment was observed for the *PPARγ* gene (P = 0.491), however, relative expression tended to increase in control females, compared to CORT

females, whilst expression levels tended to increase in CORT males compared to control males. A significant negative correlation was however identified between *PPARγ* relative expression and CSA (r = -0.419, P = 0.019). Additionally, no significant differences were observed between treatments (P = 0.809), or sexes (P = 0.724).

The relative expression in chicken BM of the *AR*, *PR* and *ER-α* did not differ between *in-ovo* treatment (P > 0.05), or sex (P > 0.05), at hatch. However, *AR* and *ER-α* relative mRNA expression were slightly elevated in CORT female birds, although not statistically significant. Additionally, no two-way interactions for the factors of sex and *in-ovo* treatment were identified (P > 0.05).

No significant *in-ovo* treatment effects were identified for the relative expression of *MyoD* (P = 0.845), *MyoG* (P = 0.351) and *Pax7* (P = 0.580). Additionally, sex did not significantly affect the relative expression of *MyoD* (P = 0.278), *MyoG* (P = 0.835) and *Pax7* (P = 0.505), nor were any interactions observed (*MyoD*: P = 0.333, *MyoG*: P = 0.967, *Pax7*: P = 0.141, **Figure 7**).

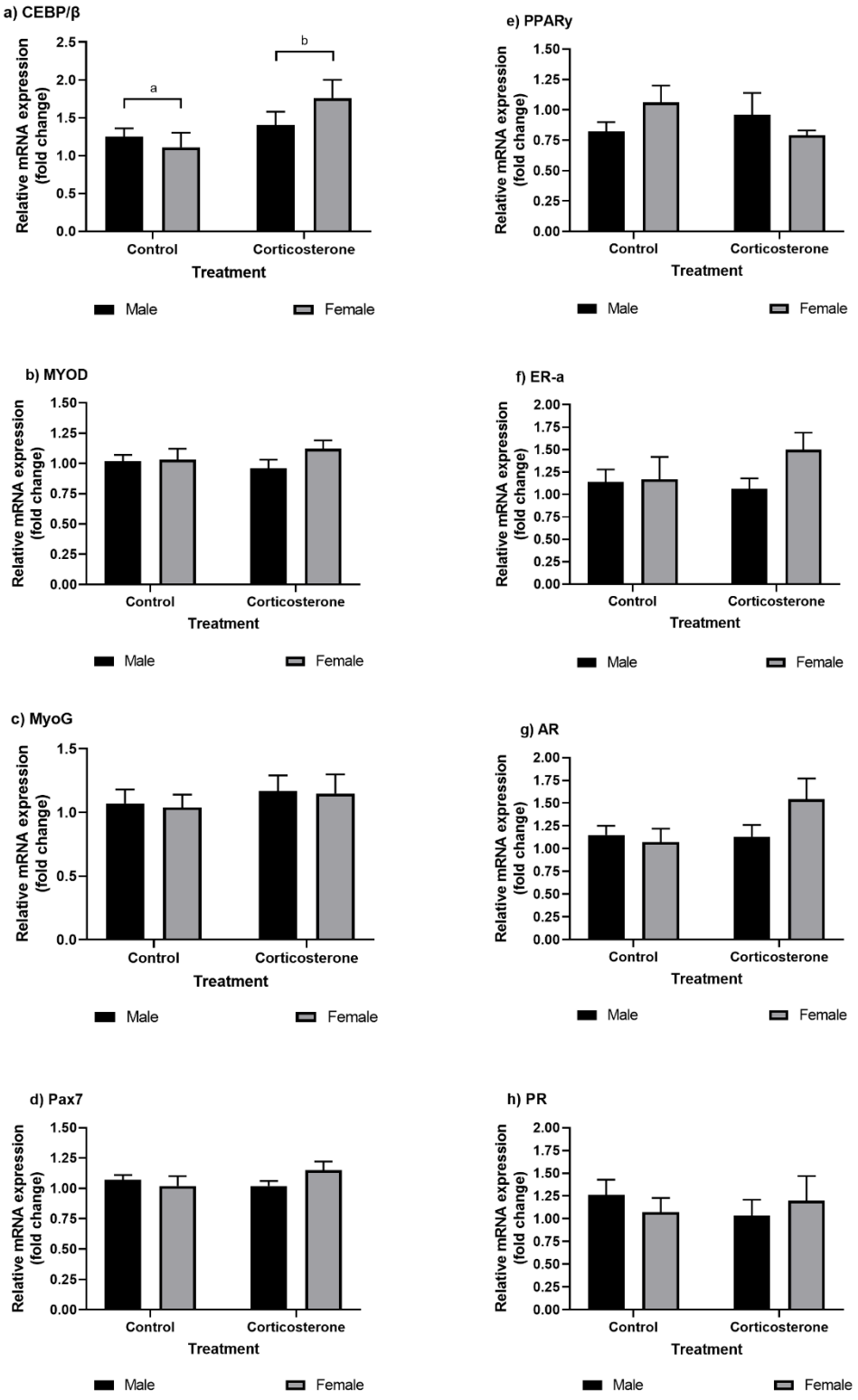

Figure 7. Relative mRNA expression of the a) *CEBP/β*, b) *MyoD*, c) *MyoG*, d) *Pax7*, e) *PPARγ*, f) *ER-α*, g) *AR* and h) *PR* gene in the breast muscle of chicken meat birds exposed to a CON or CORT solution at embryonic day 11. Samples consist of pooled left and right breast muscle, which were obtained from birds at hatch. Values are

normalised average relative mRNA expression ± SEM. Different superscripts indicate significant ($P < 0.05$) differences.

5.5. Discussion

The effects of maternal/*in-ovo* stress in production poultry have not been investigated to the same extent to that of wild birds. However such studies are pertinent given that poultry breeder birds are routinely exposed to various stressors throughout the production cycle (De Jong and Guemene, 2011). Additionally, the majority of work investigating the effects of early-life stress in commercial poultry have identified variations in growth rates (Bowling et al., 2018;Peixoto et al., 2020b), body weight (Gholami et al., 2017;Peixoto et al., 2020b), and more recently, body composition at market weight (Angove et al., 2021). However, the mechanisms regulating these phenotypic alterations in directly exposed progeny, or progeny hatched from exposed hens, remains unknown, but are of importance to determine how the *in-ovo* environment can influence progeny performance. The current study aimed to investigate whether *in-ovo* exposure to CORT would alter embryonic muscle development, and whether this correlated with differences in yolk contents of steroid hormones and/or lipid content.

Exposure to 100 µL of a 10mM phosphate buffer solution containing 1 µg of CORT dissolved in absolute ethanol at ED 11 appeared to not influence embryonic muscle development, as MFN, nor CSA, differed in response to CORT in the current study. This is somewhat surprising, as exposure to high doses of glucocorticoids (10^{-6} M), such as CORT, is documented to inhibit the ability of multipotent stem cells to proliferate and differentiate (Salloum et al., 2013), both of which are important determinants of MFN (Du et al., 2013). Additionally, increased glucocorticoid exposure also tends to promote the commitment of multipotent stems cells to adipogenic (Campbell et al., 2011), osteogenic (Kerachian et al., 2009) and chondrogenic lineages (Salloum et al., 2013). Considering the present study utilised an

'extreme' model of *in-ovo* stress (Ahmed et al., 2014b), it was expected that exposure to CORT would reduce total MFN in CORT treated birds, due to the commitment of multipotent stem cells to non-myogenic lineages (Salloum et al., 2013). Although MFN was not reduced in CORT treated birds in the present study, this may be a result of CORT exposure occurring too late in embryonic development, i.e. ED 11.

Evidence suggests multiple myogenic populations exist within developing muscles, with these populations termed, embryonic, fetal and adult (Halevy et al., 2006). Embryonic myoblasts are most abundant in the chick at ED 5, and initiate the formation of the limb bud, which provides a source of mesoderm cells which eventually differentiate into myogenic cells and subsequent primary fibres (Stockdale, 1992). Additionally, fetal myoblasts appear most abundant in the chick between ED 8 and ED 12 and form secondary fibres which become dominant in growing muscles (Stockdale, 1992). Adult myoblasts (also termed satellite cells) first appear between ED 13 and ED 16, however don't peak until 2-3 days post-hatch, after which they become quiescent until required for post hatch muscular repair and growth (Hartley et al., 1992). As the embryos in the present study were not exposed to CORT until ED 11, embryonic and fetal myoblast cell populations are likely to be set by this stage, and cell lineage re-direction may therefore not be possible. The administration of CORT at ED 11 is therefore most likely to influence the number of adult myoblasts (SC's) that form during the later stages of embryogenesis. This however appears unlikely as exposure to CORT via the chorioallantoic membrane failed to influence the relative mRNA expression of any of the myogenic regulatory genes measured at hatch in this study. Although this study's findings suggest CORT exposure is unlikely to have influenced adult myoblast numbers, the possibility that muscle fibre CSA could differ between CON and CORT treated birds during the growth phase post-hatch as result of muscular hypertrophy remains. As muscle fibre CSA is expected to increase post-hatch in chicken meat birds (Halevy et al., 2006), the finding the fibre CSA did not differ between

CORT and CON treated birds at hatch is expected. Nonetheless, the process of muscle hypertrophy and the subsequent increase in fibre CSA could be influenced by embryonic exposure to endogenous glucocorticoids, as has been previously documented (Baehr et al., 2011;Braun and Marks, 2015).

Despite CORT exposure having no apparent influence on MFN and fibre CSA, a significant difference was identified in the percentage of fascicle area occupied by muscle fibres, which was reduced in CORT treated birds. Such a result indicates the presence of another tissue type within the muscle fascicle itself other than muscle tissue in the form of fibres. The most likely source is adipose tissue, with exposure to high CORT doses previously documented to re-direct mesenchymal stem cells to the adipogenic line (Salloum et al., 2013). Whether or not the intramuscular adipocyte numbers increased in CORT treated birds was not measured in the present study, although has been identified in separate studies where intramuscular triglyceride content was increased in response to CORT (Campbell et al., 2011). However, the noted significant increase in *CEBP/β* relative mRNA expression in CORT treated birds suggests a potential upregulation of adipogenic processes at the point of hatch. *CEBP/β* is documented to promote the expression of *PPARγ*, the primary regulator of adipocyte differentiation (Wang et al., 2017). *PPARγ* expression did not differ in the present study, which may suggest the onset of adipocyte differentiation at hatch, and thus *PPARγ* expression could be upregulated at a later time point, as *PPARγ* expression tends to increase with age (Sato et al., 2004). Additionally, differences in adipogenic activity could be irrespective of *PPARγ* regulation, as *CEBP/β* is documented to interact with *CEBP/α,* another primary regulator involved in adipogenesis (Wang et al., 2017). Whether *CEBP/β* expression is influenced by glucocorticoid exposure has not been extensively investigated in the chicken, although the results from this study suggest the potential for such an interaction in the BM of birds exposed to *in-ovo* CORT. Furthermore, the phenotypic effects of *in-ovo* CORT may not be a direct effect of CORT itself, but instead

a result of CORT's documented ability to influence the synthesis and metabolism of separate endocrine factors (Tilbrook et al., 2000;Henriksen et al., 2011a;Natt et al., 2015).

In avians, the likely source of non-endogenous endocrine factors that influence early development is the yolk (Henriksen et al., 2011b;Angove and Forder, 2020). Interactions between maternal/*in-ovo* stress, yolk hormones, and subsequent progeny phenotype have been previously identified (Henriksen et al., 2011a;Ahmed et al., 2014a;Bowling et al., 2018). Most studies have focused on interactions between glucocorticoids and sex steroids, such as the androgens, gestagens and oestrogens (Rettenbacher et al., 2009;von Engelhardt et al., 2009;Henriksen et al., 2011a), due to the phenotypic differences identified in response to altered sex steroid concentrations in the yolk (Paitz and Bowden, 2008;Rettenbacher et al., 2009;Benowitz-Fredericks and Hodge, 2013;Giraudeau et al., 2017). Thus alterations to the hormonal contents of steroids within the yolk appears a plausible mechanism in which post-hatch development might be mediated in response to early-life stress.

Exposure to CORT at ED 11 had minimal influence on the relative abundance of the various hormones in the yolk measured in this study. The noted rapid reduction in testosterone and androstenedione abundance levels from ED 0 until ED 15 coincides with an increase in the relative abundance of etiocholanolone glucuronide in the yolk. Etiocholanolone glucuronide is the conjugate metabolite of the 5β-reductase metabolic pathway (Paitz et al., 2011;Kumar et al., 2019), and is perceived to be the inactivation pathway of androgenic hormones (Balthazart et al., 1990). Although this study cannot conclude testosterone and androstenedione were metabolised to etiocholanolone glucuronide, previous work identified that androgens are rapidly metabolised to etiocholanolone glucuronide during the early stage of embryonic development in birds (Paitz et al., 2011;Kumar et al., 2019). Furthermore, our study suggests a potential difference in either absorption or clearance rates of etiocholanolone glucuronide from the yolk between male and female birds, as male birds recorded a sharper decline in yolk

etiocholanolone glucuronide abundance to that of female birds. Whether yolk or yolk content absorption rates differ between sexes is not clear, but may explain why etiocholanolone glucuronide was significantly increased in CON female birds at hatch. Although only significant between CON female and CORT male birds, yolk etiocholanolone glucuronide abundance was increased in CON female birds compared to all other treatments. Additionally, yolk etiocholanolone glucuronide abundance was increased in both CON male and female birds at ED 15, with no differences observed between male birds at hatch. Therefore, the initial differences in yolk etiocholanolone glucuronide abundance at ED 15 between CON and CORT treated birds, accompanied with an apparent sex difference in absorption/metabolic rates between ED 15 and hatch may explain the increase in yolk etiocholanolone glucuronide abundance in CON female birds at hatch. Although post-hatch phenotypes were not assessed, differences in yolk absorption/metabolism of etiocholanolone glucuronide could explain the previously documented sex differences in progeny phenotypes in response to the maternal and/or *in-ovo* environment (Haussmann et al., 2012;Chang et al., 2016;Bowling et al., 2018). Whether etiocholanolone glucuronide can induce phenotypic differences in progeny is unclear, although a study by Campbell et al. (2020) identified no effect of embryonic exposure to free etiocholanolone in European Starlings. Embryos instead may metabolise androgens to etiocholanolone glucuronide to regulate their exposure to maternally sourced hormones (Paitz et al., 2011;Groothuis et al., 2019). Conversion to etiocholanolone glucuronide may also be a storage method, as glucuronides are less lipophilic, initiating uptake by the developing embryo (Paitz et al., 2011). Such absorption could initiate re-conversion back to more potent androgens (Kumar et al., 2019), although no work has identified such a pathway exists within the avian embryo.

The oestrogenic and gestagen hormones measured in the present study followed a similar metabolic pathway. The reduction in relative abundance levels of estrone throughout

embryonic development coincided with an increase in the relative abundance of 17β-estradiol. Whether estrone is metabolised to 17β-estradiol within the yolk cannot be concluded from this study, although estrone and 17β-estradiol are interconvertible (Moghrabi et al., 1997). However, previous work suggests 17β-estradiol is converted to estrone, followed by conjugation to estrone sulfonate and glucuronide (Paitz et al., 2020), a similar metabolic fate to the androgens. Thus further work is required to understand the metabolic fate of yolk oestrogens, as well as whether phenotypic variations eventuate in exposed progeny, or whether 17β-estradiol conjugates are converted back to active forms.

Similarly, reductions in the relative abundance of progesterone and pregnonelone coincided with an increase in abundance of 11-deoxycorticosterone/11β-hydroxyprogesterone, which again follows an inactivation metabolic pathway. Interestingly, the relative abundance of CORT in the yolk followed a similar pattern to 11-deoxycorticosterone/11β-hydroxyprogesterone, where both hormones act as precursors to CORT (Payne and Hales, 2004). CORT was almost undetectable in the yolk at ED 0 and ED 5, however was detected in all samples at ED 15 and hatch. Whether CORT within the yolk at ED 15 and hatch is synthesised from 11-deoxycorticosterone/11β-hydroxyprogesterone sourced from the metabolism of progesterone deposited in the yolk by the hen is not known. However, recent work suggests yolk progesterone is instead converted to the conjugate 5β-preganedione, a similar metabolic pathway to that of the androgen hormones (Paitz and Cagney, 2019). Alternatively, plasma CORT concentrations spike in the avian embryo at ED 15 and hatch (Jenkins and Porter, 2004;Angove and Forder, 2020). This coincides with the onset of a functional hypothalamic pituitary adrenal axis at ED 15 (Jenkins and Porter, 2004), and the stressors associated with the hatch process during late embryonic development (Jenkins and Porter, 2004). Whether the avian embryo is able to transfer hormones from the plasma to the yolk is not clear, although provides an alternative explanation for the presence of yolk CORT

in the later stages of embryogenesis. In fact, it is still not clear how, and when, developing embryos are exposed to steroid hormones contained within the lipophilic environment of the yolk.

Steroid hormones are highly lipophilic, and are therefore unlikely to actively transfer from the yolk to the water-rich environment of the embryo. As mentioned, conversion of free hormones to metabolic conjugates may facilitate absorption of lipophilic hormones by the embryo (Paitz et al., 2011). Alternatively, conjugation of free hormones could provide a storage mechanism until the embryo begins to absorb yolk lipids. Yolk fat absorption is minimal through the first two weeks of embryogenesis, and peaks in the final days of development (Yadgary et al., 2010). Thus, lipophilic steroid hormones may be absorbed during the period of peak fat utilisation. Interestingly, our results suggest yolk fat content is reduced in CORT treated birds at hatch, suggesting a greater fat absorption rate in these birds. To the best of our knowledge, no work has investigated whether early-life stress affects yolk fat absorption. A study by Mikec et al. (2006) did identify differences in yolk reabsorption rates in response to environmental stressors during the first 5 days post-hatch, a potential reflection of bird energy requirements in response to early life stress. Energy requirements tend to increase during periods of stress (Duan et al., 2014), and thus reductions in yolk fat could indicate a survival mechanism in response to early-life stress (Henriksen et al., 2011b). This is supported by the lack of bwt variation at hatch between CON and CORT treated birds. Similar results have been recorded in progeny exposed to CORT throughout embryonic development, with alterations in bwt and body composition identified in adult birds (Ahmed et al., 2014b;Angove et al., 2021). Additionally, yolk sac weight was increased in CORT treated birds at ED 15 and to a lesser extent, at hatch. This coupled with the reductions in yolk fat % in CORT treated birds at ED 15 suggests that embryos exposed to CORT might prioritise fat absorption more so than CON birds through the later stages of development, again as a short term survival mechanism. This is further supported by

the negative correlation identified between yolk lipid content at hatch and hatch bwt, as well as the positive correlation identified between hatch yolk sac weight (%bwt) and hatch bwt. The ramifications of altered yolk fat absorption during embryonic development are not well understood, but likely contribute to the post-hatch phenotypes subsequently displayed in adult birds across various studies in response to alterations to the *in-ovo* environment. This is because the period between ED 13 and 7 days post hatch, in which the majority of yolk fat is absorbed, coincides with the proliferation and differentiation of multipotent stem cells into adult myoblasts (Halevy et al., 2006). Yolk fat is potentially an energy source for this process, whilst also having potential to store and transport lipophilic hormones, exposing developing embryos to important endocrine factors during this developmental period.

5.6 Conclusion

The findings from this study suggest exposure to CORT at ED 11 does not influence early musculoskeletal development in commercial chicken meat birds. Instead, early-life exposure to CORT may increase adipogenic commitment of mesenchymal stem cells at the point of differentiation. This is supported by the identified increase in mRNA expression of the *CEBP/β* gene in BM tissue at hatch and decrease in fascicle percent area occupied by muscle fibres. In addition, *in-ovo* exposure to CORT reduced total yolk fat content in birds at hatch, with minimal influence on the relative abundance of the various steroid hormones measured in the yolk. To the best of our knowledge, this study is the first to identify differences in yolk fat content in response to alterations to the *in-ovo* environment encountered. However, further work is required to understand the ramifications of altered yolk fat absorption rates on subsequent performance traits, as well as yolk steroid hormone contents in chicken meat birds. An understanding of the mechanisms controlling post-hatch phenotypes as a result of the *in-*

ovo environment would allow the chicken meat industry to implement subtle manipulations to production strategies in an effort to improve performance and carcass characteristics in commercial flocks.

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Chapter Six:
General Discussion

6.1 General Discussion

The primary objective of this thesis was to provide a further understanding as to how alterations to the maternal and *in-ovo* environments encountered by breeder hens and developing embryos can lead to phenotypic alterations in adult chicken meat birds. The chicken meat industry is at the forefront of animal production regarding developmental programming and its applicability to current production methods, largely due to the pedigree breeding structure (Hynd *et al.* 2016) described in chapter 1. In addition, the *in-ovo* environment encountered is potentially a significant contributor to post-hatch performance (Halevy *et al.* 2006), as essential endocrine pathways develop during this period (Thommes *et al.* 1992; Scanes 1997; Jenkins and Porter 2004), along with the development of key musculoskeletal structures that support post-hatch performance (Halevy *et al.* 2006). Furthermore, considering genetic and nutritional gains have nearly been optimised in chicken meat birds, targeting developmental programming initiatives provides a new and innovative method to advance poultry production. However, for the chicken meat industry to benefit from the effects of developmental programming, an understanding of the contributing physiological mechanisms influencing progeny performance is required. This chapter presents the outcomes and conclusions from chapters two, three, four and five, where manipulations to the maternal/*in-ovo* environments were implemented in a bid to understand the effects on progeny performance, and the contributing physiological mechanisms. In addition, the provision of nutritional additives to both breeder hens and progeny was explored as a potential method to alleviate the consequences of maternal stress or *in-ovo* exposure to corticosterone **(CORT)**.

The initial aim was to identify whether the previously documented variations in male progeny body weight **(bwt)** as influenced by maternal stress (Bowling *et al.* 2018) and *in-ovo* exposure to CORT (Ahmed *et al.* 2014) were in response to altered fat mass, lean mass or of another biological origin. As described in Chapter 3, the animal trial was conducted under almost

identical conditions to the previously completed work by our group, in a bid to obtain the necessary samples required to explore potential physiological mechanisms contributing to altered post-hatch performance. Unexpectedly however, no differences in progeny bwt or growth rates were identified at any age. Recent work has identified significant line differences in layer chickens in response to maternal stress, where progeny growth rates, embryonic survival and behavioural differences were line dependent (Peixoto *et al.* 2020a; Peixoto *et al.* 2020b). The experiment conducted in Chapter 3 utilised eggs from a 37-week-old great grandparent broiler breeder flock sourced directly from a commercial breeder farm, whereas the previous trials conducted by our group (Bowling *et.al.* Unpublished) utilised commercial broiler chicken eggs from breeder hens of an unknown age and/or unknown genetic line (i.e. broiler breeder pedigree). Therefore, the differences in line, generation and breeder age of commercial poultry utilised between studies may explain the differences in phenotypic outcomes between trials. For instance, more distinct comparisons show that chicken meat birds exhibit superior growth rates to that of layer chickens, whilst significant variation in yolk concentrations of testosterone and triiodothyronine exists between the two types of commercial poultry (Ho *et al.* 2011). In addition, transcriptomic differences have been identified between meat and layer chickens in relation to metabolic pathways, including possible higher rates of glycolysis in meat chickens, as well as differences related to cell cycle regulation, which were noted potential mechanisms for the growth disparity between breeds (Willson *et al.* 2018). Whether such effects occur in separate genetic lines or crosses of pure line chicken meat birds is unclear. Interestingly, embryonic day **(ED)** 15 bwt in progeny from chapters 3 and 5 appeared to differ in response to *in-ovo* CORT exposure, where the primary difference between trials was line of bird being used, suggesting a potential line effect between the two trials. However, it must be noted that as the eggs utilised in trial 3 were sourced from a commercial hatchery, breeder hen age was also unknown, and could be an influencing factor contributing

to the noted differences in ED 15 bwt between trials. The effect of bird genetics is further highlighted in Chapter 4, where maternal supplementation with the yeast metabolite *saccharomyces cerevisiae* ***(SC)*** influenced ED 15 bwt in a line x sex specific manner, and significant differences in growth and body composition measures (at d 42) were identified. Therefore, it appears that genetic differences between populations of commercial chickens should be considered a potential contributing factor to the phenotypic shifts that arise from alterations to the maternal/*in-ovo* environment. Such effects must be accounted for in future studies, as phenotypic variation in response to alterations to the maternal/*in-ovo* environment are unlikely to remain consistent between genetic lines, a potential limitation for the applicability of developmental programming to the chicken meat industry.

With the exception of ED 15 bwt, no growth differences were identified in Chapters 3 and 5, however the findings suggest that *in-ovo* exposure to CORT may alter body composition, with observed increases in total fat mass over lean mass in chicken meat birds. Few studies have investigated the effects of the maternal/*in-ovo* environment on body composition measures in chicken meat birds, which is somewhat surprising considering the importance of carcass composition (Tavárez and Solis de los Santos 2016). CORT itself is well documented to increase activity of fat deposition biochemical pathways in commercial meat birds (Cai *et al.* 2011), as well as enhance protein catabolism (Scanes 2016), although the effects of maternal stress or *in-ovo* exposure to CORT on such traits is less understood. Along with the increase in fat deposition identified in female birds exposed to CORT in Chapter 3, CORT exposed birds in Chapter 5 reported a decrease in percentage of breast muscle fascicle area occupied by muscle fibres. As muscle fibre number **(MFN)** and muscle fibre cross sectional area **(CSA)** did not differ between CORT and CON exposed birds, it suggests that differences in percentage of muscle fascicle area occupied by muscle fibres is a result of increase tissue mass from another source. This source is most likely adipose tissue due to the previously identified promoter

effects of CORT on fat deposition (Cai *et al.* 2011), and the increase in CCAAT/enhancer-binding protein ***(CEBP/β)*** expression identified in chapter 5, in the breast muscle of CORT exposed birds at hatch. The prominent pathway leading to increased fat deposition is not clear, but may involve the key adipogenic regulators peroxisome proliferators-activated receptor gamma ***(PPARγ)*** and CCAAT/enhancer-binding protein alpha ***(CEBP/α)***, where the expression of both proteins is enhanced by *CEBP/β* (Wang *et al.* 2017). Although the conclusions drawn from this thesis suggest *in-ovo* exposure to CORT promotes fat deposition, whether these effects are consistent between genetic lines of birds is not clear, nor whether body composition measures differ in a sex dependent manner. As the introduction to chapter 4 describes, body composition data obtained in Chapter 3 was from a small number of female birds only (n = 10), and therefore whether the body composition differences identified in female birds were consistent between sexes could not be confirmed. The presence of sex dependent phenotypes in response to alterations to the maternal and/or *in-ovo* environment appears common across a range of performance and behavioural traits in avian (Haussmann *et al.* 2012; van Emous *et al.* 2015; Chang *et al.* 2016; Bowling *et al.* 2018) and mammalian species (Mack *et al.* 2014; Micke *et al.* 2015; Gatford *et al.* 2018). Additionally, current Australian production methods utilise mixed sex rearing strategies. Therefore, targeting the maternal environment encountered by breeder hens, along with the *in-ovo* environment of subsequent progeny, provides a novel method to improve mixed sex flock uniformity in subsequent generations of chicken meat birds.

In order to incorporate developmental programming as a tool to improve performance in the chicken meat industry, a fundamental understanding of the contributing physiological mechanisms influencing progeny production traits in response to the maternal environment is required. Therefore the second aim of this thesis was originally to identify potential physiological mechanisms that contribute to the already noted growth rate variation in broiler

progeny (Bowling *et al.* 2018; Peixoto *et al.* 2020b), as influenced by maternal stress, with particular interest in sex-dependent variation. However, due to the limited growth variations between treatment groups identified in Chapters 3 and 4, we aimed to investigate potential physiological mechanisms contributing to the increased fat mass in 35-day old CORT exposed females in chapter 3. Furthermore, we aimed to determine how maternal line influenced progeny body composition in response to alterations to the *in-ovo* environment.

Progeny phenotypes are altered in response to the maternal environment through nutritional or hormonal manipulations (Henriksen *et al.* 2011b; Groothuis *et al.* 2019) to the egg during embryonic development. Such *in-ovo* alterations can in turn mediate developmental processes in the embryo (Ho *et al.* 2011), ultimately influencing bird phenotypes post-hatch. Exposure to altered *in-ovo* hormonal concentrations has been extensively studied in avian species (Benowitz-Fredericks and Hodge 2013; Ahmed *et al.* 2014; Giraudeau *et al.* 2017; Campbell *et al.* 2020), with a vast array of phenotypic alterations identified across multiple studies, as reviewed in chapter 2. The majority of these studies however have focused on the sex steroids, including androgen (Campbell *et al.* 2020), gestagen (Paitz and Cagney 2019) and estrogen hormones (Paitz *et al.* 2020), as they appear to be readily deposited into the yolk by the hen during egg formation (Schwabl 1993; Aslam *et al.* 2013). Additionally, varied yolk concentrations of these sex steroids, primarily the androgens, has resulted in alterations to progeny growth rates (Ho *et al.* 2011), behaviour (Benowitz-Fredericks and Hodge 2013) and survival (von Engelhardt *et al.* 2006). In addition, studies have highlighted the ability of maternal stress, as well as individual stress, to influence yolk (Henriksen *et al.* 2011a) and plasma concentrations (Natt *et al.* 2015) of sex steroid hormones. Such effects are supported by the findings presented in Appendix 3, where *in-ovo* CORT increased yolk testosterone concentrations in female embryos at ED 15, as well as in Chapter 4, where increased plasma CORT concentrations in *SC* supplemented breeder hens coincided with reduced yolk

testosterone concentrations. Considering CORT's documented ability to inhibit the synthesis of the sex steroid hormones (Tilbrook *et al.* 2000) and their concentrations within the yolk of avian eggs (Henriksen *et al.* 2011a), we hypothesised that phenotypic alterations in response to maternal/*in-ovo* stress may be mediated by CORT through the actions of the sex steroids.

The results presented in Chapter 5 did not support the hypothesis that *in-ovo* exposure to CORT at ED 11 would decrease the relative abundance of steroid hormones within the yolk. Instead, *in-ovo* exposure to CORT had minimal influence on the relative abundance of any of the 9 free and conjugate target compounds detected at any time point. Free hormones, such as testosterone, androstenedione (Kumar *et al.* 2019), progesterone and pregnonelone (Paitz and Cagney 2019) are thought to be metabolised within the first 5 days of embryonic development to their inactive, conjugate forms. The findings from Chapter 5 supports this conversion, as the relative abundance of free hormones appeared to decrease rapidly after ED 5, coinciding with an increase in relative abundance of inactive free and conjugate metabolites from ED 15 onwards. Additionally, the ED 15 yolk concentrations of testosterone measured in appendix 3 appeared very low (< 0.5 ng/g), further indicating rapid early metabolism. We collaborated with the Metabolomics Australia/Australian Wine Research Institute to obtain precise and accurate hormone results using liquid chromatography-mass spectroscopy/mass spectroscopy **(LC-MS/MS)** technology, the perceived gold standard of hormone detection (Groothuis *et al.* 2019), as we detected significant discrepancies between studies using various methods. The variations in yolk concentrations of various steroid hormones in response to *in-ovo* CORT exposure within this thesis, as well as throughout the literature, may be a result of the methods used to detect the presence of such steroid hormones. As previously mentioned, LC-MS/MS is the current gold standard in hormone detection, however the majority of studies have utilised enzyme-linked or radioimmunoassay kits specifically designed to detect target compounds in particular substrates, such as plasma (Groothuis *et al.* 2019). It's been suggested that a range

of steroids located within the yolk can cross-react with antibodies used in immunoassay kits, leading to inaccurate yolk hormone concentrations being detected (Rettenbacher *et al.* 2013), and thus results being misinterpreted. In addition, the age at which embryos are exposed to target hormones (i.e. CORT) is another potential explanatory factor, with studies exposing avian embryos to target hormones at various ages of embryonic development, ranging from ED 0 – ED 11 (Ahmed *et al.* 2014; Vassallo *et al.* 2019; Paitz *et al.* 2020; Angove *et al.* 2021). If early steroid hormone metabolism does occur, target hormone detection and concentrations are likely to differ as a result of the different stages of steroid metabolism within the yolk at the various stages of development.

The early metabolism of active steroids may therefore explain why CORT had no effect on the relative abundance of free steroid hormones, as these hormones were likely metabolised before exposure to CORT at ED 11. Therefore the ED 11 time point chosen to expose embryos to CORT in trial 3 is likely a limitation as to why no significant differences in relative hormone abundance was detected at any age in trial 3. However it must be noted that ED 11 was selected to maintain consistency between trials within this thesis to ensure results were comparable between trials. Therefore, future studies may opt to expose eggs to compounds of interest at ED 0 to determine whether target treatment influences the presence of free hormones within the yolk, as well as their metabolic conversion to corresponding conjugate metabolites. In addition, exposure to target compounds at ED 0 is biologically relevant, given that developing embryos are potentially exposed to the majority of maternally sourced sex steroids almost immediately. However, the consequences of embryonic exposure to maternal lipophilic steroid hormones through deposition into yolk is still elusive, but does highlight potential new pathways and mechanisms associated with post-hatch phenotypic alterations.

One potential mechanism, although relatively unexplored, is that developing embryos are exposed to steroid hormones contained within the yolk during the process of yolk fat

absorption. The process of yolk fat absorption occurs primarily in the later stages of embryogenesis and utilises non-specific phagocytosis to transfer lipid content into the embryo (Yadgary *et al.* 2010). This non-specific phagocytosis of yolk lipids would enable steroid hormones contained within the yolk to transfer to the embryo with relative ease. Furthermore, exposure to heat and cold stress, as well as restricted access to feed, appears to influence yolk utilisation during the first five days post-hatch (Mikec *et al.* 2006), although to the best of our knowledge, no previous work has investigated the effects of early life stress on yolk fat absorption in chicken meat birds.

The results presented in chapter 5 support the additional hypothesis that embryonic exposure to CORT would decrease yolk lipid content at hatch. The reasons for such a decrease in yolk lipid content are discussed in detail in Chapter 5, but may result from altered energy requirements (Duan *et al.* 2014) and/or be a form of survival mechanism (Henriksen *et al.* 2011b). Nonetheless the ramifications of altered yolk fat content have potentially significant implications for post-hatch phenotypes in chicken meat birds, especially if it provides a source of yolk steroid hormones. This is primarily due to the time of peak yolk fat absorption coinciding with the period in which mesenchymal stem cells proliferate and differentiate into adult myoblasts (satellite cells) (Halevy *et al.* 2006), an essential component for post-hatch muscle hypertrophy. Not only do yolk lipids provide a potential energy source to enable such cell activity, but myogenic commitment of mesenchymal stem cells is significantly enhanced in the presence of the sex steroids (Herbst and Bhasin 2004). In addition, the subsequent removal of sex steroid hormones leads to satellite cell apoptosis (Collins *et al.* 2019), as well as adipogenic commitment of mesenchymal stem cells (Herbst and Bhasin 2004). Although free steroids appear to be rapidly metabolised within the yolk, the potential exists for these hormones to be re-converted back to their active form upon uptake by the embryo, meaning the conjugation process may act as a form of hormone storage. Such a pathway is of intrigue,

as it has potential to expose developing embryos and early post-hatch birds to key myogenic stimulating hormones during a critical developmental period. As satellite cell numbers peak at 2-3 days post-hatch, the potential increased exposure of birds to re-converted steroid hormones as a result of yolk conjugates could stimulate an increase myogenic commitment of mesenchymal stem cells. Such a pathway would explain the increase in total fat mass exhibited by CORT females in Chapter 3, as well as the lower percentage of fascicle area occupied by muscle fibres in CORT treated birds at hatch in Chapter 5. Additionally, alterations to yolk fat absorption rates may differ in a genetic line dependent manner, and provides a potential explanation as to why body composition traits differed both between breeds, and between sexes within breeds, as was identified in Chapter 4. However, significant work is required to not only understand the influence of maternal/*in-ovo* stress on yolk fat content, but also what the consequences of altered yolk fat absorption are on adult bird phenotypes, as it is likely that yolk fat absorption is influenced by separate biological factors (Yadgary *et al.* 2010). In addition, separate factors may influence the presence of free steroid hormones and yolk lipid content in the yolk before embryonic uptake, including breeder rearing environments, nutrition and age (Yadgary *et al.* 2010; Angove and Forder 2020), which may mediate progeny post-hatch phenotypes, despite alterations to embryonic yolk fat absorption and maternal hormone exposure.

Although the mechanisms leading to progeny phenotypic alterations in response to the maternal/*in-ovo* environment remain unclear, evidence stills suggests that the maternal/*in-ovo* environment encountered does influence post-hatch phenotypes in chicken meat birds. Therefore, methods to alleviate such alterations in performance are required. The third aim of this thesis investigated one such method, which was to use nutritional supplements to alleviate variation in progeny performance in response to maternal and/or *in-ovo* environment. However, supplementing breeder hens with *SC* during their production phase in Chapter 4 (23-40 weeks

of age), and birds exposed to *in-ovo* CORT with additional arginine in chapter 1, failed to significantly influence performance traits in commercial broiler chickens. The inability of maternal *SC* provision in chapter 4 to influence both progeny feed conversion ratio and breast muscle yield provides an example of how breeder age may influence progeny performance in relation to the maternal environment encountered. As described in Chapter 4, previous studies had identified differences in progeny performance in response to supplementing older breeder flocks (39-45 weeks of age) with *SC* during their production phase (Kidd *et al.* 2013). However supplementing younger flocks (32-35 weeks of age) resulted in no such improvements (Kidd *et al.* 2013), which coincides with the study findings presented in Chapter 4. As *SC* supplementation appeared to influence plasma CORT concentrations in Chapter 4, and to a lesser extent yolk testosterone concentration, it is plausible to hypothesise that these age dependent differences in relation to *SC* provision may in part be a result of age dependent alterations to the *in-ovo* environment. Thus, any maternal and/or *in-ovo* environment alterations induced through maternal *SC* provision may only be biologically substantial in progeny produced from older flocks. However, further work is required to understand how alterations to the maternal environment interacts with breeder flock age, and what the ramifications are for subsequently produced progeny. Nonetheless, these findings show the importance of ensuring progeny produced from flocks of differing ages are reared separately, and highlights the potential benefits that could be implemented once the effects of the maternal/*in-ovo* environment can be better understood. Furthermore, although the nutritional supplements utilised throughout this thesis failed to influence progeny growth or body composition traits, nutritional supplementation still provides an appealing and potentially cost-effective method of alleviating the consequences of maternal/*in-ovo* stress.

Final Conclusions

The results from this thesis suggest that post-hatch phenotypes in chicken meat birds are in some part a result of a complex interaction between the developing embryo and the *in-ovo* environment encountered, and is influenced by breeder age, line and genetic interactions. Our results demonstrate that *in-ovo* exposure to CORT appears to alter body composition traits in female birds, potentially through the actions of the *CEBP/β* gene. The effects of *in-ovo* CORT on male body composition traits remains to be understood, whilst both line and sex appear to be influential factors in determining the body composition traits exhibited by adult birds. The contributing mechanisms leading to enhanced fat deposition in CORT treated birds may involve the adipogenic regulators *PPARγ*, *CEBP/α* and *CEBP/β*, although further work is required to explore this pathway. Our results suggest that the absorption of yolk lipid contents may be a contributing factor, and provides a novel hypothesis in which lipophilic yolk steroid hormones are transported into the water rich environment of the embryo. Although further work is required, the period between late embryonic development and early-post hatch is a critical time point in which adult body composition traits can be influenced by the satellite cell population that develops during this period. Considering satellite cells are an essential component for post-hatch hypertrophy, alterations to both yolk fat content, as well as yolk steroid content, appear to be plausible mechanisms contributing to body composition. In addition, yolk steroid hormones appear to be readily metabolised to their inactive forms, potentially as a storage mechanism until transfer to the embryo can occur, and thus the physiological effects of conjugate hormones and their metabolic fate are essential in understanding how yolk steroids influence post-hatch phenotypes in progeny. If the physiological mechanisms contributing to phenotypic variations in progeny in response to maternal/*in-ovo* stress can be understood, then targeted nutritional supplementation to either breeder hens, progeny, or both could be implemented to negate any consequences on performance. The results presented in this thesis provide evidence to highlight the significant

effects breeder age, genetics and sex can have on performance parameters mediated through the *in-ovo* environment. Therefore, refining the maternal/*in-ovo* environments encountered by developing progeny has the potential to improve breed/line specific differences in growth, and body composition traits, as well as flock uniformity, in mixed sex chicken meat birds reared in Australia.

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Appendix 1: Supporting Publications – Conference Papers

The following is work undertaken during the PhD and presented at a domestic scientific conference. The presenting author is underlined. The format presented corresponds with the format requirements outlined by the Australian Poultry Science Symposium organisers.

Location - Australian Poultry Science Symposium, 2020, Sydney, Australia

A1.1. In-ovo corticosterone alters body composition in 35 day old chicken meat birds irrespective of dietary arginine content

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Summary

Increasing evidence suggests early-life exposure to maternal stress can permanently alter the development of an embryo. Such findings have significant application to the chicken meat industry due to the pedigree structure of the breeder sector, as transgenerational effects on progeny performance have been previously observed in relation to the maternal environment. Therefore, an *in-ovo* study was developed to investigate the effects of maternal stress in chicken meat birds on subsequent progeny performance traits. Additionally, dietary arginine (Arg) supplementation was implemented as a means of negating any consequences of maternal stress. Eggs were exposed to either the stress hormone corticosterone (CORT) or a control solution during embryonic development. Viable chicks were separated based on *in-ovo* treatment and fed either a control or Arg supplemented diet until 35 days of age. The findings from the current

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study suggest *in-ovo* exposure to CORT negatively influences the body composition of female birds by promoting fat accumulation over muscle formation, with the provision of supplementary Arg potentially alleviating these effects. Furthermore, no interactions between *in-ovo* and dietary treatments were identified in relation to body weight, body weight gain and feed conversion.

I. INTRODUCTION

Human consumption of chicken meat products has risen exponentially over the past five decades and continues to grow on a global scale. Thus chicken meat production has seen unparalleled expansion, to meet the continually increasing consumer demand (Allievi et al., 2015). Such expansion has resulted in the chicken meat industry being at the forefront of animal production, where advances in animal nutrition and genetics are near optimal. Therefore, producers are continuously looking for new and innovative methods to enhance chicken meat performance, with the maternal environment providing an economically viable method to do so. The maternal environment can be described as the overall environment a female organism encounters at the time of reproduction. Several known factors can influence the maternal environment, including maternal stress, nutrition, geographical location and individual health, ultimately altering progeny development, with permanent phenotypic effects (Reynolds et al., 2010). Previous work has shown that exposure to chronic maternal stress can negatively influence progeny performance in production animals (Reynolds et al., 2010). This is a key finding for the chicken meat industry for two primary reasons. Firstly, the chicken meat breeder industry utilises feed restriction measures in their breeder flocks. Although implemented to enhance reproductive outputs in breeder hens, an increasing body of work suggests the use of feed restriction measures induces chronic stress in breeder hens, prompted by extended periods

of prolonged hunger (Zulkifli et al., 2015). Secondly, commercial chicken meat birds now spend ~40% of their life within the *in-ovo* environment and recent findings have clearly exhibited the effects of the *in-ovo* environment on embryonic development in the chicken (Ho et al., 2011). Although there is strong evidence that suggests the maternal environment does impact offspring performance in chicken meat birds, considerable uncertainty still remains as to the precise effects environmental variations have on progeny performance. Therefore, the aim of this study was to utilise an *in-ovo* model to investigate how early-life exposure to stress may influence performance characteristics in subsequent offspring. Additionally, dietary Arg was supplemented as a means of alleviating the negative consequences of *in-ovo* exposure to CORT. Arg has been documented to enhance protein synthesis, whilst promoting the secretion of endocrine factors such as the thyroid hormones and growth hormone, both of which are involved in growth and metabolic pathways. Thus, supplementary Arg is hypothesised to alleviate, to some extent, the phenotypic consequences derived from *in-ovo* exposure to CORT.

II. METHODOLOGY

400 eggs collected from a commercial Cobb 500 broiler breeder flock were separated into two groups, with 200 eggs receiving 1 µg of corticosterone (CORT) dissolved in absolute ethanol and the remaining 200 eggs receiving a control (CON) solution. Solutions were injected into the chorioallontoic membrane at embryonic day 11. At hatch 112 CON and 100 CORT birds were weighed, then separated into four treatment groups, (1) CORT-Control, (2) CORT-Arg, (3) CON-Arg, (4) CON-Control. Birds were provided with *ab libitum* access to both feed and water. Birds fed an Arg supplemented diet received a standard chicken meat diet + 125% Arg:Lys ratio. Individual bird body weights were recorded weekly, along with pen total feed conversion ratio. A sub-sample of birds, three per treatment (n=12) were humanely killed at

day 35 and subjected to a dual-energy x-ray absorptiometry scan (DEXA) to determine total body composition. Analysis of experimental data was performed by linear mixed model analysis following the procedures of IBM®, SPSS® Statistics 25 program (Armonk, NY, USA). The data were checked for normality by the Shapiro–Wilk test. Non-normalised data was analysed using nonparametric tests including Mann-Whitney U and Kruskal-Wallis. A probability level of less than 5% ($P < 0.05$) was deemed as statistically significant.

III. RESULTS

Weight gain between day 0 and 21 did not differ between CORT or CON treated birds, nor were there any sex dependent effects in relation to *in-ovo* treatment ($P > 0.05$) (Table 1). Total weight gain from day 0 to 35 did not differ between *in-ovo* treatments; however a potential sex dependent trend appeared ($P > 0.05$). CORT treated male birds recorded superior weight gain at day 35 compared to CON treated birds. Conversely, CORT treated females recorded lower total weight gain at day 35 than CON treated birds. Additionally, CORT treated males tended to exhibit enlarged breast muscle mass (%bwt), whilst no notable variation was identified in female birds relating to breast muscle mass. Furthermore, FCR was not influenced by *in-ovo*, nor dietary treatment independently, whilst no interaction was detected between *in-ovo* and dietary treatments. Female birds fed an Arg supplemented diet tended to exhibit reduced total breast muscle yield (%bwt), whilst no interaction was observed in male birds in relation to diet ($P > 0.05$). Dietary supplementation with Arg did not significantly influence weight gain at any age ($P > 0.05$). Female birds exposed to CORT *in-ovo* exhibited greater fat mass (%bwt) and reduced total lean mass (%bwt) at 35 days of age. Conversely, CON treated female birds displayed enhanced total lean mass (%bwt) and reduced total fat mass (% bwt) (Table 2). Supplementation of Arg into the diet did not influence total bird body composition at 35 days

of age, although Arg supplementation tended to enhance lean mass and reduce fat mass in CORT treated birds.

Table 1 - Effects of *in-ovo* corticosterone exposure and dietary arginine supplementation on sex dependent performance characteristics in broiler chickens from 0 – 35 days post hatch. Values are average mean ± SEM.

Treatment	*Sex*	*Weight Gain d0 - d21 (g)*	*Weight Gain d0 - d35 (g)*	*Day 35 Breast Muscle % Bwt*
In-ovo				
CORT	Male	906.3 ± 12.58	2573.9 ± 50.61	21.2 ± 0.37
	Female	815.5 ± 10.26	1927.6 ± 29.09	20.4 ± 0.45
CON	Male	888.2 ± 15.38	2504.4 ± 58.33	20.2 ± 0.69
	Female	820.9 ± 10.26	2013.0 ± 39.29	20.5 ± 0.48
P-value				
In-ovo *x Sex*		0.387	0.082	0.325
Diet				
Arginine	Male	900.0 ± 13.58	2511.0 ± 52.37	20.9 ± 0.56
	Female	825.3 ± 9.12	2013.5 ± 28.96	19.8 ± 0.51
Control	Male	894.2 ± 14.75	2560.1 ± 63.09	20.4 ± 0.59
	Female	811.7 ± 10.91	1945.69 ± 37.12	21.0 ± 0.36
P-value				
Diet x Sex		0.739	0.531	0.089

Table 2 - Day 35 body composition of female chicken meat birds subjected to *in-ovo* CORT or CON treatment as well as birds fed an arginine supplemented diet or control diet. Values are average mean (%bwt) ± SEM.

Treatment	*BMC (%bwt)*	*Fat (%bwt)*	*Lean (%bwt)*
In-ovo			
CORT	1.14 ± .04	9.11 ± .59 [a]	88.70 ± .66 [a]
CON	1.15 ± .04	5.71 ± .59 [b]	91.77 ± .64 [b]
P-value	*0.902*	*0.007*	*0.026*

In-ovo + *diet*			
CORT control	1.13 ± 0.04	9.86 ± 1.44	87.82 ± 1.31
CORT arginine	1.16 ± 0.04	8.36 ± 0.86	89.53 ± 0.66
CON control	1.13 ± 0.11	4.76 ± 0.52	91.94 ± 1.31
CON arginine	1.17 ± 0.03	6.66 ± 0.40	91.59 ± 0.59
P - value	*0.910*	*0.088*	*0.295*

[a-b] values within a column with no common superscripts differ significantly ($P < 0.05$).

IV. DISCUSSION

Although the body of work supporting feed restriction induced chronic stress in breeder hens is relatively strong, the transgenerational effects exposure to maternal stress has on subsequent performance characteristics in progeny remains unclear. The present study utilised an *in-ovo* model to investigate the potential phenotypic consequences invoked via *in-ovo* exposure to a stressor in chicken meat birds post-hatch. Previous work by Hynd et al. (2016) reported a reduction in male progeny weight at 42 days of age in birds produced from hens subjected to feed restriction measures. These findings corresponded with feed restricted hens exhibiting elevated plasma corticosterone concentrations and heterophil/lymphocyte ratios, indicating elevated levels of stress, which had been previously reported (Zulkifli et al., 2015).

Interestingly, the results from the current study suggests exposure to maternal stress may act in a sex dependent manner. However, it must be noted that significant variability exists within the literature regarding exposure to maternal stress and its ability to disrupt offspring development. Several avian species have been exposed to maternal stress under experimental conditions, with vastly different results reported between species, as well as within species (Ahmed et al., 2016, Hayward and Wingfield, 2004). Furthermore, supplementing diets with additional Arg did not influence weight gain. Arg has been documented to promote protein synthesis as well as influence growth and metabolic pathways associated with the thyroid hormones (Ebrahimi et al., 2014) and various other growth factors in birds. However, the

chickens utilised in the current study were great grandparent birds, where performance variation is greater in birds positioned higher up the breeding pedigree. The use of such birds may have therefore unintentionally 'masked' any offspring phenotypic variation influenced by the dietary and *in-ovo* treatments administered. Thus, phenotypic variation may occur in separate strains further down the breeding pedigree, as has been reported within the literature.

The findings that female birds exposed to CORT exhibited enhanced total fat mass and reduced total lean mass compared to CON birds is somewhat novel. Such variation may eventuate as a consequence of alterations to the hormonal composition of the egg, disrupting physiological processes that influence the number of myofibres developed by the embryo, which is determined embryonically (Smith, 1963). Although previous studies reported that exposure to maternal stress could 'influence' weight gain in numerous avian species, whether such variation was attributed to muscle, fat or bone mass remained elusive. Additionally, supplementing CORT exposed birds with Arg tended to reduce the phenotypic consequences associated with early-life exposure to CORT. Although male total body composition was not measured due to insufficient numbers, these findings in female birds still suggest that the variations to the maternal environment may promote undesirable carcass characteristics in chicken meat birds. However, targeting the maternal environment provides a novel approach to improve total flock uniformity and subsequent carcass characteristics, albeit in a sex-dependent manner. Thus, future studies investigating the maternal environment and its ability to alter offspring development must incorporate total carcass composition along with weight gain and FCR, with specific interest in the sex-dependent variations that predominantly occur.

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A1.2. Performance characteristics differ between offspring from two chicken meat breeder lines, irrespective of maternal supplementation with a Saccharomyces cerevisiae metabolite

The following is work undertaken during the PhD candidature and accepted to present at an international scientific conference. However this conference was postponed until 2022 due to the global COVID-19 pandemic. The presenting author is underlined. The format presented corresponds with the format requirements outlined by the World Poultry Congress organisers.

Location – Worlds Poultry Congress, 2022, Paris, France

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Optimization of breeder diets to improve progeny performance provides a novel approach to enhance chicken meat production. Studies from the US and Brazil have reported that including yeast products in breeder diets, including *Saccharomyces cerevisiae (SC)* metabolites, reduces stress biomarkers, whilst improving progeny feed efficiency (FCR) and breast muscle (BM) yield. Thus, a study was designed to investigate the effects of *SC* metabolite supplementation in breeder diets, on progeny performance from two breederlines of meat chickens reared in Australia.

Broiler breeders (n=240) from two genetic lines (Line A & B) were separated into four experimental groups based on line (A or B) and diet (control or *SC* (1000 ppm) at 23 weeks of age. At 31 weeks, 82 eggs/treatment were collected and incubated. A sub-sample of eggs (n=7/treatment) were analysed for yolk corticosterone (CORT). At hatch, 160 chicks were separated based on maternal treatment. Chicks had *ab libitum* access to feed and water, with body weight (bwt) and FCR measured weekly. At 42d of age, eight birds/treatment were dual-energy x-ray absorptiometry (DEXA) scanned for body composition (BC). Plasma CORT concentration was analysed at d22, 37 and 42 using ELISA. Experimental data were subjected to a full factorial mixed model analysis for breed and diet factors.

The addition of *SC* increased hatchability in line A hens up until 35 weeks of age ($P = 0.03$). Yolk CORT concentrations were higher in Line B eggs ($P = 0.069$), whilst plasma CORT was increased in Line A progeny at all-time points ($P < 0.05$). Bwt and bwt gain were reduced from d 0–28 in Line A progeny ($P < 0.05$). Line A male progeny exhibited greater bwt gain between d 35-42, with no variation identified between females. Lean mass was increased in Line A male progeny compared to Line A females ($P = 0.036$), whilst fat mass was elevated in Line A females ($P = 0.043$). Line A progeny exhibited

greater BM yield (P = 0.001). Inclusion of *SC* into breeder diets did not influence performance, yolk or plasma hormone concentrations in progeny hatched from 31 week old hens.

These findings highlight performance variation between progeny hatched from two broiler breeder lines. Future investigations tailoring nutritional strategies specific to each broiler breeder line, may be effective in optimising production performance. Additionally, hen age and nutrition are critical factors that may interact to influence the mechanisms regulating progeny performance.

Appendix 2: Doctorate of Veterinary Medicine 1 Research Project

The format presented in this Appendix corresponds with the requirements of the Doctorate of Veterinary Medicine program in which Mr. Mitchell Crago was enrolled, and in which the work presented was a requirement for successful completion.

In-ovo corticosterone administration increases embryonic yolk testosterone concentration in female chicken meat birds

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ABSTRACT

OBJECTIVE Maternal stress in breeder hens has been reported to affect progeny growth and performance, however the underlying physiological mechanisms remain unclear. Secretion of the hormone testosterone, a key metabolic regulator, is reported to reduce upon exposure to increased glucocorticoids. It is suggested that testosterone has implications in the endocrine pathway leading to altered growth and performance. In this study, corticosterone was injected into the chorioallontoic membrane of fertile eggs during embryonic development to determine whether *in-ovo* corticosterone administration into the chorioallontoic membrane influences yolk testosterone concentration.

DESIGN/PROCEDURE 400 fertile eggs were divided into two groups and administered either corticosterone (CORT, 10 mg/mL) or control solution (CON) at embryonic day 11. At embryonic day 15, 40 eggs were randomly selected, embryos humanely euthanised and yolk

samples collected. Testosterone from egg yolk was extracted and quantified using a commercially available competitive inhibition enzyme ELISA kit.

RESULTS A significant interaction was observed between *in-ovo* treatments and sex, where female embyros exposed to CORT *in-ovo* had higher yolk testosterone concentrations (*P=0.032*). Similarly, CORT *in-ovo* treated embryos females had higher yolk testosterone than males (*P=0.048*).

CONCLUSIONS Results from this study shows that alteration to the *in-ovo* environment via administration of corticosterone influences embryonic yolk testosterone concentrations in a sex-dependent manner. These findings highlight the impact of the *in-ovo* environment and its ability to influence progeny development. *In-ovo* administration of corticosterone may disrupt yolk testosterone metabolism which may further impact progeny development and adult performance.

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KEY WORDS: Broiler, Developmental Programming, Androgen, Poultry, Endocrine

INTRODUCTION

Chicken meat over the last decade has become one of the most consumed animal proteins in the world due to the industry's ability to increase its production and adapt to the increasing demand for affordable lean meat.[i] Therefore, the challenge for industry is to continually produce birds to meet consumer demand, but in an economically sustainable manner. As such, studies investigating methods to sustainably optimise chicken meat production are required to advance the chicken meat industry into the future.

The industry currently utilises feed restriction practices in their breeder flocks to regulate weight gain and improve fertility.[ii] In doing so, these hens may encounter chronic hunger stress,[iii] especially during the lay period, exposing the developing embryo to these conditions and possibly altering progeny development and phenotype. Upon exposure to a stressor, the stress hormone, corticosterone, is released and triggers a stress response throughout the body.[iv] During times of reproduction, maternally elevated corticosterone has been documented to influence hormonal composition of the subsequent egg yolk,[v] thus variations to the maternal environment are documented to alter progeny developmental processes,[vi] permanently altering post-hatch phenotypes during adult life.[vii] The maternal environment is described as 'the conditions the mother experiences at the time of reproduction', this involves stress, nutrition and health status, all of which reportedly effect progeny development .[viii,ix]

Testosterone is a maternally transferred androgen, playing a pivotal role in the regulation of embryo differentiation and development. Testosterone promotes muscle and skeletal growth and can support post-natal body mass.[x] Previous studies have demonstrated that maternal plasma corticosterone levels influence reproductive hormone concentrations in the yolk, specifically a decrease in progesterone and testosterone.[xi] Other studies have also demonstrated that alterations to maternally transferred androgens may cause oxidative stress and accelerated metabolism,[xii] which together may then alter progeny growth and performance. Considering the influence of testosterone on phenotypic development in the chicken, it is possible that early-life exposure to corticosterone may alter the phenotype (body composition) of the developing progeny by decreasing skeletal muscle development and increasing adipose development. This phenotypic alteration may derive from disrupted embryonic exposure to testosterone sourced directly from the egg yolk. However, evidence linking disrupted embryonic metabolism of yolk sourced testosterone and altered adult phenotype has not been conclusively identified.

Therefore, an *in-ovo* study was developed to assess the influence of early-life exposure to corticosterone, and its influences on metabolic efficiency during embryonic development,

primarily regarding the metabolism of testosterone sourced from the egg yolk. It is hypothesised that *in-ovo* exposure to corticosterone will inhibit embryonic metabolism of yolk sourced metabolites, subsequently increasing yolk concentrations of testosterone.

MATERIALS AND METHODS

Sample collection

400 Eggs were collected from a commercial line of broiler breeder birds at 37 weeks of age and transported to the University of Adelaide Roseworthy campus where they were placed in an incubator. Incubator settings from embryonic day 0-18 were set to 37.6^{o}C and 55% humidity and from day 18-21 adjusted to 36.7^{o}C and 60% humidity, whilst eggs were rotated 90^{o} every hour.

The eggs were randomly allocated to one of two treatment groups at embryonic day 11. 200 eggs received 0.1mL of a 10 ug/mL corticosterone (CORT) solution, where corticosterone was dissolved in a 5% ethanol solution diluted in phosphate buffer solution (PBS). The control (CON) treated birds received 0.1 mL of 5% ethanol diluted in PBS.

At embryonic day 11, eggs were removed from the incubator and injected through the air sac into the chorioallontoic membrane with 100 µL of either corticosterone or control treatment. After injection, puncture sites were sealed with silicon and eggs were returned to the incubator.

At embryonic day 15, 20 eggs per treatment (n=40) were randomly selected, and embryos removed and euthanised. Yolk sacs were detached from the embryo and weighed before a subsample of yolk was collected and snap frozen in liquid nitrogen and stored at -80^{o}C.

Testosterone extraction

0.15g of yolk sample was mixed with 0.6 mL of PBS and homogenised. Samples were then frozen and stored at -80°C until further analysis was performed.

Yolk extraction was performed using methods of Henriksen *et.al.* 2011. Briefly, samples were thawed at room temperature and shaken with an orbital shaker (Ratek orbital mixer incubator) at 350 rpm at 25°C for 15 minutes. 3 mL of methanol was added to each sample and shaken again for 30 minutes. Samples were then centrifuged at 3525 rpm for 15 minutes.

1 mL of supernatant was then transferred to a new culture tube. Methanol was evaporated by placing the vial of supernatant in a 60°C water bath for 18 hours or until all liquid had completely evaporated. Samples were resuspended in 0.5 mL of PBS pH 7.4 and vortexed thoroughly. Samples were then split into two aliquots.

Testosterone ELISA

A pilot study with a testosterone ELISA (Cusabio – CSB-E12797C) to validate the method and to ensure a positive result was performed using a subsample of refrigerated and frozen samples to confirm if there was a storage effect.

The testosterone assay employs the competitive inhibition enzyme immunoassay technique. 50 µL of sample were added to each well, apart from the blanks and standard wells. The ELISA was then performed as per the manufacturer's instructions. Once testosterone detection was confirmed in the samples with the methodology used, then the ELISA was repeated, using all 40 samples with more consistency and precision to record valid data.

Data Analysis

All statistical analyses followed the procedures of IBM®, SPSS® Statistics 25 program (Armonk, NY, USA), where a mixed linear model was utilised. The data was checked for

normality by the Shapiro–Wilk test. A probability level of less than 5% ($P < 0.05$) was deemed as significant.

RESULTS

A significant interaction was observed between *in-ovo* treatments and sex, where female embryo's exposed to CORT had higher yolk testosterone concentrations in comparison to female exposed to CON (*P=0.032; Figure 1*). Similarly, in CORT treated embryo's, females had a significantly higher yolk testosterone than males exposed to CORT (*P=0.048*).

No treatment effect was observed, irrespective of sex *(Figure 2)*. All other interactions between sex and treatment groups on yolk testosterone concentration were not significant.

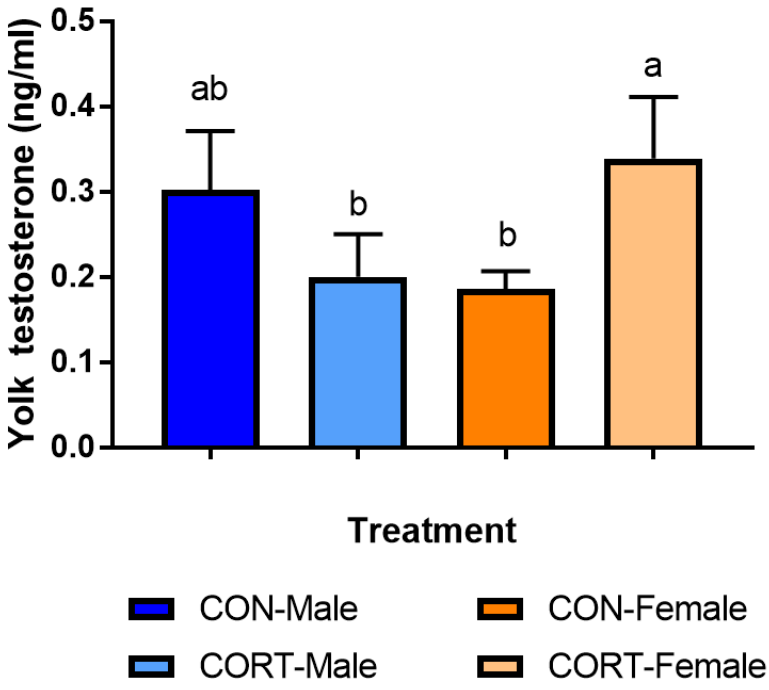

Figure 1. Yolk testosterone (ng/ml) from embryonic day 15 broiler chicken progeny (n=40) exposed to either control (CON) or corticosterone (CORT) solution. Birds were separated depending on sex and treatment group. $^{A-B}$ *values within a figure with no common superscripts differ significantly (p<0.05)*

DISCUSSION

Despite a significant body of research existing regarding maternal-mediated effects on progeny performance, the mechanisms contributing to altered offspring phenotype are yet to be unravelled. This is somewhat due to the variability that exists as to whether alterations to the maternal environment influence the concentration of critical components within the egg that are required to stimulate growth and development. The androgenic hormone testosterone has been widely touted as a mediating factor linking variations to the maternal environment and offspring development. Testosterone hormone synthesis is inhibited by corticosterone[xiii], but corticosterone's ability to influence yolk testosterone concentrations, and subsequent yolk metabolism on bird performance and body composition is not as well defined.

Corticosterone is a steroid glucocorticoid produced by the adrenal cortex via initiation from the hypothalamic-pituitary-axis, in response to a stressor.[13] Elevations in corticosterone concentrations within an individual reportedly down-regulates plasma testosterone concentrations,[xiv] and may influence yolk hormonal composition in the subsequent egg produced by passing hormones into the oocyte from maternal circulation.[xv]

Exposure to early life stress has previously been shown to influence yolk testosterone concentrations within the egg.[xvi] This may result in an altered phenotype exhibited by progeny dependent on yolk testosterone concentrations as a result of *in-ovo* stress.[7] Testosterone is classified as an androgen sex steroid produced by cells in both testis and ovaries.[xvii] The hormone itself acts on androgen receptors located throughout the body and tends to facilitate masculinising effects.[xviii] Testosterone is known to influence growth in both mammals and birds, and promotes muscle formation, bone growth and hair development.[7] Considering testosterones ability to influence growth and development of a wide range of physiological processes, it has been previously identified as a contributing factor to offspring performance variation in response to maternal stress.[xix] This led to a reduction in progeny body weight gain, however could not directly be attributed to yolk androgen concentrations. The results indicated that in female embryos *in-ovo*, a stressor contributed to the addition of corticosterone hormone

to the embryo and lead to a significant increase in yolk testosterone concentration at embryonic day 15. This finding suggests an impact on its metabolism during embryonic development, potential consequences of altered metabolism are reduced skeletal muscle development and increased tendency to develop adipose tissue.

In mammals, androgens stimulate growth by anabolic processes especially in muscle growth (muscle fibre hypertrophy) and boost post-natal body mass gain.[7,xx] Testosterone increases muscle mass with a reciprocal decrease in total body adipose tissue.[xxi] According to Herbst and Bhasin 2004, testosterone stimulates mesenchymal pluripotent cells commitment into myogenic lineage, whilst inhibiting differentiation into adipose cell lineage. Additionally, inhibition of testosterone synthesis resulted in an upregulation of pluripotent stem cells differentiating into pre-adipocytes, potentially increasing total fat mass during adult life. This may be a result of testosterones ability to interact at multiple stages of cell development, which might serve to amplify androgen effects on myogenesis and adipogenesis. Results from this study indicate a significant increase in yolk testosterone concentration at embryonic day 15 of female offspring in corticosterone treated birds. This increase signifies a decrease in metabolism of testosterone which results in decreased skeletal muscle development and increased adipose tissue development.

In-ovo testosterone metabolism in avian species is crucial to develop the myofiber number for post-natal growth.[xxii] A disruption to the metabolism of testosterone by the embryo can result in reduced myofibres of adult bird. Exposure to stress *in-ovo* may inhibit yolk metabolism as glucocorticoids are known to suppress growth and metabolic events.[xxiii] Considering chicken embryo's source nutrients directly from the yolk a reduction in metabolic events would result in reduced exposure to nutritional and hormonal components contained within the egg yolk. These are important for stimulating growth and is likely to be detrimental when not fully metabolised. Considering the previously mentioned anabolic functions of testosterone on development, muscle fibre numbers and growth would be expected to reduce when hormonal

metabolism is reduced. This would likely result in elevated yolk concentrations of testosterone, as identified in this study as the hormone is not being metabolised from the yolk.

A clear mechanism of how exposure to corticosterone inhibits testosterone metabolism is not clearly identified but may link with the activity of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11HSD1). This enzyme is a NADPH dependent reductase that converts an inactive cortisone to the active corticosterone.[xxiv] 11HSD1 is found in many tissues (liver, adipose, gonads, brain and vasculature) which are abundant in glucocorticoid rather than mineral-corticoid receptors.[24]

Previous work by our research group identified that *in-ovo* exposure to corticosterone upregulated 11HSD1 gene expression in female birds.[xxv] This could result in more active CORT being readily available. The active CORT binds to the glucocorticoid receptors[24], which may exert its effect on inhibiting yolk metabolism reducing embryonic exposure to testosterone resulting in increased adipose tissue development and decreased development of skeletal muscle. Conversely, in male birds the 11HSD1 gene expression was downregulated, CORT would likely remain inactive, and therefore unable to bind to glucocorticoid receptors and exert its metabolic effects on testosterone.[15] As such yolk metabolism occurs as expected, exposing the embryo to testosterone. The results from this study support this mechanism, as no variation between male embryos exposed to CORT or CON treatments were identified. Additionally, testosterone concentrations, primarily within the embryo itself, are significantly elevated in male progeny,[21] and thus a reduction in yolk metabolism of the hormone may not result in such severe phenotypic alterations, as has been documented in females. As body composition in males have not been measured in a field study, whether this reflects the male birds body composition still needs to be determined.

CONCLUSION

The findings from this study have identified that yolk testosterone is elevated in female birds exposed to corticosterone *in-ovo*. These findings coincide with altered body composition in female birds exposed to corticosterone, exhibiting increased fat mass and reduced lean mass. These results suggest that stress exposure *in-ovo* may potentially inhibit yolk hormone metabolism and thus reduce embryonic exposure to hormones such as testosterone. As testosterone is an important factor in determining the number of muscle fibres generated which is determined embryonically, reduced exposure may be detrimental in regards to growth and muscle yield. Further investigation is required in both females and male birds to ascertain sex-linked mechanisms behind differences in body composition.

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