Identification of biological factors that can be consistently linked to performance variation in modern commercial broiler flocks

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ABSTRACT

The intensification of the chicken meat industry over the past 50 years has resulted in a 400% increase in the growth rate of meat birds and a 50% reduction in feed conversion ratio, maintaining poultry as a cost-effective source of protein. Improvements have been a direct result of genetic selection for growth and feed efficiency (85-90%), advances in poultry nutrition and improved management practices. Despite production gains, performance variation remains both within and between broilers strains, which is a negative economic trait resulting in losses to producers and the industry alike. We therefore aimed to elucidate biological factors contributing to variations in growth and performance, particularly in meat birds.

As growth has been repeatedly shown to be an immunological trade-off, the first study investigated whether functional changes in intestinal barrier function and innate immunity could be consistently linked to the phenotypic expression of feed conversion ratio (FCR) in meat birds. Genes in the small intestine were investigated between high- and low-performing phenotypes (selected on individual FCR), collected from three separate trials. There was no evidence linking flock performance variation with basal parameters of innate intestinal immunity in the ileum in this study. Higher variation in the expression levels of two genes, Toll-like Receptor 2 (*TLR2*) and membrane protein *CD36* were of interest however, as both exhibit numerous overlapping and individual functions contributing to both innate immunity and fatty acid metabolism.

A second study was conducted to investigate whether links between innate immunity and fatty acid metabolism could be contributing to variations in growth and performance. Total carcass fat %, carcass and blood lipid composition, key genes involved in fatty acid metabolism and selected innate immune parameters were investigated in meat birds, layer birds and F1 layer x meat bird crosses at d14 post hatch. The results indicated a total upregulation of fatty acid

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metabolism in meat birds when compared to the F1 cross and layer birds, for both fatty synthesis as well as β-oxidation in the liver, suggestive of altered metabolism. There was no evidence to suggest that any birds were exhibiting cellular hepatic stress or that fatty acid metabolism was interacting with parameters of innate immunity in this study.

A third study used RNA-Seq to compare liver transcriptomes of meat birds, layer birds and their F1 cross. The objective was to identify differentially expressed (DE) genes between differing growth phenotypes to identify genes and biological pathways contributing to growth variations. Of the total genes identified, 155 were DE between all three groups. Transcriptional differences between the groups were large, particularly between meat birds and layers. Of the genes analysed, 19% were DE between meat birds and layers; 9.6% of genes DE between meat birds and cross; and 1.6% of genes DE between cross and layer birds. The most significant finding was the repeated enrichment of the FoxO signalling pathway, particularly genes related to cell cycle regulation and the insulin receptor. There was also a high correlation between FoxO pathway genes and bodyweight, as well as genes related glycolysis and bodyweight.

In summary, this thesis explores several biological factors associated with growth and performance variation in commercial meat birds. The results indicated that intestinal barrier/innate immune function was not associated with the phenotypic expression of FCR nor was altered immune function detected with differential fatty acid metabolism between birds differing in growth potential. There was however significant evidence implicating the FoxO signalling pathway (via cell cycle regulation and altered metabolism) as an active driver of growth variations in chicken. We recommend further functional characterisation and analysis of this pathway, in meat birds in particular, to further characterise variations in growth and performance.

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 Performance Variation

The structure of the chicken meat industry in Australia (often termed broiler industry, which is used interchangeably throughout this thesis), can be summarised typically in the following steps, as reviewed by (Tallentire et al., 2016). Breeding companies own and control the pure breeding lines, from which all the companies' chicken meat products will descend. These birds are subject to intense genetic selection, and give rise to great-grandparent breeding stock, which undergo trait selection. In the earlier days of industry intensification, growth rate was the primary selection trait, however in recent decades greater emphasis has been placed on increasing breast muscle yield and feed efficiency (Emmerson, 1997). The resultant grandparent lines are cross-bred to produce the parent breeding stock, which are distributed to integrated producers. The hybrid parent stocks are cross-bred to produce the meat birds raised for slaughter by production companies. This vertically integrated system has been in place since the 1950s and has allowed for great advancement in the chicken meat industry pertaining to improved genetics and overall production efficiency.

The most recent comparisons of heritage line meat birds (unselected since the 1950s), with modern meat birds shows that meat chicken growth rates have increased by over 400% (Zuidhof et al., 2014). Much of this improvement has been attributed to genetic selection alone (85-90%), with the remainder attributed to advances in poultry nutrition and improved management practices (Havenstein et al., 2003a, Havenstein et al., 2003b, Zuidhof et al., 2014). Production gains have maintained chicken meat as a cost effective source of protein. Genetic selection for growth however, has been coupled with negative metabolic disturbances. Modern meat birds are commonly predisposed to; excessive fat deposition, particularly abdominal fat

(Foud and El-Senousey, 2014), increased skeletal defects (Bessei, 2006), metabolic disorders including pulmonary hypertension and sudden death syndrome (Julian, 2005, Olkowski et al., 2007), as well as altered immune function (Cheema et al., 2003), especially when compared to slower growing lines such as layers and heritage line meat birds. Additionally, performance variation remains both between strains and within flocks, particularly in regards to efficient use of feed (Emmerson, 1997, Tallentire et al., 2016).

Feed efficiency in the poultry industry is generally measured by feed conversion ratio (FCR), that is, the amount of feed required (kg) to produce 1 kg of bodyweight. Variations in mean broiler FCR values within flock have a negative impact on economics. For example, feed costs account for ~70% of total costs in a chicken meat enterprise production (Aggrey et al., 2010). To put the importance of FCR variation into an economic context, a bird with an FCR of 1.5 would require 3 kg of feed in order to reach a slaughter bodyweight of 2 kg. If FCR was increased by 10% to 1.65, then the bird requires 3.3 kg of feed to reach a slaughter weight of 2 kg. Whilst a 300 g difference in feed may not appear dramatic on first consideration, if applied on an industry scale the economic implications are enormous. For example, in 2015, ~591 million chickens were slaughtered for meat production (Figure 1) in the Australian Chicken Meat Industry (ABS, 2016a, ABS, 2016b). A 10% increase in FCR (300 g) on an industry scale (~591 million birds), would require an additional 177,300 tonnes of feed to produce birds to 2 kg of live weight. In monetary value, if poultry feed was $300/tonne for example, an additional 177,300 tonnes of feed equates to $53,190,000. This is a conservative example of the economic implications of variations in efficiency. Additionally, consequence of variations in FCR stretch beyond the producer and industry, as there are also environmental and sustainability impacts, related to production of feed. These include increased agricultural land use for crop production,

as well as increased greenhouse gas emissions from fossil fuels used for crop production (Tallentire et al., 2016).
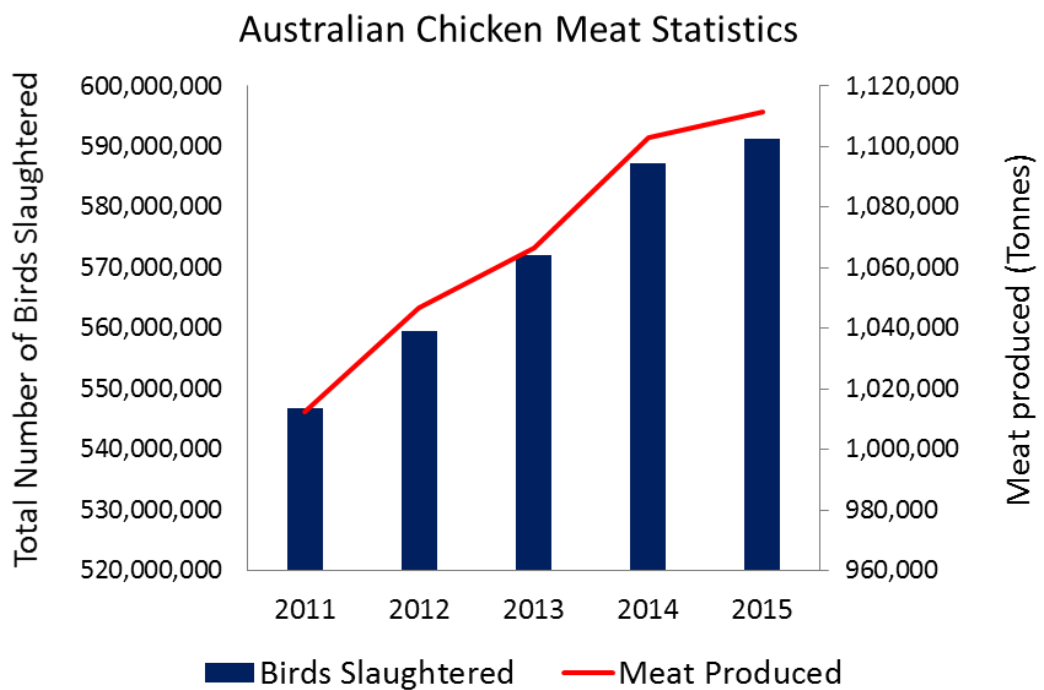
Figure 1 Australian Chicken Meat Industry production statistics from 2011-2015 complied from the Australian Bureau of Statistics (ABS, 2016a, ABS, 2016b). Total number of birds slaughtered on the left axis and volume of meat produced presented of the right axis.

Despite significant production gains over many years of research, much information remains to be elucidated on the biology driving variation in performance. Understanding the biology is critical economically, and, for sustainable meat bird production to continue, particularly with global projected population increases. Our aim was to add to the current understanding by investigating several biological factors that have been negatively associated with growth and performance variation in poultry, with particular emphasis on meat bird production.

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CHAPTER TWO: Literature Review Part A

Biological factors linked to performance variation in commercial broiler flocks: Understanding the role of intestinal innate barrier function in poultry

2.1 Abstract

The avian gut hosts a diverse complex of both commensal and pathogenic microbiota. The maintenance of gut health is reliant on intestinal homeostasis, maintained by precise co-ordination of the 'first-line' defence, comprising the innate immune system and the intestinal barrier. Differences in host innate immune responses to both commensal and pathogenic microbiota may underlie flock performance variability in domestic avian breeds. This review examines parameters of the intestinal barrier and the innate immune system with a primary focus on the avian small intestine. The aim was to determine whether there are functional differences that can be consistently identified and linked to variations in growth and performance between individual birds in commercial meat bird flocks.

2.2 Introduction

In intensively-produced poultry breeds, selection criteria for increased performance efficiency (growth, reduced feed conversion ratio) has resulted in selection based heavily on growth characteristics, likely in part compromising the immune system, as heavier breeds of poultry have shown to be less responsive to immune challenges (Cook et al., 1993, Lochmiller and Deerenberg, 2000). Factors such as health, breed, sex, diet, genetics and environmental conditions are all known to influence performance (Stanley et al., 2012); however, when accounted for in an experimental setting, the growth performance of individuals birds remains variable, which ultimately results in an economic loss to the producer (Stanley et al., 2012). Given that intestinal barrier function and animal production are intricately linked (Kohl, 2012), it is reasonable to postulate that individual birds performing better may have a more functionally efficient innate immune system.

The avian gastrointestinal microbiota, like other vertebrates, represents a diverse and complex ecosystem, the diversity and stability of which influence the nutritional status, immune function and performance of the host (Kohl, 2012). Intestinal homeostasis is maintained by

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precise coordination between different components of the 'first-line defence' barrier with the ability to distinguish between 'self' and 'non self', and excluding harmful pathogens to ensure an inappropriate immune response is not mounted (Medzhitov and Janeway Jr, 2002). This is concerned primarily with the mucosal gel layer, and the innate immune system, which functions at a background level; however, can be activated by microbial or antigen exposure to produce an immunological response that is rapid, non-specific and amplified (Finlay and Hancock, 2004). Intestinal homeostasis can therefore be seen as a balancing act with diminished or highly activated innate immune responses resulting in intestinal inflammation, potentially leading to decreased performance (Asquith and Powrie, 2010, Finlay and Hancock, 2004, Kohl, 2012).

There are several key factors which contribute to avian mucosal barrier integrity; 1) The overlying mucus layer, formed by secreted mucin glycoproteins (primarily the intestinal MUC2 protein) from intestinal goblet cells interspersed in the epithelium (Deplancke and Gaskins, 2001). The mucus gel layer maintains the integrity of the underlying intestinal epithelium by protecting against vigorous digestive processes, both chemical and mechanical (Deplancke and Gaskins, 2001). 2) Epithelial tight junctions, seal the paracellular space between enterocytes and are the principle determinant of mucosal permeability (Turner, 2009). 3) Secreted antimicrobial peptides such as β-defensins, cathelicidins and lysozyme, which act against a broad spectrum of microbial organisms (Boman, 1995); and 4) Immunoglobulin A (IgA) secretions into the intestinal lumen, blocking the adherence of microorganisms to the mucous membrane and entrapping antigens and microorganisms in the mucus layer thereby facilitating their removal (Mantis et al., 2011).

In avian species, innate recognition of microbes and other foreign substances (dietary antigens) in the intestine occurs through pathogen recognition receptors (PRRs) (Akira et al., 2001). These function by recognition of conserved molecular motifs (MAMPs or microbe-associated molecular patterns), which are both essential and inherent to a broad range of

bacteria, viruses, fungi and parasites (Brownlie and Allan, 2011). Of the PRRs the best characterised (and evolutionary conserved across species) are the Toll-like receptors (TLRs), 10 of which have been identified in domestic avian species (Brownlie and Allan, 2011). TLRs and subsequent signalling pathways have been shown to be involved in epithelial cell proliferation, immunoglobulin A (IgA) production, maintenance of tight junctions and antimicrobial peptide expression (Abreu, 2010, Lavelle et al., 2010).

Several studies and reviews have investigated key avian innate immune parameters including; the composition and modulation of the mucus gel layer and the expression of avian MUC genes (Byrne et al., 2007, Forder et al., 2011, Cao et al., 2012b, Lang et al., 2006); intestinal expression and function of avian TLRs (Leveque et al., 2003, Iqbal et al., 2005, Brownlie and Allan, 2011); and the expression and bactericidal activity of select avian antimicrobial peptides (van Dijk et al., 2008, Harwig et al., 1994, Evans et al., 1995). These studies have demonstrated differential innate immune profiles in several domestic avian breeds in response to pathogenic challenges, dietary modulation, and between birds differing in their resistance to pathogens. The last finding may suggest that those birds showing greater resistance to certain pathogens may have more functionally efficient immune systems. It has yet to be elucidated whether differential immune responses can be linked or attributed to variations in flock performance in domestic avian species.

This review examines the current literature examining the expression and modulation of genes and proteins involved in several key components of the avian innate immune system. This review was not an attempt to comprehensively review all areas, but rather to highlight common defining components of the innate immune system and intestinal barrier function, with a primary focus on the avian small intestine.

2.3   Avian Intestinal Barrier Structure

The intestinal mucosa is the innermost layer of the gastrointestinal tract, comprising an epithelial layer attached to the lamina propria by the basement membrane (Figure 1). The epithelial layer is protected by a multifunctional mucus gel-layer which is primarily comprised of mucin glycoproteins, antimicrobial peptides and secretory immunoglobulin A (SIgA) (Dharmani et al., 2009, Sklan, 2005). The intestinal epithelium is interspersed with goblet cells which produce and secrete mucin glycoproteins, the major component of the mucus-gel layer (Klasing, 1999). The apical and basal regions of the villi are also populated with lymphocytes known as intraepithelial lymphocytes (IELs) (Vervelde and Jeurissen, 1993). These populations consist primarily of; natural killer cells (Gobel et al., 2001), T-cells (Vervelde and Jeurissen, 1993), and a small number of B cells and heterophils (Beal et al., 2006).

Heterophils are the avian equivalent of the mammalian neutrophil and are known to produce a range of cytokines (Kogut et al., 2005), TLRs (Kogut et al., 2005, Iqbal et al., 2005) and antimicrobial peptides (Evans et al., 1994). In mammals, Paneth cells located in the intestinal crypts secrete a range of antimicrobial peptides; in avian intestine however, there is no conclusive evidence that Paneth cell types exist (Sklan, 2005). The lack of Paneth cells suggests that macrophages and heterophils may play key antimicrobial roles in the avian intestine and are likely the main source of antibacterial substances (Beal et al., 2006). Lymphoid aggregates are also found scattered throughout the avian intestine, thought to be similar to mammalian Peyer's patches (Befus et al., 1980). The overlying epithelium in these areas is specialised, comprised of M cells, with few if any goblet cells present (Burns and Maxwell, 1986). The role of M cells is both phagocytosis and to sample luminal material for presentation to macrophages and dendritic cells in the underlying lamina propria (Beal et al., 2006, Jeurissen et al., 1999).
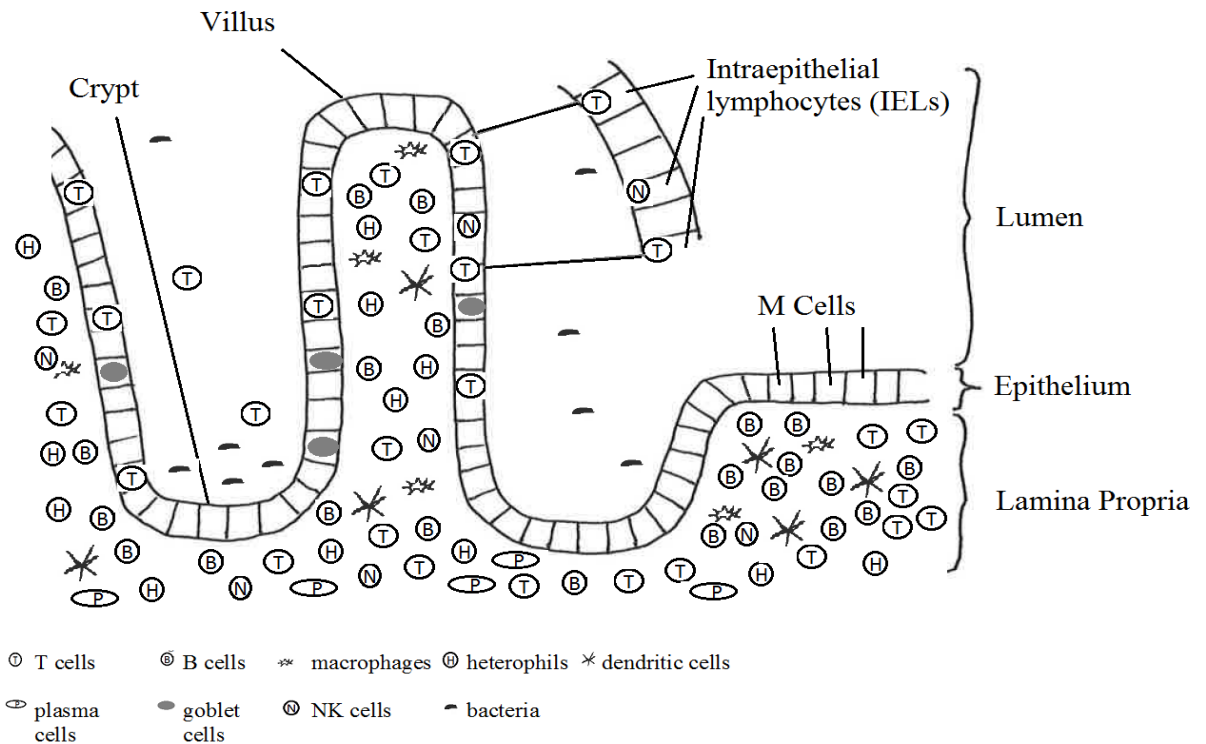
Figure 2.2 Schematic of the avian intestinal structure including the lumen, epithelium, lamina propria and the differing cell populations found in these regions. Schematic adapted from Smith and Beal (2008).

The lamina propria, is populated with a variety of leukocytes similar to the IELs, including granulocytes, macrophages and lymphocytes of B and T cell lineage (Smith and Beal, 2008, Beal et al., 2006). The lymphocyte populations found in both the epithelial lining and the lamina propria are similar in cell type, however the distribution ratios of cells present vary, and, may be altered by age, genotype, pathogen status and diet (Beal et al., 2006). Dendritic cells are also found in the lamina propria, and are specialised for antigen capture and processing, and function as messengers between the innate and acquired immune system, while the plasma cells are responsible for the production of IgA, having differentiated from B cells (Beal et al., 2006).

The combined intestinal structure, as well as chemical properties of the mucosa, function in precise co-ordination to maintain intestinal function and homeostasis.

2.4   The Mucus Gel Layer

The mucus gel layer is the integral structural component that overlies the surface of the intestinal mucosa. It is a complex mixture of water (~95%), mucin glycoproteins (~5%), cellular macromolecules, electrolytes, microorganisms and sloughed cells (Turck et al., 1993, Faure et al., 2003, Strous and Dekker, 1992). Throughout the gut, the mucus layer varies in its morphology and function. For example, the mammalian stomach contains a continuous layer of mucus, whereas the mucus layer in the small intestine is thin and discontinuous (Deplancke and Gaskins, 2001). The thickness of the mucus layer increases gradually from anterior to posterior, the thickest observed in the colon (Atuma et al., 2001). In the stomach and colon, the layer can be further divided into two sub layers; 1) an unstirred adhesive inner layer anchored to the intestinal epithelia, and 2) a loosely adherent out layer that is in direct contact with the lumen (Deplancke and Gaskins, 2001, Johansson et al., 2011, Johansson et al., 2013). Both these layers exhibit different properties and therefore functions.

The properties of the mucus gel layer as a protective barrier for the gastrointestinal mucosa are numerous (Claustre et al., 2002, Deplancke and Gaskins, 2001). The integrity of the underlying epithelium is protected against vigorous digestive process, both chemical and physical, as the mucus gel layer creates an unstirred layer for lubrication (Claustre et al., 2002, Deplancke and Gaskins, 2001, Lien et al., 2001, Johansson et al., 2013). The mucus gel layer also functions as a diffusion barrier by preventing large molecular weight compounds such as proteolytic enzymes from degrading the underlying mucosa (Lien et al., 2001, Johansson et al., 2013). Additionally, the access of microorganisms to the mucosal surface is blocked by competitive exclusion, via binding of the mucus glycoproteins with receptors on the underlying epithelial cells (Mack et al., 2003, Johansson et al., 2013). This in turn reduces colonisation and

favours their removal as the bound bacterial is removed distally with mucus turn over in the small intestine (Mack et al., 2003, Johansson et al., 2013).

2.4.1 MUC Genes and Mucins

Mucin core peptide genes (MUC genes) are responsible for expression of the mucin peptide backbone (Forstner and Forstner, 1994). These genes are characterised by the possession of tandem repeats which account for the high proline, threonine and serine content (PTS domains), as well as the production of unique mucin core proteins (Forstner and Forstner, 1994, Klinken et al., 1995, Johansson et al., 2011). The serine and threonine residues within the PTS domain are highly *O*-glycosylated, giving the mucin a filamentous or "bottle-brush-like" structure (Klinken et al., 1995, Strous and Dekker, 1992, Johansson et al., 2011).

Mucins genes encode for two type of molecules, the secretory gel forming mucins (***MUC2, MUC5AC, MUC5B*** and *MUC6*) and the transmembrane mucins (*MUC1,* ***MUC3, MUC4, MUC12, MUC13***, *MUC16* and ***MUC17***) which cover the apical surface of the enterocytes or other epithelial cells (**intestinal mucins in bold**) (Johansson et al., 2011). In the chicken genome three transmembrane mucins (*MUC4,* ***MUC13*** and *MUC16*) and four secretory mucins (***MUC2, MUC5AC, MUC5B*** and *MUC6*) have been identified (Lang et al., 2006). These genes share similar homology to human MUC genes, however the chicken has an additional gene (Between *MUC2* and *MUC5AC*) not found in mammals, that codes for a mucin protein similar to human *MUC2* but lacks a PTS domain (Lang et al., 2006).

A high level of polymorphism has been shown in both sequence and length in the PTS domain of human MUC genes and has been attributed to multiple alleles of variable number tandem repeats (VNTRs) (Jiang et al., 2013). Comparative sequencing of *MUC2* in humans and White Leghorn birds determined that the two genes are highly divergent within the PTS domain (Jiang et al., 2013). As functionality is dependent on the *O*-glycosylated state of the PTS domain

(Klinken et al., 1995), functional differences may be anticipated. This has been demonstrated *in vitro* between primary human and poultry (Cobb 500 broiler) intestinal cells infected with *Campylobacter* strains (Byrne et al., 2007). In humans, exposure to *Campylobacter* is a common cause of serious diarrhoeal disease, whereas avian exposure results in prolonged colonisation at high density but without apparent disease or inflammation (Connell et al., 2012). Byrne et al. (2007) found that mucus of chicken origin, but not human, appeared to inhibit the *Campylobacter* from interacting with epithelial cell surfaces and significantly reduced the infection of primary human intestinal cells.

2.4.2 Modulation of the Mucus Gel Layer and Effects on Barrier Integrity/function

Secretion of mucins from goblet cells is constitutive (i.e. continuous) however can be accelerated with the acute release of stored mucin granules from goblet cells in response to exposure to stimulants such as irritant gases, nerve activation, reactive oxygen species, inflammatory mediators and changes in the micro-biophysical environment (Smirnova et al., 2003, Deplancke and Gaskins, 2001). Changes in both the secretion and the composition of mucin structures can have varying effects on the intestinal barrier, which if breached leads to inflammation. The composition and amount of mucus produced is a balance between luminal mucus degradation by chemical and physical forces, and the renewal of mucins by goblet cell secretions in the intestinal crypts (Lien et al., 2001). Mucus barrier integrity can be affected by drug-induced changes in response to illness (Slomiany et al., 2001), as well as stressors such as starvation, shown to decrease intestinal mucin quality in both the rat (Sherman et al., 1985) and the chicken (Smirnov et al., 2004). Dietary modulation (inclusion of growth promoters, probiotics, probiotics and phytogenic feed additives), has also been documented to alter not only the intestinal bacterial populations, but also the mucin dynamics, including mucin gene expression, secretion and mucin monosaccharide composition (Tsirtsikos et al., 2012, Smirnov et al., 2005). Furthermore, alteration of bacterial populations can influence signalling between

intestinal microbiota and the epithelial cells, resulting in the stimulation of mucin gene expression as well as bacterial production of mucin degrading enzymes (Sklan, 2005).

Several strains of *Lactobacillus* have been shown to stimulate the up-regulation of *MUC2* expression in human Caco-2 cell-culture models (Mattar et al., 2002), induce *MUC2* expression and secretion by colonic epithelial cells (Cao et al., 2012b), and up-regulate *MUC2* expression in the chicken jejunum and ileum (Cao et al., 2012b). Poultry trials aimed at better understanding the protective properties of the mucus layer in response to *Clostridium perfringens* have shown reduced expression levels of *MUC2* and *MUC13* as well as increased levels of *MUC5AC* in the intestine in response to an *Eimeria* spp./*Clostridium perfringens* challenge (Forder et al., 2011). Interestingly, *MUC5AC* is commonly expressed in the airways and stomach rather than the intestine, however levels were also increased in the intestine of *MUC2* deficient mice when challenged with a parasitic infection (Hasnain et al., 2010). While changes in mucin dynamics are known to occur under bacterial and parasitic challenges, it is not well established whether differential mucosal compositions are seen amongst birds in an unchallenged flock setting, and whether this can be linked to performance variation (i.e. FCR and growth). As bacterial composition is likely to vary greatly amongst individual birds, it is reasonable to question these effects on the modulation of the mucus gel layer.

2.5   Epithelial Tight Junctions

Epithelial tight junctions are the principal determinant of mucosal permeability. These multi-protein complexes seal the paracellular space between enterocytes and are composed of transmembrane proteins (claudins), peripheral membrane (scaffolding) proteins (zona occludens proteins), and regulatory molecules, including kinases, which play an important role in the sorting and assembly of tight junctions (Turner, 2009, Stuart and Nigam, 1995). Paracellular transport across the tight junctions occurs through two routes (Van Itallie et al., 2008). The first is the leak pathway, characterised by no charge selectivity and allows for the

transport of large solutes (although whole bacteria are excluded) and the limited flux of proteins (Turner, 2009). The second pathway consists of small pores defined by claudin proteins. The combination of types of claudins, as well as the ratio of their expression, is thought to determine variations seen in junction tightness (Gonzalez-Mariscal et al., 2003). It appears that the number of pores, rather than pore size, differs among cell types influencing intestinal barrier permeability (Van Itallie et al., 2008). For example, the induction of claudin-2, but not -4, -14 or -18, has been shown to increase the number of small pores in both MDCK II and MDCK C7 (canine kidney) cell lines (Van Itallie et al., 2008). The effects in avian intestinal cells however, have not been determined.

Cytokines, such as interferon-γ (IFNγ) and tumor necrosis factor (TNF-α) have been shown to increase flux across tight junctions and increase barrier dysfunction in Caco-2 cells, correlated with changes in claudin-1 (*CLDN1*), occludin and zona occludens 1 (*ZO1*) distribution within the tight junctions (Watson et al., 2005, Wang et al., 2005). TNF-α induced activation of the myosin light chain kinase pathway (MLCK) is also thought to increase paracellular flux through the leak pathway by increasing myosin II regulatory light chain phosphorylation (Wang et al., 2005). MLCK activation has been identified as an intermediate in barrier dysfunction and therefore can be considered a common final pathway of acute tight junction regulation in response to a broad range of immune and infectious stimuli (Turner, 2009).

Investigations into the developing chick intestine focused on claudins -3, -5 and -16 and determined that expression begins in the embryonic intestine during the third week of development, peaks just prior to hatch before then decreasing significantly by 2d post hatch (Ozden et al., 2010). *In vivo* (SCBN cell line) and *in vitro* (White Leghorn) experimentation showed claudin-4 integrity and barrier function to be disrupted after incubation with *Campylobacter jejuni* (Lamb-Rosteski et al., 2008). In human and mouse studies, barrier

defects associated with intestinal disease including Crohn's disease and ulcerative colitis commonly result in the up regulation of claudin-2, MLCK activation and the down regulation of occludin (Blair et al., 2006, Heller et al., 2005, Zeissig et al., 2007). Tight junction homeostasis relies on a balance between immunoregulatory and pro-inflammatory responses, therefore disruption can occur if there are exaggerated responses to pro-inflammatory cytokines. It has also been suggested these responses may be associated with mutations in the endoplasmic reticulum (ER) stress response transcription factor, the X-box-binding protein (*XBP1*) (Turner, 2009), which will be discussed in section 2.6.5.

2.6   Other Key Components of Intestinal Innate Immunity

2.6.1   Toll-like Receptors

The ability to recognise microorganisms depends largely in part on Toll-like Receptors (TLRs). TLRs are evolutionary conserved across species and are the best characterised of the pathogen recognition receptors (PRRs) (Cormican et al., 2009). They function by recognition of conserved molecular motifs (MAMPs; or microbe-associated molecular patterns) which are both inherent and essential to a broad range of bacteria, viruses, fungi and parasites (Brownlie and Allan, 2011). Toll-like Receptors are structurally comprised of an extracellular N-terminal domain, containing leucine-rich repeats (LRRs) and one or two cysteine-rich regions, a transmembrane domain, and a highly conserved cytoplasmic Toll/IL-1 receptor (TIR) domain (Cormican et al., 2009). The organisation of the LRRs (which varies between both TLRs and species) provides the protein framework to allow for specific interaction with respective MAMPs (Brownlie and Allan, 2011). A specific signalling cascade is initiated upon interaction between a MAMP and a specific TLR, resulting in the activation of transcription factors and the expression of innate immune response genes (Brownlie and Allan, 2011). The TIR domain of the TLR interacts with adapter proteins such as MyD88 which results in the activation of nuclear factor κB (NK-κB) and the mitogen-activated protein (MAP) kinase signalling cascade

(Barton and Medzhitov, 2003). There are five avian adaptor proteins (MyD88, TIRAP/MAL, TRICAM-1, TRICAM-2 and SARM) which can be recruited upon TLR activation (Cormican et al., 2009) Most TLRs can signal through multiple adaptor proteins and all avian TLRs, except *TLR3*, can signal through the adaptor molecule MyD88 (Cormican et al., 2009). Toll-like Receptor 3 signals solely through MyD88 independent pathway, instead recruiting the TICAM-1 adaptor molecule (Cormican et al., 2009).

The regulation of TLR signalling in the intestine is critical, as prolonged and/or excessive activation can lead to uncontrolled inflammation (Shibolet and Podolsky, 2007). Negative regulation of TLR signalling prevents this; however, it is yet to be determined if this is a general immune response or more tissue or cell specific (Shibolet and Podolsky, 2007). Although TLRs recognise the ligands of pathogens, many commensal bacteria also produce the same ligands and it is not well understood how the two are distinguished (Rakoff-Nahoum et al., 2004). As pathogenic bacteria are equipped with TLR detectable virulence factors that allow them to pass through epithelia barriers, it is likely the separation of the indigenous microbiota from the epithelium by the mucus layer plays an important role in the prevention of TLR activation by commensal bacteria (Rakoff-Nahoum et al., 2004). Defects in TLR signalling have been shown in MyD88-/- mice, demonstrating a homeostatic imbalance of intestinal epithelium which reveals an increase in cellular proliferation in the middle and upper regions of the colonic crypts additional to the base of the crypts (Rakoff-Nahoum et al., 2004). Toll-like receptor signalling, particularly TLR2, functions to maintain intestinal epithelial homeostasis and protection from epithelial injury (Lavelle et al., 2010), while TLR4 signalling is important for leukocyte signalling and disease prevention (Humphrey and Klasing, 2004). To date, ten avian TLR genes have been confirmed (Table 2.1) five of which have clear orthologues to those found in humans and mice, (*TLR2a, TLR2b, TLR4, TLR5* and *TLR7*). *TLR15* appears to be unique to avian species and an additional putative orthologue to *TLR21,* found in fish and amphibians, and present in avian species.

Table 2.1 Avian Toll-like receptors (Brownlie and Allan, 2011)

TLR	Other names	Agonist	Pathogen
TLR1La	TLR1.1, TLR1/6/10,TLR 16		
TLR1Lb	TLR1.2	Lipoprotein	Mycoplasma
TLR2a	TLR2.1	Peptidoglycan	G + Bacteria
TLR2b	TLR2.2		
TLR3		dsRNA	Viruses
TLR4		LPS	G- Bacteria
TLR5		Flagellin	G- Bacteria
TLR7		Imiquimod, ssRNA	Viruses
TLR21		CpG motifs, chromosomal DNA	Bacteria and viruses
TLR15		Unknown?	

Toll-like receptor expression (Table 2.2) has been identified in avian heterophils, macrophages and intestinal tissue (Kogut et al., 2005, Iqbal et al., 2005, Lu et al., 2009). Constitutive expression of *TLR1La, TLR2a, TLR2b, TLR3, TLR4, TLR5*, and *TLR7* has been shown in isolated heterophils of healthy day old Leghorn chickens (Kogut et al., 2005). In the same experiment, heterophils stimulated with TLR agonists showed differential expression of cytokines (IL-1β, IL-6 and IL-8), providing evidence that the TLRs expressed by heterophils are functional (Kogut et al., 2005). Given Paneth cells are unconfirmed and thought absent in avian species, the role of heterophils and their functional efficiency in assessing efficacy of innate immunity is of increased interest.

Table 2.2 Summary of TLR mRNA expression results in heterophils, macrophages and the small intestine of 8-week SPF Leghorn chickens. Table amended from Iqbal et al., (2005)

	TLR1La	TLR2a	TLR2B	TLR3	TLR4	TLR5	TLR7
Immune cell subsets							
Heterophils	+/-	++	+	+	++++	+++	+/-
Macrophages	+	-	+	-	++++	+	+
Intestinal tissue							
Duodenum	+++	+	+	++++	++	++	+
Jejunum	+++	+	+	++++	++	++	+
Ileum	+++	+	+	++++	++	++	+

Relative level or RT-PCR product denoted by – (undetectable signal) to ++++ (strong signal) scale.

Ferro et al. (2004) reported that more functionally efficient heterophils, with significant up-regulation of interleukin-1 beta (*IL-1β*), interleukin-6 (*IL-6*) and interleukin-8 (*IL-8*) mRNA, were found in lines of chickens less susceptible to extra-intestinal *Salmonella enteritis* infections. It was suggested the up-regulation could be responsible for more efficient and effective immune responses, although TLRs were not assessed in this study. The responses of *TLR1La, TLR2a* and *TLR15* have however been explored in ileal tissue in response to a *Clostridium perfringens* challenge in Ross broilers. *TLR1La* mRNA was up-regulated d2 post infection in the intestine while *TLR2a* and *TLR15* expression was down regulated d4 post infection. Iqbal et al. (2005) also used RT-PCR to explore the entire small intestine for TLR expression with *TLR3* expression found to be the highest. *TLR1La* was moderately expressed, with the patterns of expression along the entire small intestinal tract remaining unchanged (Table 2.2). Although mRNA TLR expression is helpful, there are limitations as it is semi-quantitative estimation to the biological activity in the intestinal epithelium. Thus total mRNA estimations may not confer an accurate assessment of intestinal epithelial cell expression of

TLRs due to the fact that whole gut segment homogenates will contain multiple cells types (enterocytes, heterophils, macrophages, and dendritic cells), all capable of TLR expression.

The overall importance of TLRs, their associated pathways and involvement with intestinal homeostasis has been acknowledged due the fact that they have been highly conserved across a wide range of species. Comparative studies of TLRs and subsequent TLR signalling pathways have revealed that the TLR signalling pathways show little variation between chicken and zebra finch, and any variation observed is limited to the TLRs themselves (Cormican et al., 2009). In the same study, differences were observed in the range of the antimicrobial peptide (AMP) genes coded for by the chicken and zebra finch. As the induction of AMPs is also a critical outcome of TLR signalling, these differences are suggestive that there is species variation in microbial detection and tailored antimicrobial responses (Cormican et al., 2009).

2.6.2   Antimicrobial Defence Peptides

Antimicrobial host defence peptides (AMPs) are compounds with a broad antimicrobial spectrum against both pathogenic organisms and those which are often normally associated with the host (Boman, 1995). They function without high specificity of memory and avoid the problem of self-destruction using either cellular compartmentalisation, or, by identification of a microbial target which is normally absent from the host (Boman, 1995). AMPs are classified into five chemically different groups with two of the major classes found in avians, the defensins and cathelicidins (Boman, 1995).

2.6.2.1   *Defensins*

Defensins are sub-divided into three subfamilies; α-, β-, and θ-defensins (Ganz, 2003). Each subfamily differs in the length of the peptide segments between the six cysteines and the pairing of the cysteines that are connected by disulphide bonds (Ganz, 2003). *In silico* studies have revealed the chicken genome encodes a total of 14 avian β-defensins (AvBDs, formerly termed Gallinacins) (Lynn et al., 2004, Xiao et al., 2004, Lynn et al., 2007). Comprehensive genome screening has also revealed that unlike many vertebrates, birds do not appear to encode for α-

defensins (Xiao et al., 2004). Expression levels of avian β-defensin (Table 2.3) have shown to be variable and in multiple tissues including the large and small intestine, leukocytes and bone marrow (van Dijk et al., 2008).

Table 2.3 Chicken tissue specific beta-defensin gene expression by RT-PCR (van Dijk et al., 2008)

Tissue	AvBD1	AvBD2	AvBD3	AvBD4	AvBD5	AvBD6	AvBD7	AvBD8	AvBD9	AvBD10	AvBD11	AvBD12	AvBD13
Small intestine	-/w	-/m	-	-/~	-	-/~	-/~	-	-/w	-	-	-	-/s
Large intestine	-/w	-/m	-/~	-/~	-	-/m	-/~	-	-/~	-	-	w	-
Leukocytes	s	s		-	-	w	w	-	-	-	-	-	-
Bone marrow	s	s	w	m/s	w/s	s	s	-	w	-	-	-	-

Relative level or RT-PCR product denoted by – (undetectable signal), ~ (trace), w (weak), m (moderate), s (strong).

Both Lynn et al. (2004) and Higgs et al. (2005) found expression of *AvBD1*, *AvBD*2, *AvBD4, AvBD6, AvBD7* and *AvBD13* in the small intestine of three week male Cobb 500 broiler chickens. This expression however was not detected by Zhao et al. (2001) who investigated *AvBD1, AvBD2* and *AvBD3* in a single three month old chicken, or by Xiao et al. (2004) who investigated the expression of AvBD4-13 in a single two month old chicken (strain not specified). It remains questionable as to whether intestinal epithelial cells are producing AvBDs in poultry or whether it is heterophils amongst whole tissue homogenates, as heterophils have been shown to express AvBDs (Evans et al., 1995, Evans et al., 1994, Harwig et al., 1994, van Dijk et al., 2008). The development of the innate immune system in the chick gut has been studied in healthy, uninfected newly hatched broiler chicks for the first week post hatch (Bar-Shira and Friedman, 2006). It was found that *AvBD1* and *AvBD2* were elevated at hatch and

then declined during the first week post hatch. While AvBD mRNA detection was found in the gut, again it was difficult to distinguish whether expression was from gut epithelial cells, or, from an influx of immature heterophils from circulation (Bar-Shira and Friedman, 2006). To further demonstrate contrasting findings, in birds, non-myeloid β-defensin expression has been found in surface epithelial cells of the oviduct using RT-PCR and *in situ* hybridisation (Ohashi et al., 2005); these findings are again in contrast to Zhao et al. (2001) who found no expression.

Variation in detection and expression levels of AvBDs could be attributed to a number of factors such as; age, breed, sex, immune status, and may even vary considerably between individuals (van Dijk et al., 2008). Despite varying expressional results, it is known that avian β-defensins have potent antimicrobial activity and are essential to innate immunity (van Dijk et al., 2008). There has been some investigation into the antimicrobial activities of selected avian β-defensins in chicken and turkey heterophils and antimicrobial activities have been demonstrated against a number of common occurring poultry and human pathogens including *Escherichia coli, Salmonella enteritis* and *Campylobacter jejuni* (Evans et al., 1995, Harwig et al., 1994). While it is suggested that there are specifically tailored antimicrobial detection and responses between species (demonstrated between the zebra finch and the chicken), the extent to which this variation occurs within species is less well known.

2.6.2.2 *Cathelicidins*

Cathelicidins are a family of highly diverse AMPs and have a wide spectrum of antimicrobial activity against bacteria, fungi and viruses (Lynn et al., 2004, Goitsuka et al., 2007). Both the species origination (considerable species differences exist) and the type of mature peptide expressed will alter the microbial activity against a particular microbe (van Dijk et al., 2005). Four avian cathelicidin-like peptides have been described for the chicken, termed CATH-1, 2 and -3 and CATH-B1 (Table 5) (van Dijk et al., 2011). Analysis of the zebra finch genome has not identified cathelicidins; however, three cathelicidins sharing a high level of identity with

chicken CATH-1,-2 and -3 respectively have been found in the ring-necked pheasant (*Phasianus colchicus*, Pc-CATH-1, -2 and -3) (van Dijk et al., 2011).

Table 2.4 Cathelicidin-like peptide expression levels detected in chicken intestine

Cathelicidin	Other names	Intestinal tissue mRNA expression
CATH-1	Cathelicidin-1	Moderate (Lynn et al., 2004)
	Fowlicidin-1	Nil (Goitsuka et al., 2007)
		High (Achanta et al., 2012)
CATH-2	Chicken myeloid antimicrobial peptide 27 (CMAP27)	Low (van Dijk et al., 2005)
		Nil (Goitsuka et al., 2007)
	Fowlicidin-2	High (Achanta et al., 2012)
CATH-3	Fowlicidin-3	Nil (Goitsuka et al., 2007)
		High (Achanta et al., 2012)
CATH-B1	Cathelicidin-B1	Nil (Goitsuka et al., 2007)
		High (Achanta et al., 2012)

Results are conflicting regarding the intestinal expression of cathelicidins. Lynn et al. (2004) used RT-PCR to assess mRNA levels of *CATH-1* and found moderate expression in the small intestine of a single three week old male chicken (Cobb 500), whereas Goitsuka et al. (2007) found no *in situ* expression of cathelicidins in the chicken small intestine (age and breed not defined). Conversely, Achanta et al. (2012) found mRNA expression of *CATH-1, -2, -3* and *CATH-B1* in the duodenum, jejunum and ileum of four week old Cornish Rock broiler chickens. The apparent expression of cathelicidin mRNA in the intestine however appears to reduce as bird age increases, indicating they could play a more critical role in early innate immunity (Achanta et al., 2012).

2.6.3   Immunoglobulin A (IgA)

Avian species express three immunoglobulin classes, IgY, IgA and IgM, all of which are homologous to the corresponding mammalian isotypes (Zhao et al., 2000). Most knowledge of the avian immunoglobulins has been derived from studies on chickens. Less is known about non-galliform species, of which most work has focused on ducks (Lundqvist et al., 2006). IgA is found in chicken serum, secretions and the majority of gut plasma cells, which is typical of mammalian IgA (Lebacq-Verheyden et al., 1974). Secretory IgA (sIgA) enhances innate defence mechanisms of the host by two main mechanisms; 1) blocking epithelial receptors inhibiting the adherence of microorganisms to the mucous membrane thereby preventing infection, and 2) entrapping antigens and microorganisms in the mucus which facilitates their removal (Mantis et al., 2011). Additionally, IgA improves bactericidal function by neutralising bacterial toxins and viruses (Azzam et al., 2011, Schneeman et al., 2005).

IgA production occurs in plasma cells (differentiated from B-cells) in mucosal-associated lymphoid tissue (Beal et al., 2006). Formation of sIgA is the result of a coordinated process between both the plasma cells and the epithelial cells, which transport the IgA into the intestinal lumen (Norderhaug et al., 1999). The polymeric immunoglobulin receptor (pIgR) mediates transport of IgA across epithelial cells by transcytosis and is expressed on the basolateral surface of epithelial cells (Johansen and Kaetzel, 2011). Once translocated, a portion of pIgR is covalently linked to IgA and secreted in the form of sIgA (Cao et al., 2012a). As transport requires one molecule of pIgR, external sIgA secretion is limited by the availability of pIgR (Johansen and Kaetzel, 2011).

Stimulation of intestinal IgA production and secretion as well as *pIgR* up-regulation is influenced heavily by multiple mediators including cytokines, hormones and bacterial products (Norderhaug et al., 1999). It has also been suggested that the microbiota, through MAMP recognition, stimulate *pIgR* expression by epithelial cells and consequently enhance the

production of sIgA, which, in turn regulates the intestinal microbial composition and function creating a homeostatic loop (Johansen and Kaetzel, 2011). Schneeman et al. (2005) demonstrated with the HT29 cell line, that expression of *pIgR* can be up-regulated in response to dsRNA or LPS by signalling through *TLR3* and *TLR4*, and although up-regulation was relatively slow, it was sustained. Conversely, they demonstrated that up-regulation of pro-inflammatory gene expression, including *IL-8*, was rapid. Cytokines including IL-4, TGF-β, IL-5, IL-6 and IL-10 are also know to stimulate sIgA production, with TGF-β and IL-10 required for maintaining mucosal induced tolerance (Mantis et al., 2011).

Numerous animal trials looking at modulating the immune response using probiotics and antimicrobial peptides have shown differential expression of IgA in the intestine. Investigation into the growth performance and mucosal immunity in broilers supplemented with pig antimicrobial peptides showed both an increase in growth parameters as well as an increase in sIgA secreting cells (Bao et al., 2009). Connell et al. (2012) identified several differential patterns of gene expression between mRNA isolated from colonisation-resistant and colonisation-susceptible birds from a single population of *Campylobacter jejuni* infected chickens (Barred Rock chickens). The birds with nil-colonisation were found to have increased expression of many genes involved in the production of immunoglobulin, which was associated with resistance to colonisation. Whilst it is clear the sIgA plays an integral role in intestinal homeostasis and can be modulated, it has yet to be elucidated whether sIgA can be linked to performance variation healthy unchallenged individuals. In recent years research has focused on mammalian regulatory T-cells (Tregs) and evidence suggests, additional to IgA alone, that there is a major role for the Treg cell-IgA axis in controlling intestinal homeostasis (Feng et al., 2011).

2.6.4 Avian Regulatory T-cells

Regulatory T-cells (Tregs) are often associated with the acquired arm of immunity rather than innate; however, avian Treg research is in relatively early stages compared to mammalian counterparts and is of growing functional interest. Tregs function to suppress activated immune cells once inflammation subsides, protecting the host from excessive immune responses (primarily through increased IL-10 and decreased IL-2 production) (Selvaraj, 2013). Conversely however, overactive Tregs can impair immune function and have been implicated in pathogen resistance and impaired microbial defences (Selvaraj and Shanmugasundaram, 2013). Tregs have been best characterised in both humans and mice, and shown to constitutively express surface proteins. These markers however are not exclusive to Tregs in any particular species, or present in all species (Selvaraj and Shanmugasundaram, 2013). Forkhead box P3 (*FoxP3*) is a transcriptional factor (expressed in the nucleus of Tregs) and is essential for the development and function of mammalian Tregs (Selvaraj and Shanmugasundaram, 2013). It is a commonly used marker for the identification of Tregs, however to date has not been identified in chickens or other avian species (Selvaraj and Shanmugasundaram, 2013, Selvaraj, 2013). CD25 is a second marker of Tregs (expressed on the cell surface) and has been used to study avian $CD4^+$ $CD25^+$ cells for Treg suppressive properties (Selvaraj, 2013). These cells are predominately located in the mucosa of the intestine and respiratory tract and ensure immune host responses are not mounted against commensal bacterial and food antigens (Selvaraj, 2013).

$CD4^+$ $CD25^+$ cell populations are of functional interest due to their key roles in the maintenance of intestinal homeostasis (Feng et al., 2011). Dysregulation of Tregs has been linked to the pathogenesis of coccidial infection in chickens by increasing $CD4^+$ $CD25^+$ cell populations (hyperactivity), as well as salmonellosis in other species (Selvaraj, 2013). Chicken $CD4^+$ $CD25^+$ cells challenged *in vivo* with *Salmonella* LPS have been shown to suppress host immune cells (Selvaraj, 2013) Furthermore, dysregulation of $CD4^+$ $CD25^+$ cell have been linked with IgA regulation. Depletion of $CD25^+$ cells in mice has been shown to result in a decrease

of IgA$^+$ B-cells in the lamina propria and reduce antigen-specific IgA secretion for commensal bacteria (Feng et al., 2011). It is evident from limited research that avian Treg dysfunction may be involved in pathogenesis of key avian diseases. It is unknown however whether unbalanced Treg function can be at attributed to performance variations of the host at a sub-clinical level rather than in a disease state.

2.6.5 Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) stress response is not part of the innate immune system *per se*, but it may contribute to weakened function. The ER is the site of synthesis, modification and delivery of proteins destined for secretion, including mucins (Schroder and Kaufman, 2005). Endoplasmic reticulum stress (ER stress) occurs when the cell synthesis of proteins exceeds the folding/processing capacity of the cell, leading to the accumulation of unfolded proteins (Schroder and Kaufman, 2005). The unfolded protein response (UPF) is a collective term for signalling processes which recovers the ER to a normal state by translational attenuation, refolding of unfolded proteins and degradation of irreversible unfolded proteins (Tsuru et al., 2013). A key transmembrane ER stress sensor is inositol-requiring enzyme 1 (*IRE1*), which is highly conserved across all eukaryotes (Calfon et al., 2002, Schroder and Kaufman, 2005). Unlike *IRE1α*, *IRE1β* expression is limited to the gastrointestinal tract with the essential function to unconventionally splice X-box binding protein (*XBP1*) mRNA, resulting in the production of the transcriptionally active XPB1s (XPB1u is the inactive, un-spliced isoform) (Tsuru et al., 2013). XBP1s induces transcriptional up regulation of a large number of target genes to reduce ER stress (Tsuru et al., 2013).

As mentioned previously, MUC2 is the major component of the mucus layer and is translocated into the ER lumen where it is folded (Johansson et al., 2011). Folding of MUC2 can prove challenging for the cell due to the abundant cysteine residues and consequently there is a need for chaperone proteins which aid in the folding and assembly of proteins in the ER,

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such as anterior gradient homolog 2 (AGR2) (Park et al., 2008). The ER stress response increases the number of chaperones in the ER, and it has been suggested that the system needs to be partially activated to maintain mucin folding, as a lack of the main ER regulator *XBP1* as well as *AGR2* have been demonstrated to cause an accumulation of mis-folded mucins (Johansson et al., 2011, Backstrom et al., 2013). Furthermore, intestinal inflammation can originate solely from *XBP1* abnormalities in intestinal epithelial cells and depletion has been shown to result in spontaneous enteritis (Kaser et al., 2008). Whilst limited information is available for the ER stress response in the intestine in birds, it would be of interest to characterise avian ER stress response genes in conjunction with other parameters of innate immunity, particularly with MUC2.

2.7   Innate Immunity and Performance Variation

It is well established that gastrointestinal homeostasis is dependent on the functionality of innate immunity, the balance of which is critical not only for maintaining avian health but also performance (Kohl, 2012, Humphrey and Klasing, 2004). The sub-therapeutic use of in-feed antimicrobials in animals was banned by the European Commission on the 1st January 2006 due to concern that they were contributing towards the emergence of microbial cross resistance with antibiotics used in human medicine (Huyghebaert et al., 2011). Poultry and other livestock often show improved growth performance and feed efficiency when antimicrobials are included sub-therapeutically in the diet, particularly for those animals living in less sanitary environments (Lochmiller and Deerenberg, 2000). At sub-therapeutic levels, antimicrobials are thought to function primarily through microbial alteration of the gut, including reduced antigenic challenges as well as altered metabolism (Lochmiller and Deerenberg, 2000). It is thought that mounting an immune response, even maintaining a competent immune system is nutritionally challenging, with a trade-off between nutrient demands for growth, reproduction, temperature, work and immunity (Lochmiller and Deerenberg, 2000), hence the efficacy of antimicrobial supplementation. Although it is almost impossible to adequately measure the metabolic cost of

innate immunity maintenance (Lochmiller and Deerenberg, 2000), it is reasonable to hypothesise that a less functionally-efficient immune system could impose a higher metabolic demand for energy, and may contribute to performance variability seen between animals raised in the same environment. For poultry, particularly meat birds, performance (widely assessed commercially by feed conversion ratio) of a single flock is of paramount concern to the producer and known to be influenced by factors such as health, breed, sex, diet, genetics and growth environment (Stanley et al., 2012). When many of these factors are experimentally eliminated however, performance variation remains. The extent to which (if it all) avian innate immunity and variation in flock performance can be consistently linked is not known. What is clear from the literature and aspects of immunity discussed, is that a broad range of studies demonstrate altered innate responses to dietary, antigen, and pathogenic challenges, all resulting in decreased performance. The major gap however, is that there is no single characterisation amongst healthy individuals in relation to maintenance of individual innate immunity, and whether or not functional variations exist that can be linked to individual variations in performance.

2.8   Conclusion

The innate immune system is critical to maintaining intestinal homeostasis and highly activated or diminished function can have a detrimental effect on both the health and performance of the bird. Variations in growth and performance, particularly in domestic meat chicken breeds, is an economic cost to the producer and is known to be influenced by many factors. This review has explored various arms of innate immunity, to determine intestinal expression of innate immune parameters for candidate gene selection, and to establish known intestinal innate immune function in birds. It is apparent that characterisation of avian innate immune parameters has been largely limited experimentally to cell culture studies, which is impaired by the lack of a chicken intestinal epithelial cell line, and mRNA expressional studies, which demonstrate multiple contradictory findings in experimental results. Consequently, knowledge regarding

cell-specific and intestinal expression of key innate parameters is still limited. Furthermore, current studies of avian innate immunity have been performed on a number of domestic breeds, however, the differences in age, sex and breeds of the birds used between experiments (all factors known to affect immune gene expression), as well as variation in study design does not confer an accurate assessment of the parameters reviewed in any single breed. A broad characterisation of avian intestinal innate immunity is required to validate expression of many genes in a single breed and to determine basal immune gene expression in unchallenged healthy populations.

It was therefore hypothesised that functional changes in innate immunity and intestinal barrier function may exist between unchallenged individuals, and contribute to variations in FCR commonly seen within commercial meat bird flocks. The following chapter explores the expression of 16 innate immune genes for investigation in unchallenged birds, phenotypically categorised as either high-or low- performing based on individual FCR values.

2.8 Acknowledgements

2.9 References

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CHAPTER THREE

Selected innate immune genes reveal no consistent association with the phenotypic expression of feed conversion ratio in broilers

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Selected innate immune genes reveal no consistent association with the phenotypic expression of feed conversion ratio in broilers

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Immunology, Health and Disease

3.1   Abstract

As growth has been shown repeatedly to be an immunological trade-off, the aim of the current experiment was to investigate selected innate immune genes, to determine whether any functional changes in innate intestinal barrier function could be consistently linked to the phenotypic expression of feed conversion ratio (FCR); a common measure of performance variation within broiler flocks. In order to replicate results consistently, three experiments were conducted; experiment one and two (P1E1 and P1E2) were exact replicates with all birds fed standard commercial broiler feed, whilst experiment three incorporated the addition of zinc bacitracin. For each experiment, 'high' ($n=12$) and 'low' ($n=12$) performing broilers were selected based on their individual FCR values at d25 post hatch. A total of $n=96$ ileal samples were assayed for 16 candidate genes using Real-time PCR (RT-PCR). Expression levels were normalised against the genomic average of housekeeper genes *TBP* and *RPL19*. Real-time PCR data, FCR, bodyweight and feed intake data were analysed by one-way ANOVA in SPSS (IBM SPSS). Birds deemed to be high performing had lower FCR values ($P < 0.05$) in all experiments. High performing bird were heavier in all experiments with the exception of P1E1 ($P = 0.481$). Feed intake was not different between the high- and low-performing birds in any experimental group ($P > 0.05$). RT-PCR results indicated greatest variation in the expression of the antimicrobials avian β-defensin 1 (*AvBD1*) and avian β-defensin 2 (*AvBD2*). High-performing birds had higher expression of *AvBD1* ($P = 0.039$) and *AvBD2* ($P = 0.028$) in P2E1 (control birds). Tight junction proteins claudin 5 (*CLDN5*) and zona occludens 2 (*ZO2*) were differentially expressed in P1E2 ($P = 0.038$) and ($P = 0.017$). No other genes were differentially expressed in any experiment. Despite finding four differentially expressed genes, these findings were not replicated across repeated experiments. The results of this study therefore do not provide evidence that broiler flock performance variation can be consistently linked to select innate immune parameters investigated in the small intestine.

Key words: Broiler, feed conversion ratio, innate immunity, ileum, Real-Time PCR.

3.2 Introduction

The intensification of the poultry broiler industry since the late 1940s and early 1950s has meant that the cost of chicken meat as a sustainable protein source has remained relatively constant. This has been largely attributed to genetic selection for improved growth rates and feed efficiency (Schmidt et al., 2009). Despite the historical improvements in growth traits, there still remains considerable variance both between, and within, broiler strains for feed conversion, bodyweight and growth rate (Emmerson, 1997). From the perspective of a commercial poultry producer, birds with low efficiency reduce profitability as feed accounts for approximately 70% of the total cost of production (Aggrey et al., 2010).

Genetic selection for growth and efficiency has likely been in part an immunological trade-off for growth, with heavier breeds of poultry demonstrating reduced responses to immune challenges (Cook et al., 1993, Lochmiller and Deerenberg, 2000). More recently, van der Most et al. (2011) conducted a meta-analysis on data from 14 studies of genetic selection for body mass and immune function in poultry, providing strong evidence that selection for growth significantly decreases resistance and responses to immune function challenges. Given that intestinal barrier function and animal performance traits, such as growth, are intricately linked (Kohl, 2012), it may be reasonable to postulate that individual birds performing better may have a more functionally efficient immune system.

Both the nutritional status and immune function is known to be influenced by the diversity and stability of the avian intestinal microbiota (Kohl, 2012). Maintenance and homeostasis of the intestinal environment requires precise co-ordination of innate immune function. This includes the ability to distinguish between 'self' and 'non-self'; exclude harmful pathogens; and to ensure inappropriate immune responses are not mounted (Medzhitov and Janeway Jr, 2002). Intestinal homeostasis can therefore be considered a balancing act, with diminished or highly activated innate immune function resulting in intestinal inflammation,

potentially leading to decreased performance (Asquith and Powrie, 2010, Finlay and Hancock, 2004, Kohl, 2012). Intestinal innate immunity and mucosal barrier integrity is multifaceted incorporating but not limited to: 1) The epithelial mucus gel layer, formed primarily by intestinal mucin 2 (*MUC2*) (Deplancke and Gaskins, 2001). 2) Immunoglobulin A (IgA) secretions into the intestinal lumen, preventing the adherence of microorganisms to the mucous membrane (Mantis et al., 2011). 3) Epithelial tight junctions, sealing the paracellular space between enterocytes (Turner, 2009) and 4) Secreted antimicrobial peptides such as β-defensins, which act against a broad spectrum of microbial organisms (Boman, 1995). Additionally, innate recognition of intestinal foreign substances (i.e. microbes and pathogens), occurs through pathogen recognition receptors (PPRs), including the Toll-like receptors, 10 of which have been identified in domestic avian species (Brownlie and Allan, 2011). TLRs and subsequent signalling pathways have been associated with the modulation of; epithelial cell proliferation, immunoglobulin A (IgA) production, maintenance of tight junctions and antimicrobial peptide expression (Abreu, 2010, Lavelle et al., 2010).

Several studies and reviews have investigated key avian innate immune parameters including: the composition and modulation of the mucus gel layer and the expression of avian MUC genes, particularly in response to pathogenic challenges (Byrne et al., 2007, Forder et al., 2012, Cao et al., 2012, Lang et al., 2006); Intestinal expression and function of avian TLRs (Lavelle et al., 2010, Iqbal et al., 2005, Brownlie and Allan, 2011); and the expression and the bactericidal activity of select avian antimicrobial peptides (van Dijk et al., 2008, Harwig et al., 1994, Evans et al., 1995). These studies have demonstrated differential innate immune profiles in several domestic avian breeds at various ages in response to pathogenic challenges, dietary modulation, and also between birds differing in their resistance to pathogens. These genes, however, have not been collectively investigated in a single species to determine whether innate immune function is associated with the phenotypic expression of feed conversion ratio (FCR) between individuals.

Previous studies by our colleagues have investigated and characterised the cecal microbiota in carefully controlled trials, to eliminate trial to trial variation in the overall structure of microbiota often observed between studies (Stanley et al., 2013). Their results highlight the variability in microbiota structure across replicate trials, and between animals within a single uniformly derived flock (Stanley et al., 2013) and, indicated association of intestinal microbiota with differential feed conversion efficiency in chickens (Stanley et al., 2012). The effects of antibiotics on both microbial composition and performance measures were also investigated, again demonstrating variability in microbial structure and showing associations between gut microbiota and performance (Crisol-Martinez et al., 2017).

As immune function is known to be influenced by the diversity and stability of the avian intestinal microbiota we hypothesised that functional changes in innate immunity and intestinal barrier function may exist between unchallenged individuals, and contribute to variations in FCR commonly seen within commercial meat bird flocks. Utilising samples from Stanley et al., (2012, 2013) and Crisol-Martinez et al., (2017), our aim was to investigate selected innate immune genes to determine whether mRNA expressional changes in innate intestinal barrier function could be consistently linked to the phenotypic expression of FCR between high- and low-performing birds.

3.3 Methods and Materials

3.3.1 Birds and Management

3.3.1.1 Protocol One: Experiment One (P1E1) and Experiment Two (P1E2). Experimentally, protocols for each experiment one and two (P1E1 and P1E2) were identical. The animal experiments were performed separately and previously described by Stanley at al., (2013). Briefly, 120 male Cobb 500 broiler chickens (Baiada Hatchery, Willaston, South Australia, Australia), were raised in a rearing pen on wood shavings in a temperature and climate controlled facility. All procedures involving animals were approved by the Animal Ethics

committees of the University of Adelaide (approval no. S-2010-080 and S-2011-218) and the Department of Primary Industries and Resources, South Australia (approval no. 08/10 and 25/11).

Identical diets (Table 3.1) were fed *ad libitum* and birds had unrestricted access to water via a nipple drinker line. Diets were formulated to meet or exceed the National Research Council guidelines for broiler chickens (NRC, 1994). The lighting schedule was; Day 0-3, 23 h; Day 4, 21 h; Day 5, 18 h; Day 6, 15 h; Day 7-25, 12 h. Birds were placed into metabolism cages at d13 post hatch (*n*=48 cages, *n*=96 randomly selected chickens from original 120 birds, and transferred in pairs). Following a 2 day adaption period (d15 post hatch) birds were individually caged for seven days and fed *ad libitum*, in full visual and vocal range of the other birds. Feed conversion ratio (FCR; g feed eaten/ g weight gain), bodyweight and bodyweight gain were monitored for this period. Birds were euthanised by cervical dislocation at d25 post hatch.

3.3.1.2 Protocol Two: Experiment One (P2E1 control and P2E1 ZnBc). Protocol two (P2E1) introduced the variable of the addition of the antibiotic zinc bacitracin to the diet. Briefly, 120 male Cobb 500 broiler chickens (Baiada Hatchery, Willaston, South Australia, Australia) were randomly assigned and raised in two separate rearing pens (*n*=60 birds/pen; separated based on diet). Experimental protocols were as outlined previously for P1E1 and P1E2 above with the exception of diet. Two dietary treatments were used; a control diet (P2E1 control) of the same formulation as P1E1 and P1E2; and a second diet formulated to the same specs with the addition of zinc bacitracin (500ppm; P2E1 ZnBc).

Table 3.1 Composition of broiler chicken rearing diet

Ingredient	% Inclusion
Wheat	44.4
Barley	15.0
Peas	5.0
Soybean meal	17.0
Canola meal	10.0
Meat meal	3.2
Tallow	3.0
Limestone	1.0
Salt	0.35
Lysine HCl	0.25
DL-methionine	0.23
Threonine	0.07
Vitamin and mineral premix[1]	0.5

[1]Included xylanase and phytase enzyme products

3.3.2   Sample Collection

Upon euthanasia, 1 cm segments from the midpoint of ileum were collected from the $n=12$ highest and $n=12$ lowest performing birds based on individual FCR for; P1E1 ($n=24$), P1E2 ($n=24$), P2E1 control birds ($n=24$) and P2E1 zinc bacitracin fed birds ($n=24$). A 1x segment was segment frozen in liquid nitrogen and stored at -80°C for mRNA gene analysis.

3.3.3   Isolation and Quantification of Total RNA from Chicken Intestinal Samples

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Approximately 100 mg of frozen (-80°C) ileal tissue was homogenised in 2 mL of Trizol reagent (Invitrogen, Carlsbad, CA). Aliquots (1 mL) of the Trizol homogenate were combined with 200 μL of chloroform and centrifuged for 15 mins at 4°C. The upper aqueous phase (300 μL) was collected and mixed with 300 uL of 70% ethanol and transferred onto RNeasy columns. The remaining

collection and wash steps were performed to the manufacturer's specifications. An on-column RNase-free DNase treatment step (Qiagen, Hilden, Germany) was included and the RNA was eluted in 100 μL of EB buffer (Qiagen). The integrity of RNA samples were confirmed with agarose-gel electrophoresis across a random selection of samples. Purity and concentration were determined using UV spectrophotometry (Nanodrop 1000; Thermo Scientific, Wilmington, DE).

3.3.4   Design and Testing of Real-Time PCR Assays

Oligonucleotides for quantitative PCR assays were designed using the GenBank (National Centre for Biotechnology Information: NCBI) database and the Ensembl chicken genome browser (Gallus gallus; Build 75 F 2014). Target genes included *TLR1La, TLR2 (2), TLR3, TLR4, AvBD1, AvBD2, XBP1, pIgR, CD36, CD4+, chCD25+ (ILR2A), CLDN1, CLDN5, ZO1, ZO2* and *MUC2* (Table 3.2). Exon-intron boundaries were manually marked on the chicken cDNA sequences, and suitable pairs of exon-intron spanning primers were selected using Primer3 design software (http://bioinfo.ut.ee/primer3-0.4.0/). Amplicon sizes were kept to approximately 100 bp, and primer pairs spanned exon-intron boundaries greater than 500 bp in length. The following criteria were applied for validation of the qPCR assays: slope between -3.6 and -3.1, efficiency between 90 and 110%, $R^2 > 0.99$.

Table 3.2 Real-time PCR primers designed against chicken cDNA and genomic DNA sequences identified from RNA target searches using Ensembl and GenBank databases

RNA Target	Gene Name		Oligonucleotide sequence (5'-3')	Accession no.[2]
TLR1La	Toll-Like Receptor 1La	F[1]	CCAAAGGAGAGGAGCAAGCA	NM_001007488
		R	TCAAAGGATGTCGGCAGCTT	
TLR2(2)	Toll-like Receptor 2 (2)	F	TGCCATTTCTCAAGGAGCTGT	NM_001161650
		R	GCTGATCGACATGGCCACTA	
TLR3	Toll-Like Receptor 3	F	AGCAACACTTCATTGAATAGCCTT	NM_001011691
		R	CAGTATAAGGCCAAACAGATTTCCA	
TLR4	Toll-Like Receptor 4	F	GATGCATCCCCAGTCCGTG	NM_001030693
		R	CCAGGGTGGTGTTTGGGATT	
AvBD1	Avian β-Defensin 1	F	TGCCCTTCCCTCACTCTCAT	NM_204993
		R	GCTTGGGATGTCTGGCTCTT	
AvBD2	Avian β-Defensin 2	F	TTCCGTTCCTGCTGCAAATG	NM_204992
		R	GCCTGGAAGAAATTTTCAAAGCTC	
XBP1	Xbox Binding Protein	F	TTGAAGACAGAGCCGGAGTG	NM_001006192
		R	TGCTGCAGAGGAACACGTAG	
pIgR	Polymeric IgA Receptor	F	ATTTGTCACCACCACAGCCA	NM_001044644
		R	GAGTAGGCGAGGTCAGCATC	
cHCD25+	cHCD25+	F	GCAAGACAAACCCAAAGCCC	NM_204596
		R	CTCAGAGAGGCATGTGGGAC	
CD4+	CD4+	F	GATGGAGAGGTGTGGAGCAG	NM_204649
		R	CCTCCTTTCCTGCAATCCCA	
CD36	Fatty acid translocase	F	GAATTGCTGTGGAAGTGCTG	NM_001030731
		R	TGGTCCCAACAGACTCACTG	
MUC2	Mucin 2	F	ATGCGATGTTAACACAGGACTC	BX930545
		R	GTGGAGCACAGCAGACTTTG	
CLDN1	Claudin-1	F	TCGGGCCTTCTATGACCCTT	NM_001013611
		R	AGCAAGGCCAGAGAAGCG	
CLDN5	Claudin-5	F	AGATTTTGGGGCTGGGACTG	NM_204201
		R	TCACGTCGATGAAGGCTGAC	
ZO1	Zonula occludens 1	F	GGAAACAAAATGTCTGCCAGGG	XM_413773
		R	AAACCCAAATCCAGGAGCCC	
ZO2	Zonula occludens 2	F	GCCCAGAAGCATCCAGACAT	NM_204918
		R	TCACTGCTGACATGGATGCT	
RPL19	Ribosomal protein L19	F	AGACAAAGCTCGCAAGAAGC	NM_001030929
		R	TTCGAGAGGGTCTTGATGATTT	
TBP	TATA-binding protein	F	TCAGCAGCTATGAGCCAGAA	NM_205103
		R	CTGCTCGAACTTTAGCACCA	

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

[2]GenBank accession number

3.3.5   Synthesis of cDNA from Chicken Intestine

RNA concentrations of n=96 ileal samples were normalised to 300 ng/μL with the aid of a liquid-handling robotics system (EpMotion 5075; Eppendorf, Hamberg, Germany). Complementary DNA was synthesised using the High Capacity cDNA Synthesis kit (Applied Biosystems, Carlsbad, CA), and run to the manufacturers specifications. Additional to the kit components, 20 U of RNase inhibitor (RNaseOUT; Invitrogen) and 100 nM of oligotT primer ((5′-TTTTTTTTTTTTTTTTV-3′; where V=A, C and G) were included in the cDNA synthesis reactions. Reactions were incubated at 39 ^{o}C for 2 h and the reverse transcriptase was subsequently inactivated at 60 ^{o}C for 30 min. cDNA stocks were diluted 1:4 with 10 mM Tris (pH 8.0; Ambion) and stored at -80 ^{o}C.

3.3.6   Real-time PCR Assessment of Gene Expression Levels in Chicken Intestine

Stock cDNA (1:4) was diluted five-fold (1:20) with 10 mM Tris (pH 8.0) (Ambion) before use in real-time PCR. Diluted (1:20) cDNA (6 μL) was combined with 19 μL of SYBR-based PCR reagent. The cDNA/SYBR (5 μL) mixture was transferred in triplicate to a 384-well MicroAmp plate (Applied Biosystems). A total of n=96 cDNA preparations were examined with an 8 point standard curve, prepared by pooling a portion of four random (1:4) cDNA samples. Standard curves were prepared fresh before each real-time PCR run using 8 consecutive 2-fold dilutions in 10mM Tris (pH 8.0; Ambion; 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 dilutions of pooled cDNA and 10mM Tris blank). Quantitative PCR measurements were performed on 384-well real-time PCR machines (7900HT, Applied Biosystems) for 40 repeats using the following cycle parameters: Stage 1: 95 ^{o}C for 10 min; Stage 2: 95 ^{o}C for 15 s, 60 ^{o}C for 20 s, 72 ^{o}C for 40 s; Stage 3: 95 ^{o}C for 15 s, 60 ^{o}C for 15 s.

3.3.7 Real-time PCR Data Processing, Normalisation and Statistical Analysis

Data were processed using in-house computer software, qEXPRESS (Forder et al., 2012). Tab delimitated text files from each real-time PCR run were exported from the SDS 2.3 software (Applied Biosystems) and imported into qEXPRESS. Briefly, the reaction efficiency of each assay was determined by the standard curve and applied to a ΔCt quantification model to calculate relative quantities between samples. Non-normalised relative quantification data were imported into GenEx (MultiD, Gothenburg, Sweden) to validate the stability of the reference genes *RPL19* and *TBP* using the NormFinder application. Target gene measurements were then normalised within qEXPRESS against the genomic average of *TBP* and *RPL19*. Normalised RT-PCR data, FCR, bodyweight and performance data were analysed using a one-way ANOVA in SPSS (IBM SPSS). Two-tailed Pearson's correlations were tested between individual genes and bodyweights for each experiment using SPSS (IBM SPSS). $P < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Feed Conversion Ratio, Bodyweights and Performance Data

Feed conversion ratios were calculated from d15-d25 for $n=96$ birds in individual metabolism cages in each experiment. Post d25, the $n=12$ highest and $n=12$ lowest performing birds (based on individual FCR) were selected as the phenotypic measure of performance variation for each experiment, FCR data is presented in Figure 3.1. Birds deemed to be high-performing had lower FCR values ($P < 0.05$) indicating the birds were more efficient, therefore deemed high-performing. FCR was different ($P < 0.05$) for all experiments; P1E1, P1E2, and P2E1 (control diet) and P2E1 (zinc bacitracin diet).

Bodyweight, bodyweight gain and feed eaten is presented in Table 3.3. Bodyweight at d15 post hatch was not statistically different between any of the birds in any experiment. By d25, the high-performing birds were significantly heavier than the low-performing birds for

P1E2 ($P = 0.027$); P2E1 (control diet; $P = 0.007$) and P2E1 (zinc bacitracin diet; $P = 0.023$). Bodyweight gain was also significant ($P < 0.05$). Despite FCR being significantly different between the high- and low-performing birds in P1E1, initial bodyweight, final bodyweight and bodyweight gain differences were not detected between the high- and low-performing birds in this experiment. There were no differences detected in feed intake between the high- and low-performing birds in any experiment ($P > 0.05$).
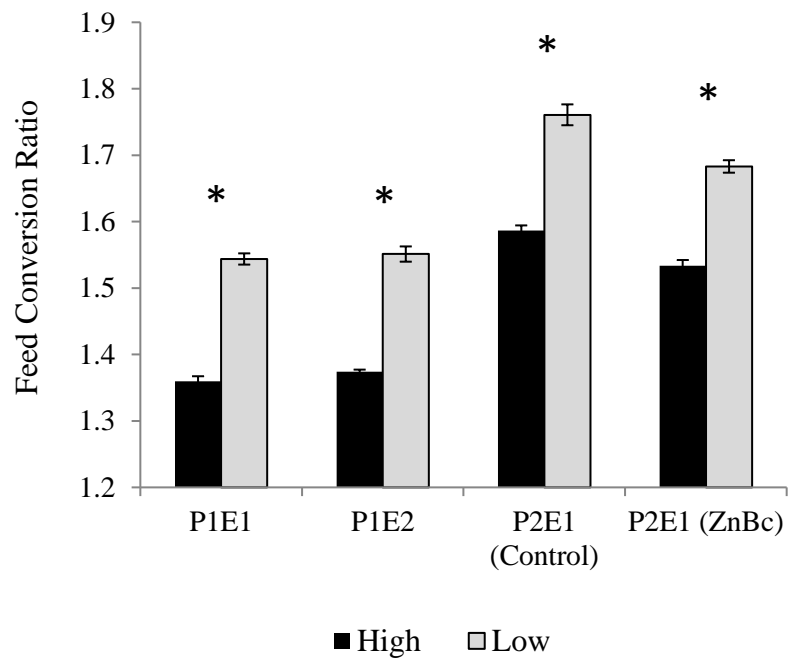
Figure 3.1 Feed conversion ratio (FCR) of n=12 high-performing (lower FCR) and n=12 low-performing birds (higher FCR) for each experiment a) P1E1, b) P1E2 c) P2E1 (control), birds fed a commercial standard diet and d) P2E1 (ZnBc), birds fed a commercial standard diet with the inclusion of zinc bacitracin at 50ppm. Values are mean ± SEM *Significance at $P < 0.05$

Table 3.3 Bodyweight (BW) d15; BW d25; Bodyweight gain (BW gain; d15-d25) and feed eaten (d15-d25) for high-performing (H; *n*=12) and low-performing (L; *n*=12) birds selected on individual feed conversion ratio (FCR) for experiments; P1E1, P1E2, P2E1 (Control), P2E1 (zinc bacitracin).

		BW d15 (g)	*P*-Value	BW d25 (g)	*P*-Value	BW gain d15-25 (g)	*P*-Value	Feed Eaten d15-25 (g)	*P*-Value
P1E1	H	379 ± 19	0.512	1174 ± 44	0.481	795 ± 28	0.112	1082 ± 39	0.296
	L	397 ± 18		1134 ± 35		737 ± 22		1138 ± 34	
P1E2	H	385 ± 11	0.434	1229 ± 34	0.027 *	844 ± 25	0.001 **	1160 ± 35	0.487
	L	398 ± 11		1125 ± 29		727 ± 20		1127 ± 30	
P2E1 (c)	H	520 ± 9	0.791	1406 ± 19	0.007**	886 ± 13	0.001 **	1406 ± 20	0.58
	L	516 ± 12		1326 ± 20		809 ± 15		1425 ± 29	
P2E1 (ZnBc)	H	537 ± 10	0.321	1454 ± 27	0.023 *	916 ± 19	0.008 **	1405 ± 27	0.746
	L	522 ± 11		1364 ± 25		842 ± 17		1417 ± 27	

(g) = grams; (c) = control; (ZnBc) = zinc bacitracin

* Significance at *P* < 0.05, ** Significance at *P* < 0.01

Values are mean ± SEM

3.4.2 Real-time PCR

Target gene expression remained consistently uniform across experiments for *MUC2, XBP1, pIgR, CD4+, chCD25+, TLR1La, TLR3*, and *TLR4 (*Table 3.4); with similar expression levels relative to the expression ratio of house keeper genes *TBP/RPL19*. The highest expression levels were consistently seen for *TLR2* for both high- and low-performing broilers, however not significantly different ($P > 0.05$; Table 3.4). Avian β-defensins *AvBD1* and *AvBD2* were the most variably expressed genes of the 16 genes explored, with the activity of each highly correlated with one another ($P < 0.001$). *TLR2* and *CD36* were the third and fourth most variably expressed genes respectively, whilst the remainder of the genes showed little variation in their expression levels between either high- and low-performing birds. Two-tailed Pearson's correlations between target genes and individual bodyweights were not significant for any experiment ($P > 0.05$).

Table 3.4 Normalised expression of 16 innate immune genes assayed across experiments P1E1, P1E2, and experiment P2E1 separated by dietary treatment

(Control) and P2E1 (ZnBc) birds.

Gene ID	P1E1		P1E2		P2E1 (Control)		P2E1 (ZnBc)	
	High (*n*=12)	Low (*n*=12)	High (*n*=12)	Low (*n*=12)	High (*n*=12)	Low (*n*=12)	High (*n*=12)	Low (*n*=12)
AvBD1	16.00 ± 5.80	43.01 ± 12.36	11.00 ± 3.04	13.97 ± 2.57	28.53 ± 5.32	12.28 ± 2.61	9.81 ± 2.09	16.98 ± 4.67
AvBD2	46.32 ± 22.15	69.06 ± 21.60	14.97 ± 4.08	18.16 ± 3.43	37.49 ± 8.19	12.87 ± 2.47	17.76 ± 6.70	22.09 ± 5.51
CD36	3.83 ± 0.70	5.33 ± 1.06	5.64 ± 1.05	4.87 ± 1.28	2.59 ± 0.31	3.17 ± 0.55	3.02 ± 0.54	3.21 ± 0.71
CD4	2.52 ± 0.18	3.09 ± 0.23	2.24 ± 0.20	2.04 ± 0.11	2.47 ± 0.09	2.22 ± 0.20	2.64 ± 0.21	2.26 ± 0.17
chCD25	1.89 ± 0.11	1.99 ± 0.13	1.76 ± 0.15	1.62 ± 0.13	1.78 ± 0.07	1.86 ± 0.14	1.95 ± 0.14	1.91 ± 0.16
XBP1	1.72 ± 0.07	1.67 ± 0.08	1.82 ± 0.13	1.78 ± 0.09	1.83 ± 0.08	1.71 ± 0.07	1.52 ± 0.10	1.63 ± 0.07
MUC2	1.71 ± 0.13	1.67 ± 0.10	2.10 ± 0.13	2.06 ± 0.15	1.78 ± 0.14	1.78 ± 0.07	1.70 ± 0.08	1.72 ± 0.08
pIgR	1.72 ± 0.10	1.65 ± 0.11	2.11 ± 0.17	2.33 ± 0.17	2.46 ± 0.24	2.12 ± 0.18	2.07 ± 0.15	1.82 ± 0.16
TLR1La	2.28 ± 0.18	2.67 ± 0.11	2.21 ± 0.16	2.37 ± 0.18	2.26 ± 0.12	2.04 ± 0.21	2.40 ± 0.22	2.20 ± 0.23
TLR2	47.81 ± 2.89	42.07 ± 2.48	37.44 ± 3.34	45.07 ± 3.09	34.03 ± 4.05	33.40 ± 8.30	37.22 ± 3.72	36.87 ± 1.85
TLR3	4.08 ± 0.64	3.84 ± 0.59	2.99 ± 0.25	2.59 ± 0.30	2.68 ± 0.31	2.59 ± 0.39	2.74 ± 0.18	3.63 ± 0.47
TLR4	1.71 ± 0.07	1.73 ± 0.09	1.86 ± 0.15	1.76 ± 0.20	1.74 ± 0.11	1.88 ± 0.16	1.55 ± 0.07	1.69 ± 0.09
CLDN1	1.57 ± 0.13	1.59 ± 0.08	2.14 ± 0.26	1.97 ± 0.13	2.00 ± 0.27	1.96 ± 0.18	2.06 ± 0.20	1.99 ± 0.12
CLDN5	1.64 ± 0.13	1.58 ± 0.13	2.08 ± 0.22	1.54 ± 0.11	1.68 ± 0.14	1.73 ± 0.11	1.55 ± 0.11	1.85 ± 0.14
ZO1	1.63 ± 0.10	1.55 ± 0.11	2.03 ± 0.14	1.63 ± 0.07	1.62 ± 0.07	1.59 ± 0.09	1.45 ± 0.09	1.64 ± 0.05
ZO2	1.48 ± 0.09	1.52 ± 0.07	1.82 ± 0.08	1.70 ± 0.09	1.69 ± 0.06	1.61 ± 0.11	1.52 ± 0.10	1.71 ± 0.07

Values are mean ± SEM

P1E1. No differences ($P > 0.05$) were detected for any of the genes assayed, Figure 3.2a. The highest variation was detected in the antimicrobials *AvBD1* and *AvBD2*. Lower performing birds had higher levels of both *AvBD1* and *AvBD2* however; the differences seen were not determined to be significant. There was a large variation in the expression of *AvBD1* and AvDB2 in individual birds, which is reflected in the large SEM (Table 3.4).

P1E2. The expression pattern of *TLR2* was opposite to that seen in the other experiments, Figure 3.2b, with high performing birds having lower *TLR2* expression; however, this was not significant and does not appear to be linked to any expressional changes within other genes. Low-performing birds had significantly higher expression levels of claudin 5 (*CLDN5*; $P = 0.038$), whilst high-performing birds had higher expression level of zona occludens 1 (*ZO1*; $P = 0.017$).

P2E1 (Control Birds). The expression patterns of both *AvBD1* and *AvBD2* had reversed from patterns previously observed, Figure 3.2c, with high-performing birds having significantly lower expression levels than low-performing broilers for *AvBD1* ($P = 0.039$), and *AvBD2* ($P = 0.028$). No other significant differences were detected ($P > 0.05$). The highest expression levels were detected for *AvBD1*, *AvBD2* and *TLR2*.

P2E1 (Zinc Bacitracin Birds). Expression levels of the antimicrobials *AvBD1* and *AvBD2* showed less overall expression variation in comparison to birds fed diets with no antimicrobial inclusion, Figure 3.2d; however, no differences were detected for any of the genes assayed between high- and low-performing broilers ($P > 0.05$). *TLR3* deviated from the previous expression patterns seen in P1E1, P1E2 and P2E1 (control birds), with high-performing birds having lower *TLR3* expression levels. The variation in the expression levels of *TLR3* was also more pronounced than in the previous sub-sets, however, not significant ($P > 0.05$).
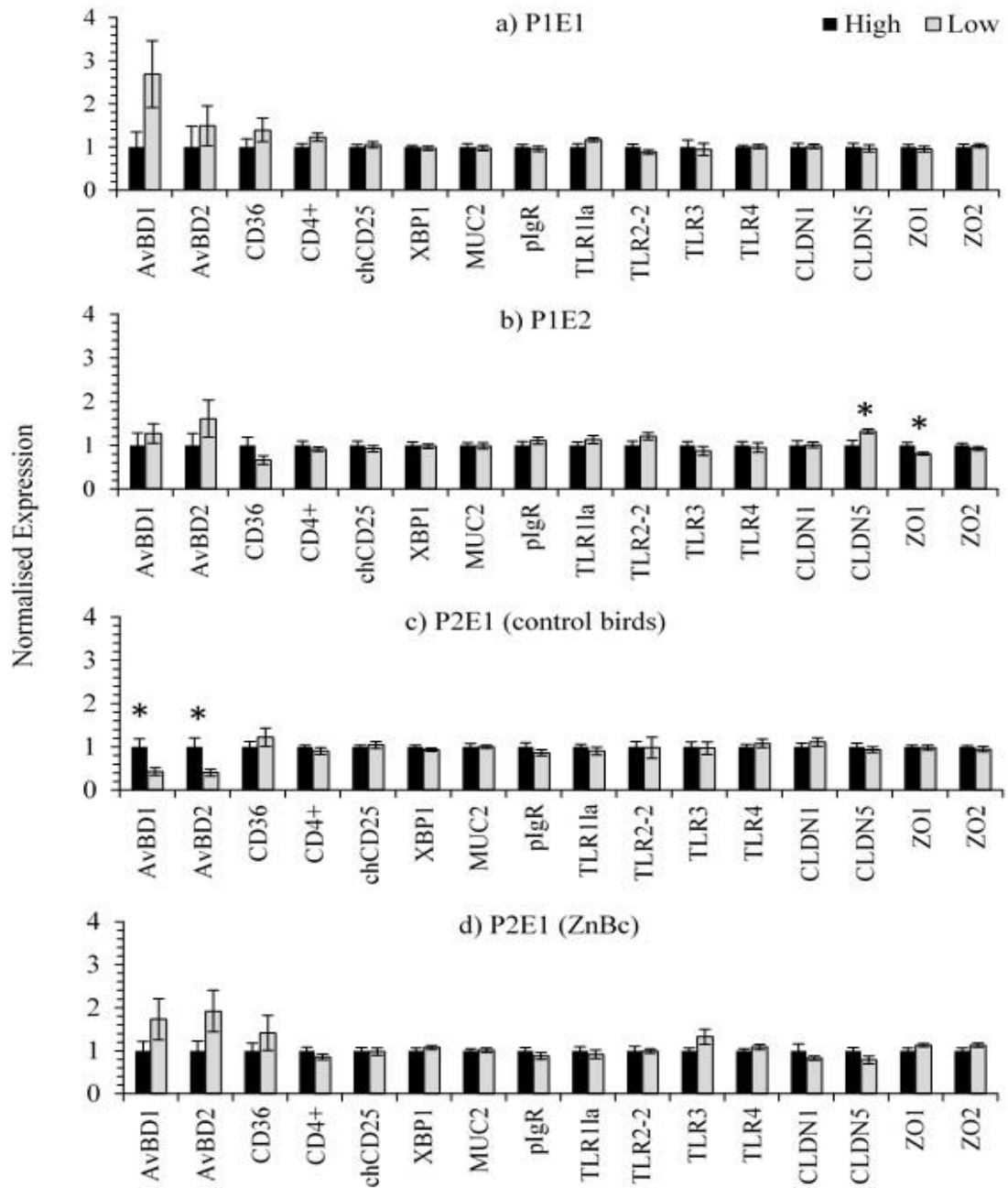
Figure 3.2 Relative gene expression levels in the ileum of the $n=12$ high-performing birds and $n=12$ low-performing birds selected on feed conversion ratios (FCR) for protocol one experiments; a) P1E1, b) P1E2 and protocol two experiment; c) P2E1 (control birds) and d) P2E1 (Zinc Bacitracin), birds fed a commercial standard diet with the inclusion of zinc bacitracin. The high-performing group was set to an arbitrary value of 1.0 and the low-performing group were expressed relative to this value. Values are mean ± SEM *Low-performing birds significantly different from high-performing birds within each gene ($P < 0.05$).

3.5   Discussion

The notion that diminished or highly activated innate immune responses are nutritionally demanding on the host is not new and has been demonstrated to result in reduced feed intake as well as redirection of resources from other functions, such as growth, thermoregulation and reproduction (Lochmiller and Deerenberg, 2000). As growth has been shown repeatedly to be an immunological trade-off (van der Most et al., 2011), we aimed to investigate selected innate immune genes to determine whether any functional changes in innate intestinal barrier function could be consistently linked to the phenotypic expression of FCR. It was important to determine the basal immunological innate function between high- and low-performing broilers, unchallenged, as this has been largely unexplored.

The results of this current study align with a recent study by Vigors et al. (2016), which investigated whether an intestinal innate immune response was contributing to the divergence of feed efficiency in pigs phenotypically selected for high- or low-residual feed intake. Characterisation of the expression of over-lapping genes between the two studies, including Toll-like receptors *TLR2* and *TLR4*, *MUC2*, and tight junction *ZO1* showed no differentiation between high- and low- performing pigs or broilers, with the exception of tight junction protein *ZO1*. *ZO1* was found to be differentially expressed, as was claudin 5 (*CLDN5*), in P1E2 in the current study. Although the differentiation of these two genes was significant between high- and low-performing broilers, an important basis of this study was to determine whether differences could be consistently detected, which they were not. The differentiation suggests the possibility of a slight shift in the distribution of these tight junction proteins, however, the relatively stable expression of all other innate immune genes in P1E2, as well as X-box 1 binding protein (*XBP1*), an endoplasmic reticulum stress response transcription factor, indicates no disruption to intestinal barrier function.

The inclusion of *XBP1* as a candidate gene was based on its known interactions with multiple branches of the innate immune system. Mutations in *XBP1* have been suggested to cause disruption to tight junction homeostasis (Turner, 2009), while intestinal inflammation can originate solely from *XBP1* abnormalities in intestinal epithelial cells and depletion has been shown to result in spontaneous enteritis (Kaser et al., 2008). *XBP1* depletion has also been demonstrated to cause an accumulation of mis-folded mucins in the endoplasmic reticulum (Johansson et al., 2011, Backstrom et al., 2013). Despite all these interaction, *XBP1* was one of the most stably expressed genes in all experiments, as was the primary component of the mucus gel layer, *MUC2*.

Additional to mucins, the mucus gel layer also contains Immunoglobulin A secretions (sIgA), which function to enhance innate defence mechanisms (Mantis et al., 2011). Differential increased expression of sIgA has been associated resistance to *Campylobacter jejuni* infection in Barred Rock chickens (Connell et al., 2012), and increases of sIgA secreting cells have been demonstrated in broilers supplemented with pig anti-microbial peptides in an attempt to evaluate performance and mucosal immunity (Bao et al., 2009). ELISA's were performed to determine the secretory component in the ileum, however; such tests proved unsuccessful on the retained ileal samples and therefore eliminated from the analysis. Polymeric IgA receptor (*pIgR*) was included in the candidate gene selection as an alternative assessment of IgA activity due to its key role in mediating transport of IgA across epithelial cells, and, as transport of IgA requires one molecule of pIgR, external secretion of sIgA into the mucus layer is limited by the availability of pIgR (Johansen and Kaetzel, 2011). While ileal sIgA concentrations between high- and low-performing broilers were not been established in this study, the stable expression of *pIgR* gives no indication that levels of sIgA may differ.

The gene results discussed thus far were exceptionally tight in their expression levels across all experiments. The antimicrobials *AvBD1* and *AvBD2* however, were not, and were the

most variably expressed genes. Like the tight junction proteins in P1E2, *AvBD1* and *AvBD2* were found to be differentially expressed in P2E1 (control birds), however, not consistently, and with large variation in individual expression levels which is reflected in the large standard error of the means. A plausible explanation for the large differences in expression and variation observed could align with the suggestion that differences in antimicrobials are tailored antimicrobial responses occurring in animals within a species; an idea which has been also suggested to occur between differing bird species (Cormican et al., 2009). The high individual cecal microbial variation identified by Stanley et al., (2013), as well as the birds fed zinc bacitracin (Crisol-Martinez et al., 2017) utilised in this study would further support individual tailored responses accounting for *AvBD* variability. Although *AvBD1* and *AvBD2* expression in P2E1 control birds were significantly different between high- and low-performing broilers, they were not detected between high- and low-performing broilers supplemented with the antibiotic zinc bacitracin (P2E1, ZnBc). This result however, cannot be attributed to the addition of the zinc bacitracin to the diet, as no significant differences in AvBD expression levels in either P1E1 or P1E2 were detected, with no antibiotic supplement administered to birds in these experiments.

Toll-like receptor 2 (*TLR2*) was the third most variably expressed gene across experiment data, and had highest mRNA expression levels relative to the housekeeper genes. Although there was no association of *TLR2* with high- and low-performance variation detected in any experiment, the variation in the expression levels of *TLR2* are of interest. There is suggestion that in addition to maintaining intestinal epithelial homeostasis, TLRs, particularly *TLR2*, could participate in the sensing of the energy state of the body and to the subsequent control of food intake (Wolowczuk et al., 2008). Additionally *CD36*, which was the fourth most variably expressed gene, has been linked to facilitating *TLR2* signalling (Wolowczuk et al., 2008), lipid transport (Hoebe et al., 2005) and fat deposition (Shu et al., 2011). Although we saw differences in final bodyweights between high- and low-performing broilers at d25, we

have no information on the carcass composition of the broilers and therefore cannot speculate further on the current data in relation to this. However, given the links between *TLR2* and *CD36*, these findings warrant further investigation into links between innate immunity and lipid metabolism in broilers in relation to performance variation.

Four genes were found to be differentially expressed between high- and low-performing broilers; two genes in P2E1 (antimicrobials *AvBD1* and *AvBD2* in the control birds) and two genes in P1E2 (tight junction proteins *CLDN5* and *ZO1*), however, the differential expression could not be replicated. The differential expression found therefore does not provide evidence that broiler flock performance variation can be consistently linked with basal parameters of innate intestinal immunity investigated in the ileum in this study. It is of interest to investigate further the link between innate immunity and fatty acid metabolism in broilers, as this work has largely been studied in mammals. Consideration also needs to be given to a major limitation in this experimental work, which was the small (although significant) differences observed in FCR values between the "high FCR" birds and "low FCR" birds. The experimental differences in such a controlled environment are unlikely reflective of the true variation seen in a commercial setting, and therefore, greater variation and range in FCR values are desired experimentally for biological or functional changes to be identified in future studies.

3.6   Acknowledgements

3.7   References

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CHAPTER FOUR: Literature Review Part 2

Is avian lipid metabolism and its links to innate immunity associated with variations in growth performance of meat birds?

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Is avian lipid metabolism and its links to innate immunity associated with variations in growth performance of meat birds?

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Running title: Lipid metabolism, innate immunity and avian performance variation

4.1 Abstract

The meat chicken (broiler) industry has undergone intense genetic selection over the past 50 years resulting in historical improvements for growth and feed efficiency, however, significant variation remains for performance and growth traits. Production improvements have been coupled with unfavourable metabolic consequences, including immunological trade-offs for growth, and excess fat deposition. Excess fat deposition results in decreased feed efficiency, which is an economic cost to the producer, and also results in decreased processing yields through the removal of visceral fat. Broilers make for an interesting model of obesity, as birds are naturally hyperglycaemic, yet, are relatively insensitive to insulin. Given the correlation between obesity and chronic inflammation, it is reasonable to postulate that the performance variation seen in modern commercial broiler flocks could be attributed to interactions between altered fatty acid metabolism (including excess fat deposition), innate immunity, and the resultant activation of inflammatory pathways by lipid ligands. The scope of this review was to explore the current biology of avian lipid metabolism, and to investigate fatty acid interactions with innate immunity to elucidate possible mechanisms contributing to the performance variation commonly seen in commercial broiler flocks.

Key words: Chicken, Innate Immunity, Lipids, Obesity, Performance Variation

4.2   Introduction

Over the past 50 years, the intensification of the broiler (meat chicken) industry and concurrent genetic selection, primarily for growth and feed conversion ratio by major breeding companies, has resulted in a ~400% increase in the growth rates of commercial broilers (Zuidhof et al., 2014). Despite these advances there still remains significant performance variation (>10%) within strains of genetically-similar animals (Emmerson, 1997). Production gains have not been without unfavourable consequences to the broilers and the industry alike. One major consequence of selection for growth has been increased fat deposition which has been suggested to have peaked in the 1970s, but since reduced by ~10% due to additional increased selection emphasis on efficiency (Tallentire et al., 2016). Increases in fat are mainly stored as abdominal fat, and highly correlated with total carcass fat (Griffiths et al., 1978). Excessive adipose tissue not only decreases feed efficiency during production, but also results in economic impairment due to 1) the decrease in feed efficiency, thus increasing the economic cost of production and 2) removal of abdominal fat by evisceration, decreasing processing carcass yields (Daval et al., 2000, Choct et al., 2000).

It is estimated between 15-18% of commercial broiler total bodyweight is fat and it has been suggested that 85% of this stored fat is nonessential to normal physiological functioning (Daval et al., 2000, Choct et al., 2000, Leenstra, 1986). The negative association of carcass fat with avian performance has prompted investigation into the genetic mechanisms underlying fat deposition in poultry. Major models used for much of this work began in the early 1980s with the development of genetically 'fat' or 'lean' lines. Selection criteria was based either on high or low abdominal fat (Leclercq et al., 1980, Leclercq and Simon, 1982), or very low density lipoprotein (VLDL) plasma concentrations (Whitehead and Griffin, 1984). Comparisons of these lines, regardless of nutritional status, shows that total plasma lipids and lipoprotein levels are higher in the fat lines (FL), suggesting a higher rate of hepatic lipogenesis in FL broilers (Hermier et al., 1984).

In avian species, plasma lipid substrates for triglyceride storage are primarily derived either directly from the diet, in a concentrated form in high-energy poultry rations (6-10%), (Hermier, 1997, Cherian, 2007); or, through hepatic lipogenesis. Early avian *in vivo* and *in vitro* studies in the pigeon concluded that the liver was the main site of fatty acid synthesis, and determined that as much as 96% of total body lipogenesis was occurring in the liver, with just 4% occurring in the adipose tissue (Goodridge and Ball, 1967, Goodridge and Ball, 1966). Investigations into the lipogenic capacity of isolated adipose tissue of male cross-bred chicks (New Hampshire males x Columbian females) supported the notion that avian adipose tissue plays a minor secondary role in overall lipogenesis (O'Hea and Leveille, 1968). Additionally, it was found that adipose tissue was insensitive to insulin, and that pyruvate and acetate were utilised preferentially over glucose, which is in direct contrast to findings in rats and mice. In the liver however, the insulin-signalling cascade parallels that seen in mammals (Ji et al., 2012). Recent work on avian adipose tissue using RNA-seq has shown that avian adipose tissue is much more biologically active than initially thought, and that its 'negligible' role in fatty acid synthesis may need to be reviewed (Resnyk et al., 2015).

Relative to egg-laying or wilder strains, some now consider commercial broilers to be 'obese', which could be considered true as obesity is generally defined by an excess of fat content in the body (Lin et al., 1980). The term 'obese' however must be used with caution in reference to broilers, as this classification is not derived from an anatomical or body mass index classification. Obesity is correlated with a state of low-grade chronic inflammation in humans, characterised by the synthesis of pro-inflammatory cytokines from both adipocytes and immune cells such as macrophages (Fresno et al., 2011). Lipid metabolism and innate immunity are thought to be closely intertwined, as many lipid themselves are ligands for transcription factors such the peroxisome proliferator activated receptors (PPARs); and sterol regulatory element binding proteins (SREBPs). PPARs and SREBPs directly regulate fatty acid metabolism gene transcription as well as inflammatory responses (Forman et al., 1997, Brown and Goldstein,

1997). Additionally several lipids have been shown to be ligands for Toll-like receptors (TLRs), particularly TLR2 and TLR4, with activation initiating downstream inflammatory responses and innate immune dysregulation, consequently interrupting insulin signalling (Fresno et al., 2011). Whilst insulin resistance is generally associated with obesity, the mechanisms behind the resistance remain somewhat unclear, but are hypothesised to also include adipokine production, accumulation of intracellular lipids, endoplasmic reticulum stress (ER stress), and activation of innate inflammatory responses (DiAngelo et al., 2009). Chickens make for an interesting obesity model as they naturally exhibit hyperglycaemia and decreased insulin sensitivity relative to mammals, however spontaneous insulin resistance is not observed in birds (Đaković et al., 2014). Although relatively insensitive to insulin, particularly in adipose tissue, studies of fat (FL) and lean line (LL) chickens demonstrate a glucose-insulin imbalance in the FL broilers (Simon and Leclercq, 1982).

Given the problem of excess fat deposition in modern broilers and the associated economic impacts, the scope of this review is to explore the biology behind avian lipid metabolism and fat deposition, and to investigate the interactions between innate immunity and lipid metabolism (Figure 4.1). The objective is to further elucidate possible mechanisms contributing to the performance variation and excess fat deposition commonly seen in commercial broiler flocks.

Figure 4.1 Summary of review scope: "Is avian lipid metabolism and links to innate immunity associated with variations in growth of meat birds?" Section 4.4 Summarises avian fatty acid metabolism; Section 4.5 Explores the regulation of avian fatty acid metabolism; and Section 4.6 Investigates Fatty Acid Metabolism and Innate Immunity.

4.3 Avian Fatty Acid Digestion, Absorption and Transport

Like mammals, lipids ingested by avians arrive intact in the duodenum where the presence of food stimulates the release of cholecystokinin, contraction of the gallbladder and release of pancreatic juice to emulsify dietary triglycerides and other fat-soluble nutrients. Insoluble amphiphiles, such as monoglycerides, glycerol and free fatty acids (medium chain and unsaturated long chain) swell and interact with bile salts resulting in the spontaneous formation of mixed micelles (Krogdhal, 1985). The hydrophobic core of these micelles acts as liquid crystals, aiding to solubilise diglycerides and long chain unsaturated fatty acids (Krogdhal, 1985). The jejunum is the most active site for lipid absorption in the chicken (and mammals), however, with increasing levels of fat, the ileum has a more active role in lipid absorption in comparison to mammals (Griminger, 1976). This is thought to be due to the location of the pancreatic and bile ducts, which enter at the proximal end of the duodenum in mammals, but the distal end in avians (Renner, 1965).

In the enterocytes, monoglycerides and free fatty acids are re-esterified in combination with free and esterified cholesterol and phospholipids to form lipoproteins (Baião and Lara, 2005). In mammalian species, specialised lipoproteins (termed chylomicrons) are secreted into the lymphatic system and carried by the thoracic lymphatic duct to the vena cava where they enter circulation (Nelson and Ackman, 1988). The route of absorption depends on the fatty acid carbon chain length, with short chain fatty acids (fewer that 8-12 carbons) absorbed and passed into the portal vein, thus going directly to the liver without incorporation into chylomicrons (Nelson and Ackman, 1988). Compared to mammals, avians have a poorly-developed lymphatic system and the route of absorption of lipoproteins is instead through the portal venous system directly to the liver, and therefore termed portomicrons (Bensadoun and Rothfeld, 1972). Early work has suggested that portomicrons are not likely metabolised by the liver due to their size (Fraser et al., 1986), and that rapid catabolism of portomicrons occurs in

extrahepatic tissue. During absorption and transport, no alterations are made to the composition of fatty acids, therefore dietary lipids, and stored body fat are similar (Baião and Lara, 2005).

4.4 Avian Fatty Acid Metabolism

4.4.1 Overview of de novo Hepatic Fatty Acid Synthesis

Avian fatty acid synthesis occurs in the cytosol, catalysed by two enzyme systems: the biotin containing enzyme acetyl-CoA carboxylase (ACC) and the multi enzyme complex Fatty Acid Synthase (FASN). These two enzyme systems have been comprehensively reviewed by Volpe and Vagelos (1973). Synthesis starts with acetyl-CoA, derived from 1) the oxidative carboxylation of pyruvate, an end product of glycolysis; 2) the breakdown of ingested or previously synthesised fatty acids; or 3) catabolism of certain amino acids (Griminger, 1976). Acetyl-CoA must initially be transferred from the mitochondria to the cytosol via the tricarboxylate transport system (citrate shuttle). Acetyl-CoA is bound to a four-carbon molecule of oxaloacetate to form citrate for transfer across the mitochondrial membrane. In the cytosol, the reaction is reversed by the enzyme ATP-citrate-lyase, leaving the acetyl-CoA free for fatty acid synthesis (Bensadoun and Rothfeld, 1972). The oxaloacetate produced by this reversal is converted to malate, by malate dehydrogenase. An additional reaction proceeds where malate can be converted to pyruvate, by malic enzyme, producing NADPH and CO_2 (Bensadoun and Rothfeld, 1972). Both malate and pyruvate are shuttled back to the mitochondria, with the resultant NADPH and CO_2 utilised for fatty acid synthesis.

For fatty acid synthesis to proceed in the cytosol, acetyl-CoA must first be converted to malonyl-CoA, catalysed by ACC (Griminger, 1976). This is an irreversible reaction and is the first committed step in fatty acid synthesis. Acetyl-CoA carboxylase has been found in limited amounts in embryonic chicken liver, but increases to adult levels by approximately 20 days post hatch (Arinze et al., 1970). The overall reaction requires 1 acetyl-CoA, 1 ATP, 1 CO_2 as well as ACC (Figure 4.2a). Following the formation of malonyl-CoA, the elongation of fatty

acids occurs by a series of repetitive cyclic reactions, driven by the multi-enzyme complex FASN. The *FASN* gene is a highly efficient system, as a singular gene encodes seven enzymes and a site for the acyl carrier protein required for fatty acid synthesis (Smith, 1994). *FASN* is a αβ dimer in animals with the α-subunit containing the β-ketoacyl-ACP synthase and β-ketoacyl-ACP reductase enzymes, whilst the β- subunit contains the acetyl transferase domain, the malonyl transferase domain, the β-hydroxyacyl dehydrogenase domain and the enoyl reductase domain. Initially, two simple acyl carrier protein (ACP) complexes are formed, acetyl-ACP (Figure 4.2b) and malonyl-ACP (Figure 4.2c), catalysed by acetyl transferase and malonyl transferase respectively.
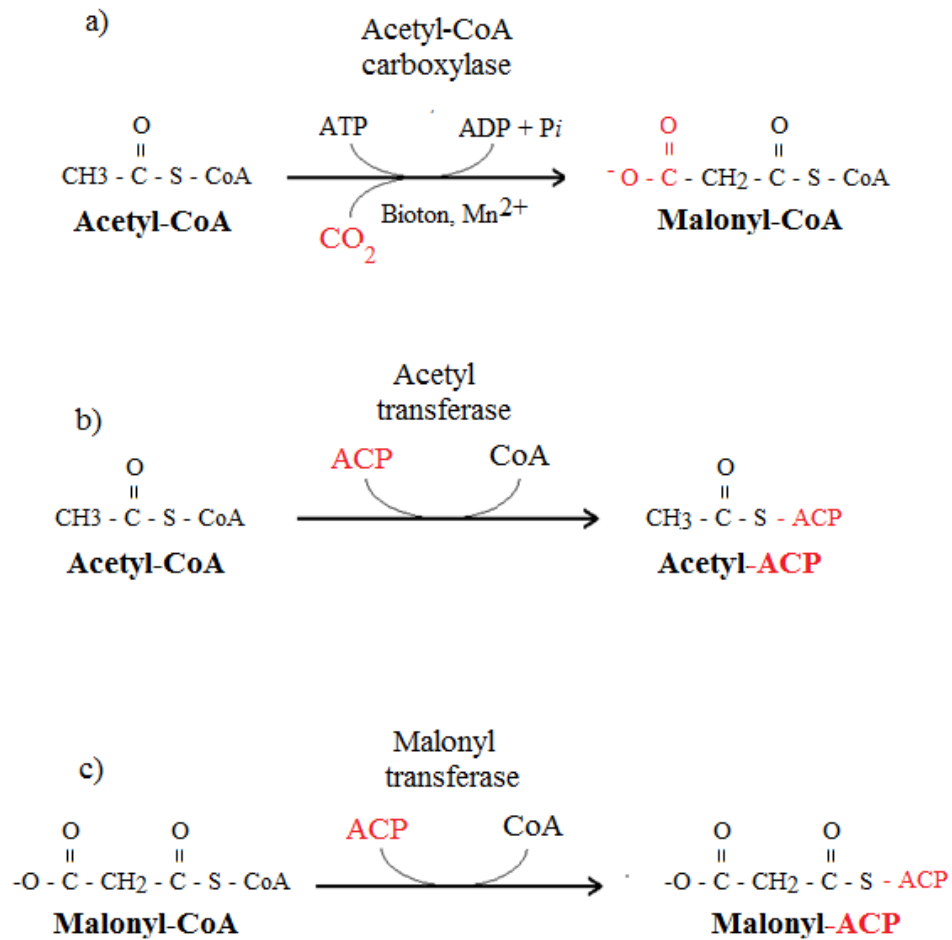
Figure 4.2 Carboxylation of acetyl-CoA to form malonyl-CoA with the biotin containing enzyme acetyl-CoA carboxylase. Acetyl-CoA carboxylase is the first rate limiting enzyme of fatty acid synthesis (4.2a). Acetyl-CoA and an acyl carrier protein (ACP) are joined to form acetyl-ACP, catalysed by acetyl transferase (4.2b). Formation of malonyl-ACP requires malonyl-CoA and an acetyl carrier protein (ACP). The reaction is catalysed by malonyl transferase, specific for malonyl-CoA (4.2c).

The elongation of fatty acids occurs in four sequential steps (Figure 4.3). Volpe and Vagelos (1973) and Wakil et al. (1983) have each reviewed the synthesis of fatty acids extensively. In brief, the four steps of elongation are; Step 1) condensation of acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP, catalysed by β-ketoacyl-ACP synthase (Figure 4.3.2.1). Step 2) the keto group is reduced to alcohol by NADPH, catalysed by β-ketoacyl-ACP reductase to form β-hydroxybutyryl ACP (Figure 4.3.2.2). Step 3) dehydration of the alcohol produced in step two, (catalysed by D-β-hydroxyacyl ACP dehydratase) introduces a double bond into the molecule, forming crotonyl-ACP (Figure 4.3.2.3). Step 4) removal of the double bond by hydrogenation with NADPH (catalysed by 2, 3- Trans-Enoyl-ACP Reductase), to produce butyl ACP (Figure 4.3.2.4). This first two-step process produces a four-carbon fatty acid chain (C_4). The four-step cycle then continues with the addition of another malonyl-ACP and repeated for a further six cycles. The final product is palmitate; a 16 carbon (C_{16}) saturated fatty acid, which is cleaved from the enzyme complex by palmitate thioesterase.

Phospholipid
Triacylglyceride

Phosphatidic acid

Glyceroneogenesis
Glycolysis ——→ DHAP

Gly-3-P
Acyltransferase

HA Lipase

Gly-kinase
Glycerol ——→ Glycerol-3-P

Malonyl-ACP

Elongation

Palmitate (C_{16})
$CH_3 - (CH_2)_{11} - CH_2 - CH_2 - CH_2 - C - O^-$
O

→ Modification

Palmitoyl-CoA (C_{16})
$CH_3 - (CH_2)_{11} - CH_2 - CH_2 - CH_2 - C - S - CoA$
O

Acyl-CoA synthetase

AMP   ATP
           CoA-SH

Desaturation
Cannot do:
Linoleic acid (n-6)
Linolenic acid (n-3)

Fatty acid released:
Thioesterase

Butyryl-ACP (C_4 Fatty acid)
$CH_3 - CH_2 - CH_2 - C - S - CoA$
O

Carnitine Transport System

Mitochondria   **Cytosol**

1. FA β Oxidation

Palmitoyl-CoA (C_{16})
$CH_3 - (CH_2)_{11} - CH_2 - CH_2 - CH_2 - C - S - CoA$
O

2,3-Trans-Enoyl-ACP
reductase

NADP$^+$

NADPH/H$^+$

2.4 Hydrogenation

1.1 Oxidation

Acyl-CoA
dehydrogenase
*Chain specific

FAD

FADH$_2$

Crotonyl-ACP
OH   O
$CH_3 - C = C - C - S - ACP$
H

Trans-△2-eonyl-CoA
$CH_3 - (CH_2)_{11} - CH_2 - CH = CH - C - S - CoA$
O

D-β-hydroxyacyl-ACP
dehydratase

H$_2$O

1.2 Hydration

Enoyl-CoA
hydratase

H$_2$O

2.3 Dehydration

β-Hydroxybutyryl-ACP
H   O
$CH_3 - C - CH_2 - C - S - ACP$
OH

L-3-Hydroxyacyl-CoA
OH   O
$CH_3 - (CH_2)_{11} - CH_2 - CH - CH_2 - C - S - CoA$

Repeat

1.3 Oxidation

β-hydroxy Acyl-CoA
dehydrogenase

NAD$^+$

NADH/H$^-$

β-Ketoacyl-ACP
reductase

NADP$^+$

NADPH/H$^+$

2.2 Hydrogenation

Acetoacetyl-ACP
O   O
$CH_3 - C - CH_2 - C - S - ACP$

3-Ketoacyl-CoA
O   O
$CH_3 - (CH_2)_{11} - CH_2 - C - CH_2 - C - S - CoA$

2.1 Condensation

β-Ketoacyl-ACP
synthase

ACP + CO$_2$

2. FA Synthesis

1.4 Clevage

Thiolase

HS-CoA

Acetyl-ACP
O
$CH_3 - C - S - ACP$

+

Malonyl-ACP
O   O
$^-O - C - CH_2 - C - S - ACP$

Multi-enzyme complex
Fatty Acid Synthase (FAS)

Cycle continues:
Six more Malonyl-CoAs added for C_{16} fatty acid (Palmitate)

Myristol-CoA (C_{14})
$CH_3 - (CH_2)_{11} - CH_2 - C - S - CoA$
O

+

Acetyl-CoA
$CH_3 - C - S - CoA$
O

Tricarboxylate Transport System

Acetyl
transferase

CoA

ACP

Malonyl
transferase

CoA

ACP

Acetyl-CoA
$CH_3 - C - S - CoA$
O

Malonyl-CoA
O   O
$^-O - C - CH_2 - C - S - CoA$

Acetyl-CoA → Citrate   Citrate → Acetyl-CoA

OAA   OAA   → Glycolysis

NAD$^+$

Malate

Pyruvate ← Pyruvate   NADPH

→ FA Synthesis

Acetyl-CoA
carboxylase

ADP + P$_i$

ATP + CO$_2$

*Rate limiting
enzyme

Acetyl-CoA
$CH_3 - C - S - CoA$
O

Figure 4.3 Overview of avian hepatic fatty acid metabolism; 2.1) Fatty Acid (FA) β-Oxidation occurring in the mitochondria; 2.2) Fatty Acid (FA) Synthesis, occurring in the cytosol of the cell.

Shorter chain fatty acids can be generated by releasing the fatty acid before reaching 16 carbons of length. Further modification of fatty acids results in additional elongation, or desaturation. Modification generally occurs the endoplasmic reticulum (ER) rather than the mitochondria, with the addition of malonyl-CoA, but by different enzymes. Monounsaturated fatty acids (MUFAs) are primarily obtained from the diet; however, *de novo* synthesis does occur in the ER. The first critical step in the biosynthesis of MUFAs is the insertion of the first double bond between the 9th and 10th carbon, an oxidative reaction catalysed by stearoyl-CoA desaturase (SCD1) (Ntambi, 1999). The precursors for MUFA production are the saturated fatty acids palmitate (C16:0) and stearate (C18:0), producing the ω-7 palmitoleate and the ω-9 oleate respectively (Figure 4.4) (Ntambi and Miyazaki, 2004). Much investigation into the role of SCD1 has been carried out in murine models, in which four isoforms of SCD1 have been identified, differing in tissue distribution (Miyazaki et al., 2003). In avians, thus far only one isoform has been identified (Dridi et al., 2007). The MUFAs synthesised by SCD1 are used as major substrates for phospholipid, triglyceride and cholesterol esters, with increasing evidence that SCD1 plays a major role in body weight control and lipid metabolism in mammals (Dridi et al., 2007).

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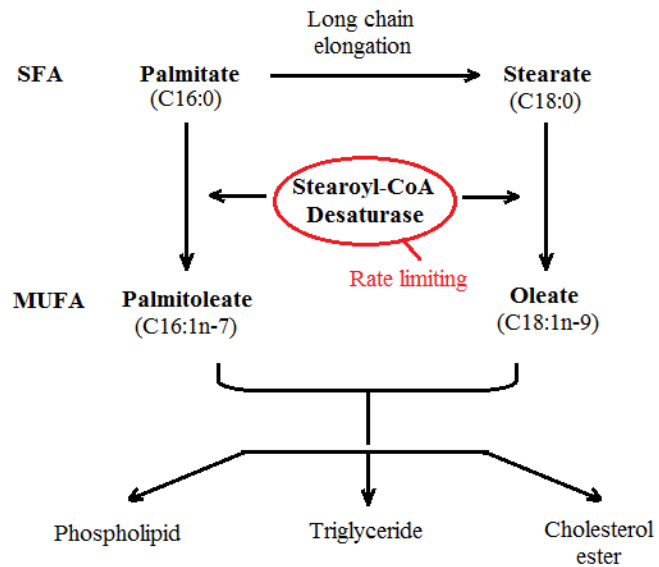
Figure 4.4 Long chain saturated fatty acids (SFA) palmitate and stearate undergo an oxidative reaction catalysed by the enzyme stearoyl-CoA desaturase. The monounsaturated fatty acids (MUFA) produced are palmitoleate and oleate respectively. Stearoyl-CoA desaturase is rate limiting for MUFA production. Adapted from Ntambi and Miyazaki (2004).

4.4.2 Fatty Acid β-Oxidation

Long chain fatty acids are catabolised via the β-oxidation pathway in the mitochondria. In this process, a saturated acyl-CoA is degraded by a recurring sequence of four reactions (Figure 4.3.1). Before β-oxidation can occur, fatty acids must be converted to CoA thioesters, catalysed by acyl-CoA synthetase (Schulz, 1991). The mitochondria is only permeable to acyl groups of fatty acids if linked to carnitine, of which there are two proteins, carnitine palmitoyltransferase (CPT); CTP1 and CTP2. These proteins work in reverse, with CTP1 residing on the outer, and CPT2 residing on the inner side of the mitochondrial membrane respectively (Lopez-Marques et al., 2015). CTP1 has been shown to be rate-limiting, as it is inhibited by the fatty acid synthesis intermediate, malonyl-CoA (Lopez-Marques et al., 2015).
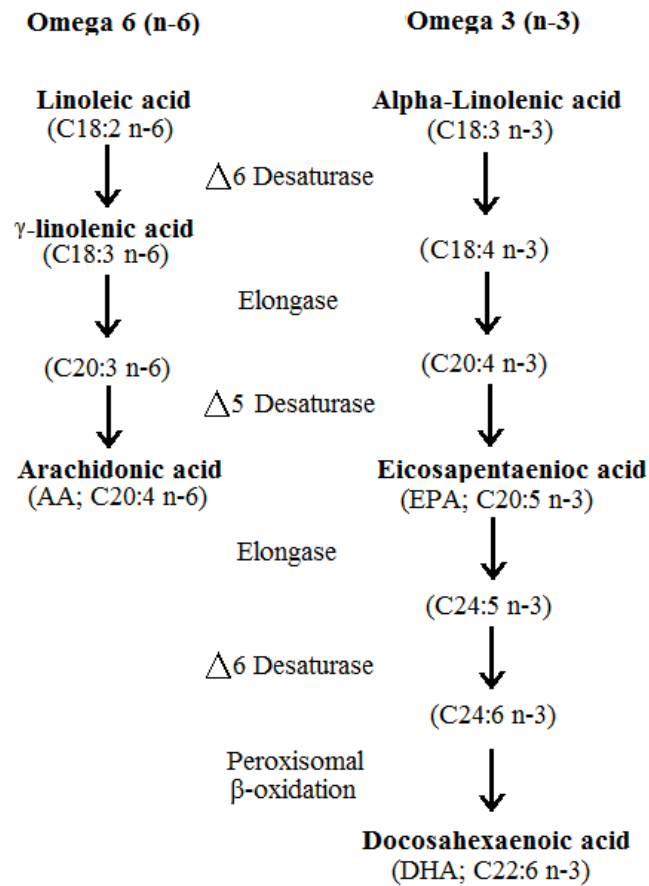
Once in the mitochondrial matrix, a sequential cycle of four reactions takes place, resulting in the removal of 2 carbon atoms in the form of acetyl-CoA. In brief, the four steps of β oxidation are; Step 1) the oxidation of acyl-CoA to 2-trans-enoyl-CoA (Figure 4.3.1.1). The enzymes used differ depending on the chain length of the fatty acid being catabolised (Schulz, 1991). Step 2) 2-trans-enoyl CoA is hydrated to L-3-hydroxyacyl-CoA, (Figure 4.3.1.2). Step 3) oxidation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA (Figure 4.3.1.3), and Step 4) the cleavage of 3-ketoacyl-CoA thioesters, resulting in a shortened fatty acid chain by two carbons, and acetyl-CoA (Figure 4.3.1.4) (Schulz, 1991). This four step series of reactions is repeated on the resultant shortened fatty acid until the complete breakdown.

4.4.3 Essential Fatty Acids-Polyunsaturated Fatty Acids

Aves, like mammals, require essential fatty acids (EFAs) for normal physiological functioning. Polyunsaturated fatty acids (PUFAs) are 18 carbons or more in length and contain two or more double bonds. PUFAs are split into two major groups, ω-6 and ω-3, classified by the position of the first double bond proximal to the methyl end of the fatty acid (Leonard et al., 2004). Two

major PUFAs that must be derived from the diet are; ω-6 linoleic acid (C18:2n-6) and ω-3 α-linolenic acid (C18:3n-3). Although linoleic, α-linolenic and arachidonic acids are all generally considered metabolically essential in poultry, a direct supply of dietary linoleic acid is considered sufficient to meet EFA requirements (Watkins, 1991). The inclusion level of dietary linoleic acid has been estimated at ~1%, however Zornig et al. (2001) have suggested this could be an overestimation, and that EFA requirements can be met with linolenic inclusion levels as low as 0.20% if the diet contains adequate levels of total lipids and energy.

Both linoleic acid and α-linolenic acid compete in a shared pathway of desaturation and elongation enzymes (Figure 4.5) (Nakamura and Nara, 2003). The ω-6 linoleic acid is initially converted to arachidonic acid (AA). Arachidonic acid can be further metabolised to form either docosapentaenoic acid (C22:5n-6) or functions as a precursor to eicosanoid production, including 2-series prostaglandins (i.e. PGE_2) and 4-series leukotrienes (i.e. LTB_4), having both pro- and anti-inflammatory functions (Poudyal et al., 2011). In the same pathway, the ω-3 α-linolenic acid is converted to eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3). EPA and DHA are also precursors of eicosanoid production, however the end products, such as 3-series prostaglandins (i.e. PGE_3) and 5-series leukotrienes (i.e. LBT_5), are known to have 'less' inflammatory effects than the eicosanoids produced from the ω-6 eicosanoid precursors (Cherian, 2007). In this common anabolic pathway, the Δ6 desaturase enzyme has a higher affinity for α-linolenic acid, however, if the dietary ratio of ω-6 to ω-3 favours high levels of ω-6, which is common in commercial poultry diet formulations, then long chain ω-3s can be depleted (Horrocks and Yeo, 1999, Watkins, 1991). In poultry it has been shown numerous times, that the composition of fatty acids in the tissue, is responsive to alterations of the ω-6 to ω-3 dietary ratio (Lopez-Ferrer et al., 2001, Carragher et al., 2016).

```
        Omega 6 (n-6)                    Omega 3 (n-3)

       Linoleic acid                  Alpha-Linolenic acid
        (C18:2 n-6)                       (C18:3 n-3)
            |                                 |
            |         △6 Desaturase           |
            ↓                                 ↓
      γ-linolenic acid
        (C18:3 n-6)                       (C18:4 n-3)
            |                                 |
            |           Elongase              |
            ↓                                 ↓
       (C20:3 n-6)                        (C20:4 n-3)
            |         △5 Desaturase           |
            |                                 |
            ↓                                 ↓
     Arachidonic acid               Eicosapentaenioc acid
      (AA; C20:4 n-6)                  (EPA; C20:5 n-3)
                                          |
                        Elongase          |
                                          ↓
                                      (C24:5 n-3)
                                          |
                   △6 Desaturase          |
                                          ↓
                                      (C24:6 n-3)
                   Peroxisomal            |
                   β-oxidation            ↓
                               Docosahexaenoic acid
                                 (DHA; C22:6 n-3)
```

Figure 4.5 The anabolic pathway of linoleic acid (C18:2 n-6) to arachidonic acid (C20:4 n-6) and alpha-linolenic acid (C18:3 n-3) to EPA (C20:5 n-3) are the same. Both compete for the same enzymes with the $\Delta 6$ desaturase having a higher affinity for alpha-linolenic acid. Further conversion of EPA to DHA (C22:6 n-3) also uses the $\Delta 6$ desaturase enzyme which has a lower affinity compared to linoleic or alpha-linolenic acids, resulting in low production of DHA.

4.5 Regulation of Fatty Acid Metabolism

4.5.1 Dietary Lipid Composition and FA Manipulation

The dietary fatty acid profile has been consistently shown to modulate both lipid metabolism and deposition in broilers. Crespo and Esteve-Garcia (2001) studied the effect of four different dietary fatty acid profiles: 1) SFA; 2) MUFA; 3) PUFA (high ω-6) and 4) PUFA (high ω-3) on performance parameters and abdominal fat deposition. Their results found the high PUFA diets reduced the abdominal fat deposition with no difference in final bodyweights. This is accordance with the findings of multiple studies including Sanz et al. (2000a), where fatty acid composition of the diets were reflected in the fatty acid composition of the tissues (thigh, breast and fat pad). Furthermore, the association of dietary fatty acid profile and lipid deposition has also been shown in the serum (Frttsche et al., 1991, Newman et al., 2002) as well as lymphoid tissues, such as the spleen, thymus and bursa of broilers fed differing dietary fatty acid profiles (Frttsche et al., 1991). Higher resting metabolic rates, lower abdominal fat deposition and higher breast muscle mass have also been reported in response to higher dietary PUFA incorporation in broilers (Newman et al., 2002). The effects of the saturation level of dietary fat have been associated with lipogenesis and lipolysis, ultimately influencing fat deposition. Diets containing high levels of PUFAs rather than SFA have been shown to potentially increase the rate of β-oxidation with results indicating increased levels of *CPT1*, and a decrease in fatty acid synthesis, with decreased levels of *FASN* observed (Sanz et al., 2000b).

Diets high in PUFA, both ω-6 and ω-3, are thought to alter fatty acid metabolism in a number of ways; 1) the enrichment of plasma and microsomal membranes alters hormone binding to cell-surface receptors, the effects of which will alter carbohydrate and lipid metabolism (Clarke and Jump, 1994). 2) PUFAs affect nuclear mechanisms that change the expression of key genes involved in lipid metabolism-including *FASN*, malic enzyme (*ME1*), *SCD1, ACC* and the S14 protein (Clarke and Jump, 1994). In order for a PUFA to have an inhibitory effect on the expression of genes encoding lipogenic proteins, the fatty acid chain

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must be a minimum of 18 carbons in length, with the double bonds, at the 9th and 12th positions (Clarke and Jump, 1994).

4.5.2 Maternal Effects

It is well established that dietary fatty acid composition has a significant impact on avian lipid metabolism and growth, however, it's become increasing clear that the maternal diet composition, and subsequent egg yolk lipid composition in which the progeny develops, has an even greater effect on progeny health and metabolism (Cherian, 2015, Hynd et al., 2016). This is not surprising given a chick now spends 30% of its life *in ovo*, relying on nutrients in the egg deposited by the breeder hen. White Leghorn laying hens fed diets containing either flax or canola seed (high ω-3 PUFA) were compared with hens fed control diets containing either soybean or wheat-based diets. Compared with controls the ω-3 contents of the eggs increased significantly, as did the ω-3 content in the brains of chick embryos (Cherian and Sim, 1991). Additionally, plasma levels in chicks hatched from flax-fed mothers also had significantly higher ω-3 levels, and a reduced circulating ω-6, specifically arachidonic acid (Cherian and Sim, 1991). Broiler breeder hens fed differing levels of ω-6 and ω-3 oils showed similar responses in the progeny raised on identical diets. After 2 weeks ω-6 levels in the spleens did not differ between any of the chicks, however progeny from hens fed high ω-3 retained significantly higher levels of long chain ω-3s (Wang et al., 2002). Similar experiments have also showed higher retained ω-3 levels in cardiac and hepatic tissue (Cherian, 2007), as well as immune response and PUFA derived eicosanoid production (Bautista-Ortega et al., 2009, Hall et al., 2007). All results indicate that the maternal diet composition has a unique role in modulation of progeny lipid metabolism, as well as eicosanoid metabolism, derived from either the ω-6 or ω-3 precursors, thereby influencing immune and inflammatory responses in the progeny in addition to performance.

4.5.3   Transcription Factors

Multiple transcription factors have been linked as probable targets for fatty acid regulation including but not limited to; sterol regulatory element binding proteins (SREBPs), liver-X factor-α (LXR-α), retinoid X receptor-α (RXR-α) and peroxisome proliferator activated receptors (PPARs). Peroxisome proliferator activated receptors are transcription factors that belong to a supergroup of nuclear hormone receptors. Chickens have three subtypes of PPARs (α, β and γ), with PPARα known to regulate the transcription of several target genes mainly involved in lipid metabolism leading to increased levels of FA β-oxidation (Contreras et al., 2013, Takada and Kobayashi, 2013). *PPAR-α* is highly expressed in the liver whereas *PPARγ* expression occurs more so in adipose tissue and in macrophages and appears to influence adipocyte differentiation (Yoshikawa et al., 2003). Ligands for PPAR-α activation include PUFAs (both ω-3 and ω-6), eicosanoids and hypolipidemic drugs, such as fibrates (Forman et al., 1997). Activation modulates DNA transcription through binding to specific nucleotide sequences in the regulatory region of target genes, termed PPAR response elements (PPREs) (Forman et al., 1997). For binding to occur, PPAR-α must form a heterodimer with RXR-α (Contreras et al., 2013). Once a ligand binds with PPAR-α, the PPAR-α/RXR-α heterodimer undergoes conformational changes inducing an active transcriptional complex (Contreras et al., 2013).

Sterol regulatory element binding proteins (SREBP) are a family of transcription factors known to regulate the transcription of genes encoding enzymes in the cholesterol biosynthetic pathway (SREBP-2), as well as genes encoding enzymes in fatty acid synthesis and uptake (SREBP-1) in most organs (Brown and Goldstein, 1997). Assaf et al. (2003) studied the distribution of these two genes to analyse the correlation between SREBP expression and the lipogenic capacity of the tissue. Their results showed high expression of *SREPB-1c* in the avian liver, and contrastingly low levels in adipose tissue, which would be expected given the liver is the major site of lipogenesis in birds. SREBPS are membrane bound transcription factors that

enter the cell nucleus and bind with to SRE or related sequences to activate gene transcription. SREPB-1c activates the transcription of major genes in FA synthesis, including (not limited to) *ACC*, *FASN*, *SCD1* and glycerol-3-phosphate acyltransferase (Schultz et al., 2000).

As discussed, RXR-α binds with PPAR-α, but addition to this, RXR-α also forms obligate heterodimers with liver-X receptor-α, a subclass of nuclear hormone receptors. This heterodimer has been identified as a dominant activator of SREBP1-c promotor in mice models (Yoshikawa et al., 2001). Interestingly, activation of PPAR-α represses LXR signalling and LRX mediated SREBP1-c gene expression through reduction of the RXR/LRX heterodimerisation in the liver (Yoshikawa et al., 2003), and conversely, activation of LXR supresses PPAR-α signalling (Ide et al., 2003).

The co-ordinated and reciprocal roles of PPAR-α and SREBP-1 can be best compared as to their converse function in fed and fasted states. The role of PPAR-α as a key mediator of fatty acid oxidation was demonstrated by Leone et al. (1999) using *PPAR-α* knock out mice. Fasting induced significant hepatic and cardiac expression of PPAR-α target genes for mitochondrial and extra-mitochondrial genes modulating mitochondrial β-oxidation in PPAR-$\alpha^{+/+}$ mice, but not in PPAR-$\alpha^{-/-}$ mice, confirming a critical role of PPAR-α in β-oxidation and the fasting response (Leone et al., 1999). In contrast, a reduction of *SREBP1c* expression has been demonstrated in the fasted state, and dramatically increased in the refed state inducing lipogenesis, whereas the activity of *PPAR-α* was reduced (Yoshikawa et al., 2003).

Advances in gene technology, such as RNA-seq, are providing new insights into fatty acid metabolism and regulation in broilers. Li et al. (2015), used RNA-seq to compare the livers of juvenile and laying hens and found the PPAR signalling pathway was enriched with 18 significantly differentially expressed genes. *PPAR-α* was shown to be supressed in the livers of laying hens compared to juveniles. PPAR-α pathway genes involved in fatty acid transport were

up regulated as were genes involved in lipogenesis, including *SCD1* and Δ6 desaturase (*FADS6*), in laying hens compared to juveniles. Given the laying hen needs to synthesise additional lipid to be deposited in the yolk this would be anticipated. Another study used the PPAR ligand clofibrate to activate the PPAR-α pathway to determine whether SREBP-1c activity would be inhibited in the livers of broilers (Zhang et al., 2015). Triglyceride concentration was lower in the livers of birds supplemented with clofibrate, indicating that activation of PPAR-α had reduced the transcription and activation of SREBP-1, repressed LXR-α mediated activity of SREBP-1, and, consequently reduced lipogenic gene expression. This is in agreement with findings in mice (Yoshikawa et al., 2003). Cholesterol levels in the liver of clofibrate fed birds were also decreased via a reduction in SREBP-2 dependent gene expression. Interestingly from a performance perspective, the bodyweights of the treatment groups were not significantly different, nor were their feed conversion ratios. Given that excessive adiposity is a major problem in broiler production, this finding is particularly exciting as a potential strategy for nutritional manipulation of fat accumulation in production.

4.6   Fatty Acid Metabolism and Innate Immunity

Interactions between PPARs, Toll-like receptors, adipokines and cytokines have all emerged as links between lipids and innate immunity, as has stress at a cellular level. In chapter three, we hypothesised that performance variation in broiler flocks could be attributed to functional differences in innate immunity, and that broilers with a more functionally efficient intestinal innate immune system would perform better. Our candidate gene selection included genes linked to various aspects of innate immunity including Toll-like receptors and endoplasmic reticulum (ER) stress, and focused solely on the ileum. The ileum was targeted due to our colleagues' previous identification of gut microbes associated with the phenotypic expression of FCR (Stanley et al., 2016). Our findings did not show any consistent patterns of differential innate immune gene expression between broilers for the phenotypic selection of high- or low-performance (based on feed conversion ratio). We did however see high variation in the ileal

expression of *TLR2* and *FAT/CD36*, leading to the hypothesis that variation in feed conversion ratio and performance variability could be linked to altered fatty acid metabolism and interaction with parameters of innate immunity.

4.6.1   Toll-Like Receptors and PPARs

In chapter three, *TLR2* was the third most variably expressed gene across all four data subsets, and also the highest expressed gene relative to the housekeeper genes. Despite showing no direct significance with high- and low-performance variation between individual birds, the variation in the expression levels of *TLR2* were of interest for two reasons; 1) The role in signalling and maintaining intestinal epithelial homeostasis; and 2) The suggestion that TLRs, particularly TLR2, could participate in the sensing of the energy state of the body and to the subsequent control of food intake (Wolowczuk et al., 2008).

The mechanisms of the role of TLRs in obesity and insulin resistance remains somewhat unclear, however evidence from genetically deficient animals, particularly of *TLR2* and *TLR4*, show that TLRs play an important role in the development of obesity (Fresno et al., 2011). As previously discussed, diets high in saturated fatty acids increased obesity, whereas diets high in PUFAs reduced obesity. In macrophage cell cultures, saturated fatty acids, such as stearic acid and palmitic acid, have been shown to activate TLR2 and TLR4 signalling pathways, which consequently activates down steam pro-inflammatory pathways. Conversely, PUFAs, particularly ω-3s, have been shown to inhibit *TLR2/4* expression, activation and downstream signalling (Wahli and Michalik, 2012). TLR4 signalling results in the subsequent activation of the activated B cell (NF-κB) pathway in both the liver and adipose tissue, as well as pro-inflammatory cytokine and chemokine release in monocytes and adipocytes, all promoting insulin resistance (Schäffler and Schölmerich, 2010). *TLR4* deficient macrophages show reduced TNF production and inflammatory signalling when compared with wild type

macrophages in response to saturated fatty acids, further suggesting saturated fatty acids are direct ligands of TLR4 (Suganami et al., 2007).

Toll-like receptor 2 has also been linked to obesity and insulin resistance. The fatty acid transporter apolipoprotein has been shown to be a ligand for TLR2 both *in vivo*, and *in vitro*, activating the NF-κB and pro-inflammatory cytokine expression in mouse adipocytes (Abe et al., 2010). Several studies have shown that *TLR2* deletion is associated with reductions in adipocyte hypertrophy, diminished macrophage infiltration, and inflammatory cytokine expression (Himes and Smith, 2010, Fresno et al., 2011). In broilers, the increase in abdominal fat pad primarily results from hyperplasia of adipocytes until ~4 weeks post hatch, after which, increases are attributed to hypertrophic growth (Matsubara et al., 2005). In mature broilers, the mass of the adipose tissue is generally reflected in the size of the adipose cells, not the number (Hood, 1982). It would be of interest to assess whether adipose *TLR2* expression is increased and correlates with adipocyte hypertrophy in mature broilers.

There are numerous studies that provide evidence of the TLR/lipid cross talk, particularly in mice, however few in chickens. This makes comparative biology somewhat difficult, given that adipose tissue in mice is much more active than avians. It must be noted however that recent RNA-seq analysis has shown that adipose tissue is much more active than previously thought in chicken and there is suggestion that this notion of negligible activity needs to be revised (Resnyk et al., 2015). In chapter three, the fourth most variably expressed gene was *CD36* or, *FAT/CD36*, a membrane receptor which facilitates the transport of fatty acids into cells (Hoebe et al., 2005). Interestingly, TLR2 is also known to form complexes in lipid rafts with CD36. TLR/CD36 complexes stimulate NFκB signalling and consequent release of pro-inflammatory cytokines (Cai et al., 2012). In addition to the classification as a scavenger protein, CD36, is thought to promote the synthesis of triglycerides in adipocytes, the clearance of chylomicrons from plasma, as well as mediate lipid metabolism and fatty acid transport

(Drover et al., 2005, Silverstein and Febbraio, 2009). Studies in broilers have found that active immunisation with CD36 resulted in upregulation of *CD36*, acetyl-CoA binding protein and *PPAR-γ* mRNA expression in the visceral fat of male broilers indicating that avian fat deposition has spatial and sex specific differences (Shu et al., 2011).

Many of the mechanisms linking innate immunity and fatty acids are yet to be elucidated; however, it is clear that there is a strong interaction. For PUFAs in particular, the 'anti-inflammatory' action may be due to the inhibition of *TLR2* and *TLR4*, and/or concurrent activation of PPARs. PPAR-α, as discussed previously, inhibits fatty acid synthesis and promotes fatty acid β-oxidation in the mitochondria, whereas PPAR-γ, expressed much higher in adipose tissue, has been linked to adipocyte differentiation (Sato et al., 2009). After treatment with a PPAR-γ ligand, the *PPAR-γ* mRNA expression was linearly correlated ($r = 0.67$) with abdominal fat pad weights in broilers, suggesting PPAR-γ activation is an important factor in fat deposition in chickens, as was age and nutrition (Sato et al., 2009). This is in agreement with Shu et al. (2011) and it's been reported that expression of *PPAR-γ* is regulated by CD36 dependent fatty acid uptake (Drover et al., 2005). Whist research has generally focused on the liver of birds in relation to fat deposition, the notion that the adipose tissue is perhaps more biologically active than previously thought has increased research focus on avian adipose tissue.

4.6.2 Adipose Tissue and Macrophages

Adipocytes and macrophages share many similar properties, such as the uptake of lipids, shared transcriptional regulation by similar transcription factors such as PPAR-γ and Toll-like receptors, and both secrete similar molecules (Fresno et al., 2011). In mouse models of obesity, the number of macrophages within adipose tissue have been shown to increase, contributing to the induction of inflammatory pathways (Lumeng et al., 2007). Furthermore, there is increasing evidence that pro-inflammatory macrophages (M1) are predominantly found in adipose tissue of obese animals, whereas anti-inflammatory macrophages (M2) are predominant in the adipose

tissue of lean animals (Lumeng et al., 2007). Resnyk et al. (2013) used a microarray analysis to compare the abdominal fat pads of fat line (FL) and lean line (LL) broilers, utilising the model developed in the 80's, and found differential adipokine expression between the lines. Furthermore, Đaković et al. (2014) evaluated 11 select adipokines in broilers which are known to either enhance insulin sensitivity in mammals (leptin, omentin, visfatin, adiponectin, vaspin, chemerin, and apelin), or decrease insulin sensitivity (Interleukin 6, tumour necrosis factor-α , PAI-1 (SERPINE1), and resistin). Of these genes, five were 'lost' in the chicken genome, with only five adopikines enhancing insulin sensitivity (apelin, visfatin, vaspin, chemerin, and adiponectin) and one adipokine inhibiting insulin sensitivity (Interleukin 6), found in chicken adipose tissue. Interestingly, receptors for these genes remain in the chicken genome, which has kept research interest high in finding the gene itself, particularly leptin. In 2015 leptin was finally identified in the chicken and duck, with suggestion that leptin has an autocrine/paracrine mode of action rather than circulating hormone as in mammals (Seroussi et al., 2015). If this is the case, there are implications for our current understanding of comparative physiology.

4.6.3   Physiological Stress

Acute stress is of minimal consequence to broiler production, however chronic stress results in an increase of corticosterone, increased heterophil to lymphocyte ratios as well as altered protein, carbohydrate and lipid metabolism, increasing deposition of abdominal fat (Virden and Kidd, 2009). One definition of stress is any situation that elicits the biological stress mechanisms of an animal (Virden and Kidd, 2009). Organelle stress owing to nutrient overload and processing defects result in the activation of the kinases, such as the JUN-terminal kinase (JNK), and IκB kinase-β, both leading to the disruption of the insulin signalling pathways and altered metabolic and pro-inflammatory responses (Hotamisligil and Erbay, 2008). In chapter three we analysed two genes involved in endoplasmic reticulum stress, x-box binding protein 1 (*XBP1*), and inositol-requiring kinase 1 (*IRE1*). We found no differential expression of these genes in the small intestine between our high- and low-performing phenotypes, which may be

reflective of the tissue type we selected; however, the ER has an important role in lipid metabolism. When proteins accumulate in the ER, a process known as the unfolded protein response (UPR) is initiated to upregulate chaperone proteins which promote protein folding and restore ER homeostasis (Hotamisligil and Erbay, 2008).

It has long been established that broilers are hyperglycaemic. Chronic exposure of high concentrations of glucose can result in sustained activation of *IRE1*, leading to engagement of the JNK and IKK-NFκB pathways and increased expression of pro-inflammatory cytokines (Hotamisligil and Erbay, 2008). This poses the question, are broilers under a constant state of physiological stress leading to increases in fat deposition? Saturated fatty acids have been established to trigger the UPR in hepatocytes, cardiomyocytes and macrophages, as well as links between lipid synthesis and breakdown (Hotamisligil and Erbay, 2008). In hepatocytes, *XBP1* regulates the transcription of many genes involved with fatty acid synthesis, including *SCD1* and *ACC* (Lee et al., 2008). Selective deletion of *XBP1* in mice has been shown to compromise hepatic lipogenesis, resulting in decreases in serum triglyceride levels, cholesterol and free fatty acids, however, other indicators of ER stress were not evident, suggesting *XBP1* functions as a mediator of hepatic lipogenesis, distinct from its function in ER stress and the UPR (Lee et al., 2008). ER stress in the liver has been demonstrated in obese mouse models compared to their lean controls, including dramatically increased JNK activity (Özcan et al., 2004). Liver cells treated with agents used to induce ER stress also showed marked reduction in insulin signalling (Özcan et al., 2004). Whilst it is clear that organelle stress and inflammation contribute to obesity, insulin resistance and metabolic disease, it remains to be determined which of the processes comes first.

4.7   Summary

The scope of this review was to assess avian lipid metabolism, and explore links between lipids, lipid metabolism and innate immunity to elucidate whether their complex interaction could be

attributing to performance variation commonly seen in commercial broiler flocks. In summary, obesity in modern commercial broilers is a major problem-decreasing feed efficiency during the production period and resulting in lower carcass yields during processing. Both of these consequences result in economic losses. Dietary fatty acid composition is known to modulate both lipid metabolism as well as lipid deposition in avians. It is becoming increasing evident that the maternal diet also affects lipid composition and fatty acid metabolism of progeny. Comparative biology shows avians and mammals share the same transcription pathways including the PPARs and SREBPs that directly regulate the transcription of genes involved in lipid metabolism and that dietary lipids are ligands for both. Links between Toll-like receptors, as well as physiological stress at a cellular level have been well documented to alter fatty acid metabolism, particularly in rodent models. Activation of *TLR2* and *TLR4* as well as activation of ER stress have been demonstrated to contribute to obesity. Much investigation in avians has focused on the liver, due to the liver being the primary site of lipogenesis, and there are few studies combining innate immunity and fatty acid metabolism in broilers. Comparisons between chicken and rodent studies must be made with caution, particularly in relation to obesity, as five adipokines that inhibit or promote insulin signalling in the adipose tissue have been 'lost' in the chicken genome. Furthermore, adipose tissue in rodents has a much higher lipogenic capacity than in avians, making comparisons somewhat difficult. It is evident there are many gaps in understanding the fatty acid metabolism and innate immunity-cross talk in avians. What is clear however is that there is a growing body of evidence, particularly in other species, to support the hypothesis that fatty acid metabolism and innate immunity cross talk could be a significant contributor to performance variation in commercial broiler production.

It was therefore hypothesised that interactions between fatty acid metabolism and innate immunity may be associated with variations in FCR commonly seen within commercial meat bird flocks. The following chapter investigates how genetic selection has influenced carcass lipid composition, key genes involved in fatty acid metabolism and select innate immune

parameters to further assess biological factors underpinning variations in growth performance of meat chickens.

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CHAPTER FIVE

Evaluation of fatty acid metabolism genes and innate immunity interactions between commercial broiler, F1 layer x broiler cross and commercial layer strains selected for different growth potentials

Statement of Authorship

Title of Paper	Evaluation of fatty acid metabolism genes and innate immunity interactions between commercial broiler, F1 layer x broiler cross and commercial layer strains selected for differing growth potentials
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Principal Author

Name of Principal Author (Candidate)	Nicky-Lee Willson
Contribution to the Paper	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.
Signature	Date 20/3/2017

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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Evaluation of fatty acid metabolism genes and innate immunity interactions between commercial broiler, F1 layer x broiler cross and commercial layer strains selected for different growth potentials

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5.1   Abstract

Background: The broiler industry has undergone intense genetic selection over the past 50 years resulting in improvements for growth and feed efficiency, however, significant variation remains for performance and growth traits. Production improvements have been coupled with unfavourable metabolic consequences, including immunological trade-offs for growth, and excess fat deposition. The aim was to determine whether interactions between fatty acid (FA) metabolism and innate immunity may be associated with performance variations commonly seen within commercial broiler flocks. Total carcass lipid %, carcass and blood FA composition, as well as genes involved with FA metabolism, immunity and cellular stress were investigated in male birds of a broiler strain, layer strain and F1 layer x broiler cross at d14 post hatch. Heterophil: lymphocyte ratios, relative organ weights, bodyweights and feed conversion ratios were also compared.

Results: Broiler bodyweight (n=12) was 3.5 times that of layers (n=12) at d14 post hatch. Broilers had significantly higher total carcass fat percentage ($P < 0.001$) compared to the cross (n=6) and layers which were not significantly different from each other ($P > 0.05$). The carcass and whole blood fatty acid (FA) analysis revealed differences in the FA composition between the three strains indicating altered fatty acid metabolism, despite all being raised on the same diet. The broilers had significantly higher monounsaturated fatty acids whilst the layers had significantly higher polyunsaturated fatty acids (PUFAs). Both n-3 and n-6 PUFA levels were elevated, reflecting the upregulation of the genes encoding the enzymes involved in the elongation of each process, stearoyl-CoA desaturase (*SCD1*) and fatty acid desaturase 6 (*FADS6*) respectively. Genes associated with fatty acid synthesis and β-oxidation were all upregulated in the broilers compared to the layers indicating a net overall increase in fatty acid metabolism as a whole, which may be driven by the larger relative liver size as a percentage of bodyweight in the broilers. The x-box binding protein (*XBP1*) had higher expression levels in layers compared to broilers however no other genes involved in innate immunity such as Toll-like receptors -2 and -4 and, as well as organelle stress indicators inositol-requiring kinase 1

(*ERN1*) were found to be non-significant. Additionally there were no differences in heterophil: lymphocytes ratios between any of the strains.

Conclusions: The results provide evidence that genetic selection may be associated with altered metabolic processes between broilers, layers and their F1 cross. Whilst there is no evidence of interactions between FA metabolism, innate immunity or cellular stress, further investigations at later time points as growth and fat deposition increase would provide useful information as to the effects of divergent selection on key metabolic and immunological processes.

Keywords: Broiler, cellular stress, fatty acid metabolism, innate immunity, layer, selection

5.2 Background

Over the past 50 years, the intensification of the broiler industry and concurrent commercial genetic selection for growth, feed efficiency and yield has resulted in broiler growth increases in excess of 400% (Zuidhof et al., 2014), with broilers having the capacity to reach 2 kg of live weight within 35 days (Schmidt et al., 2009, Robins and Phillips, 2011). At least 85% of production improvements has been attributed to genetic selection with meat production efficiency continually increasing by 2-3% per year through selective breeding programs alone (Gous, 2010, Zuidhof et al., 2014).

Selection for feed efficiency is largely measured by feed conversion ratio (FCR), the amount of feed intake (FI) per unit bodyweight gain. In poultry systems, feed accounts for approximately 70% of total production costs (Aggrey et al., 2010). Selection for efficiency has resulted in an FCR decrease of over 50% over the past 5 decades, maintaining poultry as a cost efficient source of protein (Zuidhof et al., 2014). Despite continued improvements, there still remains significant (>10%) variation in performance traits, including efficiency, bodyweight and growth rate within broiler strains (Emmerson, 1997). This performance variation results in an economic cost to the producer, and is problematic for modern automated processing plants.

Despite much investigation, the basis of this significant performance variation has yet to be clearly elucidated.

Maintenance of innate immunity and intestinal barrier function is one parameter thought to be nutritionally costly to the host, particularly as highly activated or diminished immune responses could lead to increased performance variation (Kohl, 2012). In chapter three, we compared high- and low-performing broilers to determine whether innate immune function could be consistently linked to the phenotypic expression of FCR. A candidate gene approach was used to determine whether functional changes in innate immune parameters could be consistently identified, the results of which, they could not. Variable expression in the pathogen recognition receptor Toll-like receptor 2 (*TLR2*) and membrane protein *CD36* also known as FAT/CD36, was however of interest, as both have been linked to each other and various roles in fatty acid metabolism. Lee and Hwang (2006) reported on links between fatty acids and TLR activation, with saturated fatty acids (SFAs) activating TLR2 and TLR4 signalling pathways and unsaturated fatty acids having an inhibitory effect on TLR-mediated signalling pathways and gene expression. Toll-like receptor 2 is known to form complexes with CD36 in lipid rafts (Hoebe et al., 2005), and CD36 has been described in facilitating TLR2 signalling, although the mechanism remains somewhat unclear (Wolowczuk et al., 2008). Furthermore, CD36 is thought to promote the synthesis of triglycerides in adipocytes, the clearance of chylomicrons from plasma, as well as mediate lipid metabolism and fatty acid transport (Drover et al., 2005, Silverstein and Febbraio, 2009). Additionally, studies in broilers have found that CD36 has a novel role in the visceral fat deposition of male broilers, and indicate that avian fat deposition has sex specific differences (Shu et al., 2011).

Fat deposition in broilers has been an unfavourable consequence of selection for growth, particularly up until the 1970s, and despite reports of a reduction in body fat content from 26.9% in the 1970s to 15.3% in commercial breeds in the past decade (see Tallentire et al. (2016) for

review), fat deposition in broilers is still excessive, particularly in comparison to layers or wild type species. Fat deposition is negatively linked to FCR, with observations that heavier chickens usually have a higher FCR and deposit a higher amount of fat (Gaya et al., 2006). Fat has been demonstrated to account for 15-18% of the total broiler bodyweight and is considered the most variable body component, with a coefficient of variation for the total body fat content between 15 and 20%, and higher again for abdominal fat, varying between 25 and 30% (Havenstein et al., 2003b, Leenstra, 1986, Daval et al., 2000, Choct et al., 2000). The major site for fat deposition in broilers is the abdominal fat pad, which is highly correlated to total carcass fat (Gaya et al., 2006, Zerehdaran et al., 2004). It must be noted however that these references reporting fat content, despite often being referred to in current literature, are >15 years old, and that total body fat content may have reduced somewhat as selection for efficiency continues. Excess fat accumulation and the variation may be considered the net balance of dietary absorbed fat, the rate of fat synthesis (primarily hepatic lipogenesis), and fat catabolism (Sanz et al., 2000). As obesity is correlated with chronic low grade inflammation (Lumeng and Saltiel, 2011), and that highly activated or diminished immune responses can result in inflammation potentially leading to decreased growth performance of the host (Lochmiller and Deerenberg, 2000), it is of interest to determine whether links between fatty acid metabolism, obesity and innate immunity could be contributing to performance variation commonly seen in broilers.

It was hypothesised that interactions between fatty acid metabolism and innate immunity may be associated with variations in FCR commonly seen within commercial broiler flocks. To investigate whether differences in innate immunity and fatty acid metabolism are contributing directly to variations in flock performance, we compared broiler and layer chicken strains that have been intensively selected for different traits; high carcass yield and growth efficiency for broilers, commercial egg production and egg efficiency for layers (Druyan, 2010). This selection over the years has seen the two strains diverge for these traits, with the bodyweight of broilers being five times that of layers by 6 wks of age (Zhao et al., 2004). In

the current experiment we compared broilers, layers, and a layer x broiler F1 cross to identify how genetic selection has influenced carcass lipid composition, key genes involved in fatty acid metabolism and select innate immune parameters to enable a better understanding of the biological factors underpinning feed efficiency, and variations in growth performance.

5.3   Methods

All animal procedures were approved by the University of Adelaide Animal Ethics committee (approval #S-2015-171) and the PIRSA Animal Ethics committee (approval #24/15).

5.3.1   Birds and Management

In total, 150 newly-hatched male chicks (n=50 broiler, n=50 F1 layer x broiler cross, n=50 layer) were obtained from the HiChick Breeding Company Pty Ltd, Bethel, South Australia (extra birds were obtained to account for any unexpected mortality). The cross progeny were produced by HiChick utilising their commercial breeding lines. Briefly, three Isa Brown roosters and 135 Isa Brown breeder hens were used to produce layer progeny, three broiler breeder roosters and 135 broiler breeder hens used to produce the broiler progeny, and three Isa Brown roosters and 135 broiler breeder hens used to produce the F1 layer x broiler cross. All progeny were produced via natural mating (broiler breeder specifications not disclosed due to commercial confidence). The F1 cross was utilised as an intermediate growth phenotype against broiler and layer strain progeny. Chicks were separated by breed and placed 25 chicks/rearing pen in a temperature and climate controlled room at the SARDI PPPI Poultry Research Unit, Roseworthy Campus, The University of Adelaide.

All birds were fed *ad libitum* (standard commercial broiler starter diet, no in-feed antimicrobials or coccidiostats added), and had unrestricted access to water via nipple drinker lines. The three experimental groups were selected for their growth potential: Fast growing (broilers; n=50) moderate growing (F1 layer x broiler; n=50) and slow growing (layer strain; n=50). Performance data was recorded weekly (bodyweight, bodyweight gain, pen FCR). On

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d0, -7, -14 and -28 post hatch, 36 birds (*n*=12 birds/breed) were randomly selected and euthanised by cervical dislocation for subsequent sampling. Day 14 was the primary sampling time point.

5.3.2   Total Carcass Lipid and Total Blood Lipid Composition

At d14, eviscerated (fat pad and feathers were not removed) carcasses (*n*=12 broilers, *n*=6 cross, *n*=12 layers) were weighed and immediately frozen at -20ºC. Whole carcasses were submerged into liquid nitrogen for 3 min, shattered with a mallet in zip lock bags to contain all fragments, and homogenised in a 1700W blender. Sub samples of homogenate were aliquoted (10 mL) and stored at -20 ºC for analysis of total carcass lipid % and carcass lipid composition. Total lipids were extracted at the Waite Lipid Analysis Service (WLAS), Waite Campus SA, using the methods of Folch (Folch et al., 1957, Kartikasari et al., 2012). Fatty acid composition of tissues was determined and quantified using a Hewlett-Packard 6890 GC (CA, USA) equipped with flame ionization detection and a capillary column (50×0.32 mm internal diameter) coated with 70% cyanopropyl polysilphenylene-siloxane with a film thickness of 0.25 μm (BPX-70, SGE, Victoria, Australia). Fatty acid transmethylation for fatty acid methyl ester (FAME) extraction, and gas chromatography analysis of FAME were run by the methods of Folch (Kartikasari et al., 2012). Fatty acid peaks were identified by comparing the retention time of each peak against the retention times of a fatty acid standard of known composition. Each peak from a trace was expressed as the relative percentage of the total FAME in the sample. The detection limit of each fatty acid was 0.05% of total fatty acids.

Total blood fatty acids were measuring using the PUFAcoat dried blood spot (DBS) card, developed by the Waite Lipid Analysis Service (WLAS), Waite Campus SA. Samples were prepared by placing a drop of blood on PUFAcoat DBS card and dried at room temperature for 5 h (Liu et al., 2014). In brief, lipids were extracted using a modified Folch method and FAME were extracted into heptane for gas chromatography. A Hewlett-Packard 6890 GC (CA,

USA) equipped with a BPX70 capillary column 50 m×0.32 mm, film thickness 0.25 µm (SGC Pty Ltd., Victoria, Australia), programmed temperature vaporisation injector and a flame ionisation detector (FID) was used. The identification and quantification of FAME were achieved by comparing the retention times and peak area values of unknown samples to those of commercial lipid standards (Nu-Chek Prep Inc., Elysian, MN, USA) using the Hewlett-Packard Chemstation data system.

5.3.3 Heterophil: Lymphocyte Ratios

Blood smears ($n=6$ broilers, $n=6$ cross, $n=6$ layers) were made by placing 1 drop of whole blood on the end of a Starfrost frosted slide (ProSci Tech). Slides were air-dried and fixed in 100% methanol for 1 min, feather side down. Slides were stained with Geisma-Wright stain on a Hema-Tek 2000. A total of 100 cells (Cell types; lymphocytes, heterophils, eosinophils, basophils and monocytes) were counted at a 40x magnification. Subsequent heterophil: lymphocyte ratios were determined.

5.3.4 RNA Extraction, Library Preparation and Sequencing

Day 14 liver samples ($n=6$ broilers, $n=6$ cross, $n=6$ layers) were randomly selected for RNA-sequencing. Total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Approximately 80 mg of frozen (-80 °C) liver tissue was homogenised in 2 mL of Trizol reagent (Invitrogen, Carlsbad, CA). Aliquots (1 mL) of the Trizol homogenate were combined with 200 µL of chloroform and centrifuged for 15 mins at 4 °C. The upper aqueous phase (350 µL) was transferred to a gDNA eliminator spin column and centrifuged at >8000 g (14,000 rpm) for 30 s. The flow through (300 µL) was collected and mixed with an equal volume of 70% ethanol and transferred onto RNeasy columns. The remaining collection and wash steps were performed according to the manufacturer's instructions. RNA was eluted in 200 µL of RNA-free water. Purity and concentration was determined using UV spectrophotometry (Nanodrop 1000; Thermo Scienfic, Wilmington, DE).

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RNA-Seq was carried out by the ACRF Cancer Genomics Facility, Adelaide, SA. The sample quality was analysed on an Agilent Bio-analyser (minimum RIN requirement of 7) and sequencing libraries were made using 2 µL of total RNA. PolyA mRNA isolation was performed using oligo dT beads. Libraries were prepared using KAPA Library Quantification Kits for Illumina platforms (KAPABiosystems, Massachusetts, USA). 2x 100nt sequencing was carried out on an Illumin HiSeq 2500 Sequencing System to generate a minimum depth of 25 million reads.

5.3.5   RNA Sequence (RNA-seq) Analysis

Reads were returned in fastq format. FastQC and adaptor sequences were trimmed from the 3' end of reads with Cutadapt. Hisat2 (Pertea et al., 2016) was used to map reads to the reference genome Galgal5.0 (ftp://ftp.ncbi.nlm.nih.gov/genomes/Gallus_gallus). Duplicate and poor quality reads were removed. Stringtie (Pertea et al., 2016) was used to define transcripts from the read mappings for each sample, and to merge the transcript definitions for all samples. Transcripts were cleaned up using in-house scripts. The number of raw read counts were calculated for each transcript and sample using the function featureCounts of the R package Rsubread (Liao et al., 2013). Another R package, edgeR (Robinson et al., 2010) was used to analyse differential gene expression using normalised counts per million transcripts (CPM) to correct for varying depth of sequence among samples. Differential expression of genes were considered significant at $P < 0.05$, false discovery rate of < 0.05, with any fold change considered. Transcript data were aggregated by gene. Genes where the maximum CPM was <1 were removed. A total of 22 candidate genes related to fatty acid metabolism and innate immunity were pre-selected from the RNA-Seq analysis for inclusion in this study (Table 5.1).

Table 5.1 Candidate genes selected for their involvement in fatty acid metabolism and parameters of innate immunity.

RNA Target	Gene Name	Accession no.[1]
ACACA	Acetyl-CoA Carboxylase	NM_205505.1
ACADL	Acyl-CoA dehydrogenase	NM_001006511.2
ACLY	ATP-Citrate-lyase	NM_001030540.1
ACSL1	Acyl-CoA synthetase	NM_001012578.1
APOA1	Apolipoprotein A1	NM_205525.4
APOC3	Apolipoprotein cIII	NM_001302127.1
CD36	FATCD36	NM_001030731.1
CPT1A	Carnitine palmitoyltransferase 1	NM_001012898.1
CPT2	Carnitine palmitoyltransferase 12	NM_001031287.2
FABP1	fatty acid binding protein 1	NM_204192.3
FADS6	Δ6 desaturase	XM_426241.5
FASN	Fatty Acid Synthase	NM_205155.2
LPL	Lipoprotein Lipase	NM_205282.1
MDH1	Malate dehydrogenase	NM_001006395.2
ME1	Malic Enzyme 1	NM_204303.1
PPARA	peroxisome proliferator-activated receptor alpha	NM_001001464.1
RXRA	Retinoic X receptor-α	XM_003642291.3
SCD	Stearoyl-CoA desaturase	NM_204890.1
TLR2A	Toll-Like Receptor 2	NM_001161650
TLR4	Toll-Like Receptor-4	NM_001030693
XBP1	X-box binding protein	NM_001006192
ERN1	inositol-requiring kinase 1	NM_001285501.1

[1] NCBI accession number

5.3.6 Statistical Analysis

Data were analysed by one-way ANOVAs in SPSS (IBM SPSS Statistics 22). Any data not normally distributed were logged ($\log_{10}$) to normalise and analysed by one-way ANOVA. Pen effect was originally fitted to the model however was not significant and removed. Statistical significance was accepted at $P < 0.05$ level after which Post Hoc tests were performed using Tukeys[HSD] to differentiate between the three groups of birds at each sampling time point. A two-tailed Pearson's correlation was applied to individual gene expression levels against individual bodyweight for the three combined groups of birds.

5.4   Results

5.4.1   Bodyweight, Bodyweight Gain and Performance Data

Bodyweights and performance data were recorded for a 28d grow-out period (Table 5.2). Starting bodyweights (mean ± SEM) at hatch were significantly different between meat bird (44.4 ± 0.4 g); cross (42.5 ± .04 g; $P < 0.008$) and layer birds (38.5 ± 0.4 g; $P < 0.001$). Bodyweights remained significantly different between all three groups of birds for the remainder of the grow-out period ($P < 0.001$).

Table 5.2 Weekly bodyweights (grams) for broiler, cross, and layer line males for d0, -7, -14, -21 and -28 post hatch

	d0	d7	d14	d21	d28
Broiler	44.4 ± 0.4 [a]	195 ± 2 [a]	560 ± 8 [a]	1,153 ± 22 [a]	2,102 ± 35 [a]
Cross	42.5 ± 0.4 [b]	137 ± 3 [b]	311 ± 8 [b]	603 ± 12 [b]	1,037 ± 31 [b]
Layer	38.5 ± 0.4 [c]	84 ± 1 [c]	159 ± 2 [c]	261 ± 3.82 [c]	403 ± 6 [c]

[a-c] Means (± SEM) within the same column with different superscripts are significantly different ($P < 0.05$)

Feed intake and FCR values (Table 5.3) are presented on a total pen basis. The broiler birds consumed significantly more feed for the duration of the 28d grow-out period. FCR values on a pen basis were not significantly different between broiler ($n=2$) and cross ($n=2$) pens at any week, being 1.47 and 1.45 from d14-d28 respectively. As expected, the layer line males consumed significantly less feed ($P < 0.001$), and had significantly higher FCR values ($P < 0.001$) than the broilers or combined cross males.

Table 5.3 Weekly feed intake per bird (grams), and feed conversion ratios (FCR) calculated on an average pen basis

	Pen ($n=2$)	d7-d14	d14-d21	d21-d28	d14-d28
Feed Intake, g/bird	Broiler	447 ± 19 [a]	872 ± 38 [a]	1397 ± 5 [a]	2269 ± 33 [a]
	Cross	371 ± 4 [b]	698 ± 21 [b]	1083 ± 8 [b]	1781 ± 29 [b]
	Layer	112 ± 1 [c]	174 ± 5 [c]	261 ± 18 [c]	435 ± 24 [c]
FCR	Broiler	1.22 [a]	1.45 [a]	1.48 [a]	1.47 [a]
	Cross	1.26 [a]	1.42 [a]	1.47 [a]	1.45 [a]
	Layer	1.52 [b]	1.69 [b]	1.85 [b]	1.78 [b]

[a-c] Means (± SEM) within the same column for each parameter with different superscripts are significantly different ($P < 0.05$).
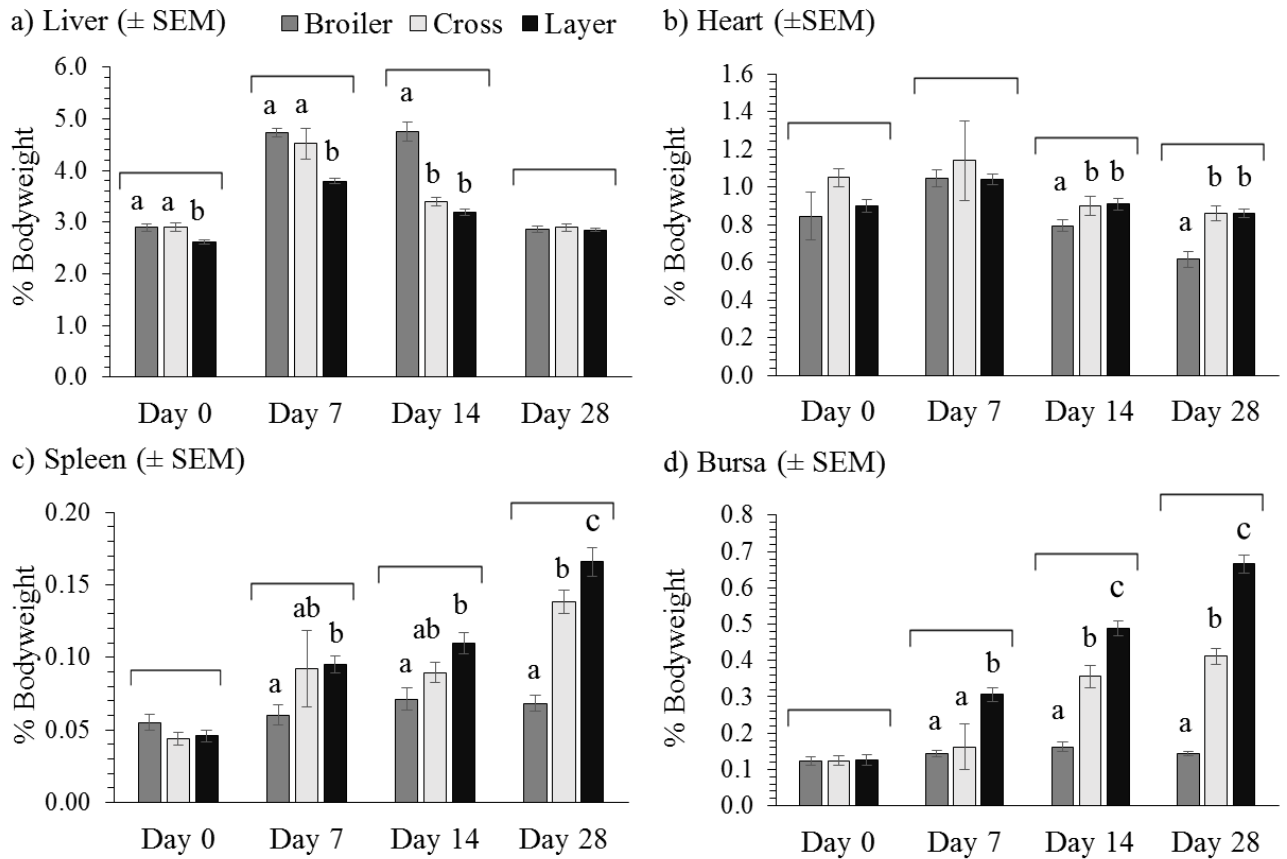
5.4.2 Organ Weights

Organ weights were expressed as a percentage of total bodyweight to account for growth differences between broilers, layers and the F1 cross (Figure 5.1). At d0 and d7 the layers had significantly lower relative liver weight percentages than the broiler and cross males ($P = 0.006$ and $P < 0.001$ respectively). Liver weight as a percentage of bodyweight peaked at d14 in the broilers, which were significantly different from both the cross and layer birds ($P < 0.001$;

Figure 5.1a), whereas the cross and layer birds reached peak relative liver weights at d7 post hatch. By d28 post hatch there were no differences in relative liver weight (~2.9% of total bodyweight) between the three groups of birds ($P = 0.852$).

The heart accounted for 0.85-1.08% of total bodyweight at both d0 and d7 with no significant differences ($P = 0.202$ and $P = 0.611$) between broiler, cross and layers birds at each time point respectively (Figure 5.1b). The relative weight of the layer's hearts remained constant for the 28d growth period, representing ~1% of total bodyweight. The broilers had significantly lower relative heart weights than the layer and cross birds at d14 and d28 post hatch ($P < 0.001$).

Relative spleen weights were not different between any of the three groups at d0 ($P = 0.233$; Figure 5.1c). Layers had significantly heavier relative spleen weights than broilers from d7 onwards ($P = 0.004$). The cross and layer spleen weights continued to increase in relative weight over the 28d period, whereas the broilers reached their maximum relative spleen weight by d14 post hatch. By d28 post hatch broiler spleens accounted for 0.07% of total body weight whereas layer spleens accounted for 0.17% of total bodyweight ($P < 0.001$).

No significant differences were found in relative bursa weight between broilers, layers and cross birds at d0 ($P = 0.997$; Figure 5.1d). Relative bursa weights peaked in broilers at d14 post hatch, exhibiting a 0.04% increase from d0-d14 (0.12%-0.16%) then reducing slightly by d28 to 0.14% of total bodyweight. Relative weights of the bursa increased in layer and cross birds at all sample time points. The increases were most pronounced in the layer birds with the bursa significantly different from both the crossed and layer birds at both d14 ($P < 0.001$) and d28 ($P < 0.001$). At d28 post hatch the bursa weights were 0.14% and 0.67% of total bodyweight for broilers and layers respectively.

Figure. 5.1 Organ weights presented as a percentage of total bodyweight (± SEM) for broiler, cross and layer line males at d0, d7, d14 and d28 post hatch. [a-c] Differing superscripts within each time point are significantly different ($P < 0.05$).

5.4.3 Total Carcass and Total Blood Lipids

Total carcass fat (%) and subsequent fatty acid compositions were evaluated on eviscerated homogenised carcasses and blood samples at d14 post hatch only. Broilers ($n=12$) had significantly higher ($P < 0.001$) total carcass fat percentage (11.3%) than the cross ($n=6$, *8.9%*) and layer line males ($n=12$, 7.7%; Figure 5.2). The cross and layer total body fat percentages were not significantly different ($P > 0.05$).
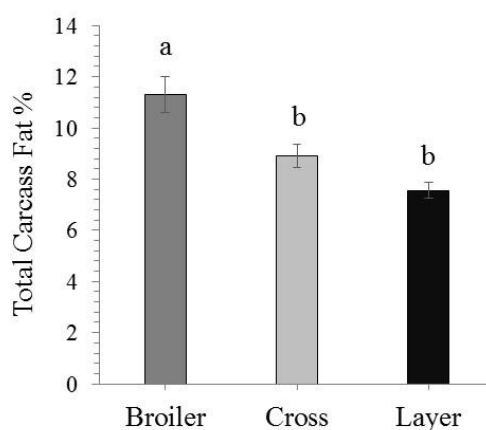
Figure 5.2 Total carcass fat % for eviscerated homogenised carcasses for broilers ($n=12$), cross ($n=6$) and layer line ($n=12$) males at d14 post hatch. [a-b] Differing superscripts are statistically different ($P < 0.05$). Values are means ± SEM.

The fatty acid composition of the carcasses varied indicating differential fatty acid metabolism (Table 5.4). The layers had higher levels of total saturated fatty acids (SFAs), followed by broilers, and then the cross, all significantly different ($P = 0.001$). The broilers had higher levels of palmitic acid (C16), whereas the layers had higher levels of stearic acid (C18), indicating increased elongation of SFAs in the layers. The same SFA pattern was seen in the

blood (Table 5.5). Total carcass monounsaturated fatty acids (MUFAs) were higher in the broilers and cross relative to the layers ($P < 0.001$), indicating increased elongation of MUFAs in the broilers and cross, this pattern also reflected in the blood. The cross and layers had significantly higher carcass percentages of polyunsaturated fatty acids (PUFAs), both omega-3 and omega-6. This was reflective both the n-6: n-3 ratio as well as the PUFA: SFA ratios between the strains. The composition of the serum and the composition of the carcass was generally the same for broilers, layers and crossed birds.

Table 5.4 Fatty acid composition (% of total identified fatty acids) in homogenised carcass samples for broiler (*n=12*), cross (*n=6*) and layer line males (*n=12*) fed the same commercial broiler diet formulation at d14 post hatch.

Fatty Acid	Broiler (*n=12*)	Cross (*n=6*)	Layer (*n=12*)	*P-Value*
Eviscerated carcass				
Total Carcass Fat %	11.3 [a]	8.90 [b]	7.56 [b]	< 0.001
Total SFA	37.7 ± 0.3 [a]	36.8 ± 0.2 [b]	38.6 ± 0.2 [c]	0.001
Palmitic acid C_{16}	27.7 ± 0.24 [a]	25.9 ± 0.19 [b]	25.3 ± .25 [b]	<0.001
Stearic acid C_{18}	7.8 ± 0.12 [a]	8.4 ± 0.15 [b]	10.0 ± 0.18 [c]	<0.001
Total TFA	0.8 ± 0.03 [a]	0.9 ± 0.05 [ab]	1.0 ± 0.06 [b]	0.038
Total MUFA	49.5 ± 0.27 [a]	48.7 ± 0.34 [a]	44.0 ± 0.41 [b]	<0.001
Palmitoleic acid ($C_{16}1n$-7)	7.8 ± 0.17 [a]	6.2 ± 0.27 [b]	4.8 ± 0.19 [c]	<0.001
Oleic acid ($C_{18}1n$-9)	38.6 ± .27 [a]	38.9 ± 0.27 [a]	35.8 ± 0.19 [b]	<0.001
Vaccenic acid ($C_{18}1n$-7)	2.7 ± 0.07 [a]	3.1 ± 0.09 [b]	3.0 ± .06 [b]	0.003
Total PUFAn-3	1.5 ± 0.01 [a]	1.6 ± 0.02 [a]	1.9 ± 0.05 [b]	<0.001
α-Linolenic acid ($C_{18}3n$-3)	1.1 ± 0.01	1.1 ± 0.00	1.1 ± 0.01	0.684
Eicosapentanoic acid ($C_{22}5n$-3)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	-
Docosahexanoic acid ($C_{22}6n$-3)	0.2 ± 0.01 [a]	0.3 ± 0.02 [b]	0.6 ± 0.02 [c]	<0.001
Total PUFAn-6	10.4 ± 0.12 [a]	12.0 ± .017 [b]	14.5 ± 0.32 [c]	<0.001
Linoleic acid ($C_{18}2n$-6)	9.8 ± 0.12 [a]	11.0 ± 0.13 [b]	12.8 ± 0.24 [c]	<0.001
Arachidonic acid ($C_{20}4n$-6)	0.3 ± 0.02 [a]	0.6 ± 0.03 [b]	1.1 ± 0.07 [c]	<0.001
n-6 :n-3 ratio	6.88 [a]	7.42 [b]	7.68 [c]	<0.001
(MUFA + PUFA) : SFA	1.61 [ab]	1.68 [a]	1.57 [b]	0.004
PUFA : SFA	0.31 [a]	0.40 [b]	0.43 [b]	<0.001

[1] Data are expressed as the percentage of identified fatty acids ± Standard error of means (SEM);

[a-c] Means within the same row for each parameter with different superscripts are significantly different (*P* < 0.05).

Table 5.5 Fatty acid composition (% of total identified fatty acids) in PUFAcoat DBS blood spot samples for broiler, cross and layer line males fed the same commercial broiler diet formulation at d14 post hatch

Fatty Acid	Broiler (*n*=12)	Cross (*n*=6)	Layer (*n*=10)	*P*-Value
Total SFA	43.7 ± 0.7	43.05 ± 0.3	46.0 ± 1.2	0.107
Palmitic acid C_{16}	$24. \pm 0.52$	22.5 ± 0.18	23.9 ± 1.78	0.424
Stearic acid C_{18}	14.9 ± 0.39 [a]	16.16 ± 0.21 [ab]	17.07 ± 0.51 [b]	0.004
Total TFA	0.85 ± 0.03 [a]	0.93 ± 0.05 [a]	1.1 ± 0.06 [b]	0.004
Total MUFA	33.55 ± 0.33 [a]	28.55 ± 0.65 [b]	23.63 ± 0.60 [c]	<0.001
Palmitoleic acid (C_{16}1n-7)	4.19 ± 0.17 [a]	2.55 ± 0.07 [b]	1.69 ± 0.13 [c]	<0.001
Oleic acid (C_{18}1n-9)	$26.53 \pm .25$ [a]	23.08 ± 0.65 [b]	19.36 ± 0.50 [c]	<0.001
Vaccenic acid (C_{18}1n-7)	1.96 ± 0.05 [ab]	2.11 ± 0.06 [a]	1.78 ± 0.10 [b]	0.036
Total PUFAn-3	2.84 ± 0.13 [a]	3.58 ± 0.18 [b]	3.66 ± 0.25 [b]	0.007
α-Linolenic (C_{18}n-3)	0.69 ± 0.02	0.71 ± 0.03	0.63 ± 0.03	0.189
Eicosapentanoic (C_{22}5n-3)	0.133 ± 0.01	0.35 ± 0.04	0.31 ± 0.03	0.628
Docosahexanoic (C_{22}6n-3)	1.59 ± 0.09 [a]	2.2 ± 0.14 [b]	2.4 ± 0.18 [b]	0.001
Total PUFAn-6	19.06 ± 0.43 [a]	$23.86 \pm .059$ [b]	25.62 ± 1.1 [c]	<0.001
Linoleic (C_{18}2n-6)	16.38 ± 0.36 [a]	19.45 ± 0.33 [b]	19.72 ± 0.72 [b]	<0.001
Arachidonic (C_{20}4n-6)	1.26 ± 0.05 [a]	2.6 ± 0.23 [b]	4.06 ± 0.35 [c]	<0.001
n-6 :n-3 ratio	6.82	6.68	7.14	0.418
(MUFA + PUFA) : SFA	1.27	1.30	1.18	0.071
PUFA : SFA	0.51 [a]	0.64 [b]	0.65 [b]	0.002

[1] Data are expressed as the percentage of identified fatty acids ± Standard error of means (SEM);

[a-c] Means within the same row for each parameter with different superscripts are significantly different (*P*< 0.05).

5.4.4 Heterophil: Lymphocyte Ratios

The cross birds appeared to have a lower number of heterophils and a higher number of lymphocytes than the broiler and layer birds, however no statistical differences were detected in the heterophil; lymphocyte ratios between any of the strains (Figure 5.3; $P > 0.05$). The differences were likely reflective of the high individual variation in cell frequencies, which is reflected by the large standard error. In addition to the heterophils and lymphocytes, basophils, monocytes and eosinophils were also counted, however; no significant differences were detected in the cell frequencies between any of the strains ($P > 0.05$).
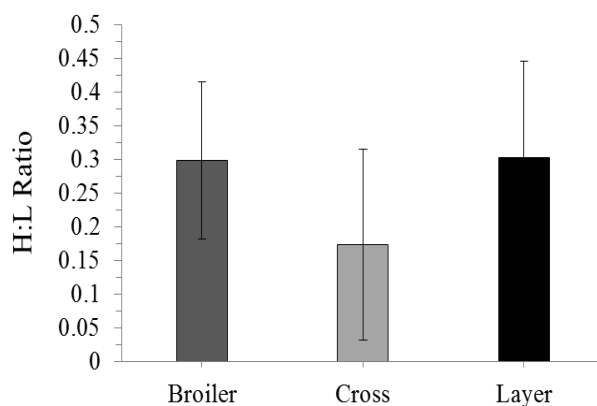
Figure 5.3 Heterophil: Lymphocyte ratios (±SD) for Broilers ($n=6$), Cross ($n=6$) and Layers ($n=6$).

5.4.5 Gene Expression

The 22 candidate genes selected (Table 5.6) revealed that broilers ($n=6$) in comparison to layers ($n=6$) had significant hepatic upregulation of genes involved in lipid transport ($P < 0.05$, *APOA1, APOC3*), lipogenesis ($P < 0.05$, *ACACA, ME1, FASN, GPAM*; $P < 0.001$, *MDH1, SCD1*), fatty acid transport ($P < 0.05$, *FABP1*; $P < 0.001$, *ACLY*) and fatty acid oxidation ($P <$

0.05, *ACADL*; $P < 0.001$, *CPT-2*), (Figure 5.4). An exception was the down-regulation of *FADS6* ($P = 0.054$) in broilers, a rate-limiting enzyme involved in the elongation of PUFAs. Broilers when compared to the cross ($n=6$) birds exhibited generalised upregulation of fatty acid metabolism, although not as pronounced as seen between broilers and layers. Significant hepatic upregulation of genes associated with lipid transport ($P < 0.05$, *APOC3*), lipogenesis ($P < 0.05$, GPAM; $P < 0.001$, *MDH1*), fatty acid transport ($P < 0.05$, *FABP1*; $P < 0.001$, *ACLY*) and fatty acid oxidation ($P < 0.05$, *ACADL, CPT-2*) were observed for broilers. Layers and cross comparisons indicated no real differential expression in fatty acid metabolism between the strains, with the exception of down regulation of lipogenic ($P = 0.003$, *SCD1*) and fatty acid oxidation ($P = 0.001$, *CPT-2, ACAA1*) genes. Layers in comparison to the cross also had upregulated expression of the transcription factor *PPARA* ($P < 0.05$), a difference not seen elsewhere.

Endoplasmic reticulum (ER) stress-related gene *ERN1* was not differentially expressed between any of the three strains ($P = 0.67$). *XBP1* was found to be significantly upregulated in comparison to both the broilers ($P = 0.002$) and cross birds ($P = 0.007$). Toll-like receptors *TLR2* and *TLR4* were not found to be differentially expressed between any of the three groups ($P = 0.951$). Pearson's two-tailed correlations with individual bird bodyweights (Table 5.6), revealed 15 of the 22 genes were highly correlated with bodyweight at $P < 0.01$, 2 genes correlated at $P < 0.05$ and 6 of the genes non-significant with bodyweight. The highest correlation detected was between malate dehydrogenase (*MDH1*) and bodyweight ($r = 0.902$).

Table 5.6 Pearson correlation coefficient (r) of target gene against individual bodyweight (BW), and mean expression levels of genes between broilers ($n = 6$), cross ($n = 6$) and layers ($n = 6$).

Gene name	R value[1]	Broiler ($n = 6$)	Cross ($n = 6$)	Layer ($n = 6$)	Regulation[2]
ACACA	0.695**	3135.4 ± 118.6 [a]	2918.5 ± 101.4 [a]	2367.5 ± 135.4 [b]	↑
ACADL	0.734**	751.3 ± 27.8 [a]	648.1 ± 15.3 [b]	620.9 ± 23.4 [b]	↑
ACLY	0.855**	3605.7 ± 201.6 [a]	2386.9 ± 117.4 [b]	1956.9 ± 163.3 [b]	↑
ACSL1	0.336	693.5 ± 61.9	553.0 ± 21.6	612.8 ± 27.1	
APOA1	0.639**	1519.0 ± 107.2 [a]	1348.5 ± 42.2 [ab]	1194.1 ± 57.3 [b]	↑
APOC3	0.736**	1859.5 ± 131.2 [a]	1472.7 ± 63.2 [b]	1307.6 ± 77.4 [b]	↑
CD36	0.593**	517.8 ± 24.6 [a]	580.4 ± 15.1 [ab]	596.5 ± 15.8 [b]	↓
CPT1A	0.044	244.5 ± 37.1	247.6 ± 15.5	233.5 ± 10.0	
CPT2	0.853**	224.7 ± 8.4 [a]	195.4 ± 6.7 [b]	151.8 ± 4.3 [c]	↑
FABP1	0.722**	998.8 ± 96.5 [a]	687.5 ± 30.3 [b]	606.7 ± 38.4 [b]	↑
FADS6	0.547*	109.6 ± 8.3	130.4 ± 11.7	145.1 ± 9.1	↓
FASN	0.769**	10794 ± 755.5 [a]	8475.9 ± 480.1 [b]	6486.9 ± 559.1 [b]	↑
LPL	0.600**	48.2 ± 22.3 [a]	90.1 ± 9.1 [ab]	117.9 ± 9.6 [b]	↓
MDH1	0.902**	667.0 ± 28.0 [a]	462.6 ± 18.8 [b]	386.7 ± 12.3 [b]	↑
ME1	0.601**	1045.0 ± 127.5 [a]	963.7 ± 75.0 [ab]	600.6 ± 92.9 [b]	↑
PPARA	0.376	447.1 ± 17.3 [ab]	434.6 ± 17.1 [a]	496.8 ± 15.3 [b]	
RXRA	0.012	65.9 ± 2.9	63.4 ± 2.8	64.6 ± 3.0	
SCD	0.817**	2785.2 ± 130.0 [a]	2322.6 ± 81.9 [a]	1413.6 ± 233.1 [b]	↑
TLR2A	0.041	21.1 ± 1.3	20.9 ± 1.9	20.4 ± 1.7	↑
TLR4	0.360	10.2 ± 0.9	9.3 ± 0.4	12.6 ± 1.2	
XBP1	0.620**	225.6 ± 9.1 [b]	231.4 ± 9.9 [b]	281.8 ± 12.4 [a]	↓
ERN1	0.578*	28.9 ± 2.5	23.9 ± 1.2	23.2 ± 1.1	↑

[1] Pearson's correlation coefficient of target gene against individual bodyweight (BW); *Sig at $P < 0.05$, **Sig at $P < 0.01$

[2] Direction of regulation: ↑Broiler upregulated (broiler > cross > layer); ↓Broiler downregulated (broiler < cross < layer)

[a-c] Means (± SEM) within the same row for each parameter with different superscripts are significantly different ($P < 0.05$).

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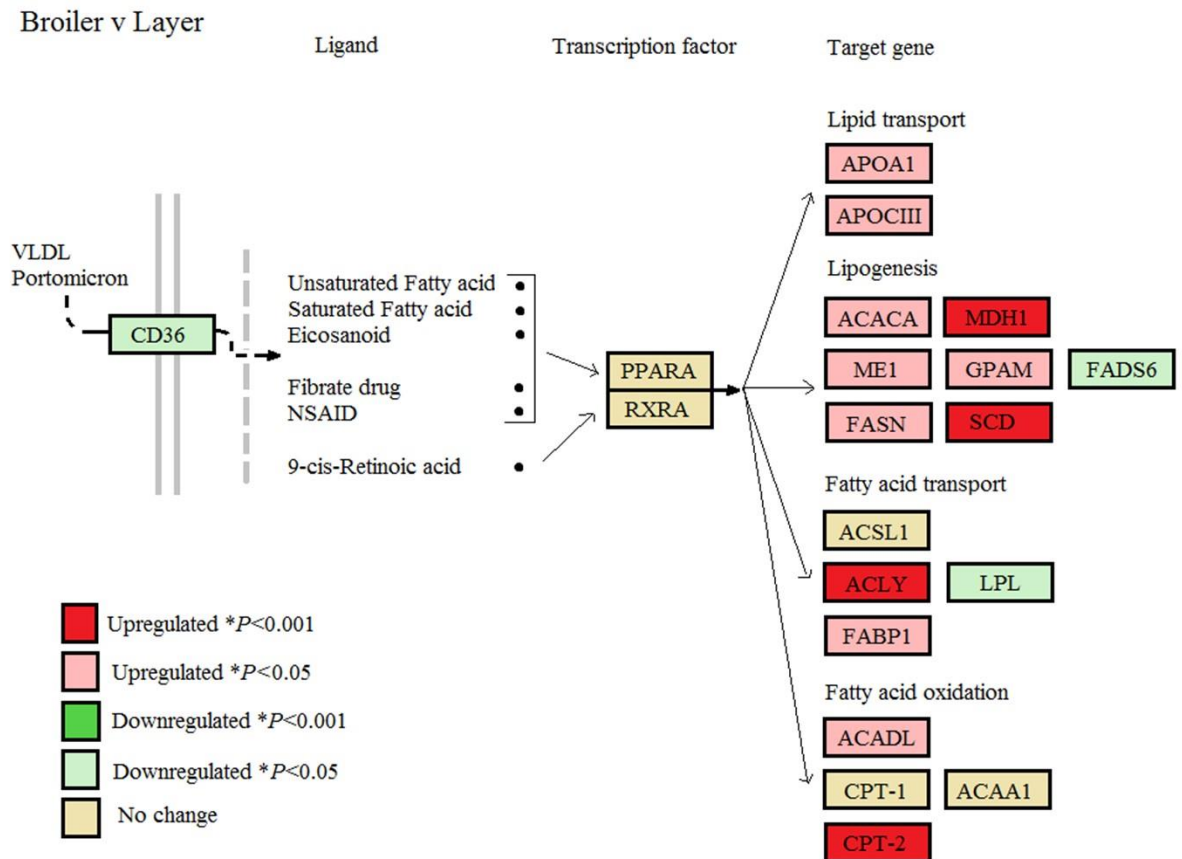
Figure 5.4 Changes in hepatic gene expression associated with the PPARA signalling pathway and fatty acid metabolism between broilers (*n=6*) and layers (*n=6*). Red boxes indicate upregulation in broilers; green boxes indicate downregulation in broilers in comparison to layers.

5.5   Discussion

Our aim was to elucidate how genetic selection has influenced carcass composition, fatty acid metabolism and select innate immune parameters. The objective was to further develop the understanding of factors that may be underpinning performance variation in modern broilers. Our previous experimental work (chapter three) did not provide sufficient phenotypic variation in feed conversion ratio within flock, thus it was decided to investigate birds with grossly different growth potentials; namely, broilers, layers and a layer x broiler F1 cross. Although samples were taken at multiple time points, d14 was selected as the primary sampling date due to the rapid growth acceleration seen in broilers from 2-3 weeks of age. By sampling at this time point, it was hoped to capture physiological changes at the beginning of the growth acceleration to further understand broiler growth rates.

As expected, the growth rates of the broiler progeny well exceeded those of the layer strain progeny. By d14, the broilers were four times the weight of the layer strain males and twice the weight of the F1 cross. The total lipid carcass percentage of the broilers was higher than both the layers and the cross, which were not significantly different from each other, despite the cross being twice the weight of the layers. Interestingly, many studies have shown that the dietary fatty acid composition is reflected in the fatty acid composition of the tissues and serum of broilers (Frttsche et al., 1991, Newman et al., 2002). Despite being raised in the same environmental conditions and fed the same diet, the fatty acid composition of the carcasses and blood spots differed between the three strains in this study, suggesting difference existed in fatty acid metabolism. The broilers had increased overall MUFA percentages, which would correlate with the significant upregulation *SCD1*, which encodes the rate-limiting enzyme converting SFAs into MUFAs (Ntambi, 1999). Comparisons of the total SFA, MUFA and PUFAs revealed layers had higher n-6 and n-3 levels, indicating two possibilities, layer strains have a higher physiological requirement for long chain PUFAs, or, layers are more efficient at converting available dietary linoleic and alpha-linolenic fatty acids to their long

chain derivatives. The gene encoding the enzyme *FADS6*, which is rate limiting in the elongation of PUFAs, was found to be upregulated in the layers in comparison to the broilers, which may support this concept.

An alternative hypothesis of altered metabolism of PUFAs between the strains could be if the maternal dietary lipid composition was different, given the large effect of avian maternal diet on progeny performance (Hynd et al., 2016). Differential PUFA composition of broiler breeder diets has been found to alter progeny lipid metabolism when the progeny were raised on identical diets, particularly in the first two weeks post hatch (Wang et al., 2002). Although this may be a plausible explanation, we cannot speculate further as we do not know the lipid composition of the maternal diets in this instance, however further investigation as to whether it is a true strain variation, or influenced by differences in maternal diets would be of interest.

Whilst it may be anticipated that the increased fat deposition is due to either increased lipogenesis and/or a decrease in fatty acid β-oxidation, we saw a net overall increase in both lipogenesis and fatty acid β-oxidation genes in the broilers compared to layers or their F1 cross. Although this could be controlled by transcription factors regulating FA metabolism, such as the nuclear receptor PPARA, we found no evidence to support this. The higher metabolic activity may therefore be reflective of d14 liver weight, which was relatively larger than that of the layers expressed as a percentage of bodyweight. The early increase in liver mass has also been observed in multiple studies, including comparisons of modern broilers and heritage lines (Schmidt et al., 2009). In the current study the layer and cross birds reached their maximum relative liver weights by d7, however the broilers had higher relative weights at d7 and reached their relative maximum weights at d14 post hatch. By d28, there were no differences in relative liver weights between the strains. Schmidt et al., (2009) propose this early increase in liver mass could correspond to increased liver capacity required in early post hatch, and that a possible effect of selection may have shifted earlier maturation of the liver in modern broiler lines. The

relative heart weights followed a similar pattern to the liver in that they were at their maximums in the first 2 wks post hatch. From d14 onwards the broiler relative heart weights had significantly reduced when compared to the cross and layers. These findings are not surprising as the reduced cardiac relative size and capacity has been well documented in broilers due to selection (Collins et al., 2014, Havenstein et al., 2003b, Schmidt et al., 2009).

Additional to differential fatty acid metabolism, it was hypothesised that innate immune parameters may also be interacting with fatty acid metabolism ultimately influencing performance variation. Modern broilers exhibit excessive fat deposition, particularly relative to layer strains, so obesity-related pathologies such as inflammation and cellular stress may be anticipated to be increased in broilers. To test this hypothesis immune organ weights (spleen and bursa), heterophil: lymphocyte ratios, as well as Toll-like Receptors (*TLR2a, TLR4*), fatty acid translocase (*CD36*) and endoplasmic reticulum stress indicator genes (*ERN1, XBP1*) were included in the current study.

The relative weight of both the spleen and bursa continued to increase in the cross and layer birds from d0 until d28 post hatch. The broilers reached maximum relative spleen and bursa weights at d14 and then decreased from there on in. There has been conflicting interpretation as to whether relative increased immune organ size equates to a better immune defence system. One study found that the size of the spleen was correlated with changes in body condition, and that size was elevated in individual birds in prime body condition (Møller et al., 1998). It could be argued that all of our birds were in good body condition for their strain, as there was no disease, parasite infection or mortality. Body condition as measure of fatness vs leanness however, as used by Møller et al. (1998), would assume the layers and the cross were in better relative condition than the broilers, and potentially reflective of the smaller immune organs. Additionally broilers have repeatedly been shown to be less responsive to immune challenges, attributed to a negative consequence of genetic selection (Lochmiller and

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Deerenberg, 2000). Although the increase in lymphoid organ weight (% of bodyweight) was observed, there is no evidence to suggest that the broilers were compromised immunologically due to increases in fat deposition in an unchallenged experimental setting. Heterophil to lymphocyte ratios were not significantly different between any of the birds, although there was a high level of variation between the individuals. The cross did appear to have a lower ratio; however, this is more likely attributed to a lower number of samples and the high variation in individual birds than a significant trend.

Whilst short-term stress is of minimal consequence to broilers, long-term stress results in increased serum corticosterone, increased heterophil to lymphocyte ratios and altered protein, carbohydrate and lipid metabolism, and increased deposition of abdominal fat (Virden and Kidd, 2009). It may be reasonable to consider a broiler as chronically stressed at a cellular level, particularly with the reduction of organ weights relative to overall bodyweight as growth increases. To investigate whether there was any evidence of organelle stress occurring, two key ER stress indicators which initiate the unfolded protein response (UPR) were included in this study (*ERN1* and *XBP1*), as saturated fatty acids have been shown to trigger the UPR response in hepatocytes and the UPR has been linked to lipid synthesis and breakdown (Hotamisligil and Erbay, 2008). Broiler, layer and F1 cross birds had differing SFA levels, and, *XBP1* was found to be upregulated in the layers in comparison to both the broiler and cross birds, however no differences were found in the expression levels of *ERN1*. Given that *ERN1* levels are showing no indication of ER stress, the differential expression of *XBP1* may align with the suggestion that *XBP1* functions as a mediator of hepatic lipogenesis, distinct from its function in ER stress and the UPR (Lee et al., 2008). It is thought to regulate the transcription of genes involved with fatty acid synthesis, including *SCD1* and *ACACA*, with deletion of *XBP1* resulting in decreased triglyceride, cholesterol and free fatty acids (Lee et al., 2008). It is difficult to conclude whether *XBP1* is exhibiting a regulatory effect on lipogenesis in the layers however the aforementioned genes are not seen to be increased in the layers compared to the broilers or the cross.

In addition to organelle stress, Toll-like receptors, including *TLR2* and *TLR4* have received attention for their roles in the development of obesity and insulin resistance, although the mechanisms by which they contribute still remain unclear. Mice lacking *TLR2* and *TLR4* genes do show however that TLRs are involved in the development of obesity (Fresno et al., 2011). In macrophage cell cultures, saturated fatty acids, such as stearic acid and palmitic acid, have been shown to activate *TLR2* and *TLR4* signalling pathways, which consequently activates down steam pro-inflammatory pathways, Conversely, PUFAs, particularly n-3s, have been shown to inhibit *TLR2/4* expression, activation and downstream signalling (Wahli and Michalik, 2012). In our current study, we found no differential expression of *TLR2a* in the avian liver in any of the three types of birds. Additionally we found no evidence in the expression levels of *TLR4* to suggest that the differing fatty acid profiles of the birds was having an effect or interaction with the expression of *TLR4* at d14 post hatch. This was also the case for *CD36*, with the exception of a down regulation in the broilers in comparison to the layers. Given the biological diversity for the role of *CD36*, this likely does not translate into down regulated facilitation of fatty acid transport given the overall upregulation of fatty acid metabolism seen in the broilers.

5.6 Conclusion

Fatty acid metabolism in broiler chickens was upregulated compared to an F1 cross and commercial layer strain. This increase was most likely a result of genetic selection for growth, with the overall increase resulting in increased FA synthesis as well as β-oxidation in the liver. There was no evidence to suggest at d14 post hatch that broilers were in a state of cellular hepatic stress or demonstrating changes in innate immune parameters such as *TLR2* and *TLR4* expression, despite broilers growing at four times the rate of the layers with significant increases in fat %. Day 14 post hatch was selected to capture the physiological changes as the broiler growth acceleration begins. It is possible that the d14 sample time point was too early in relation to fatty acid metabolism and innate immunity/cellular stress interactions to capture changes that

may ultimately be driving performance. Analysis at additional time points in the grow out phase could better reveal indicators of chronic stress as the organ weights continue to decrease by relative weight, contributing to metabolic stress and altering metabolism. The current study does however provide a valuable data set with a full transcriptome analysis between broilers; layers and their F1 cross to allow further investigations into biological factors that ultimately may be contributing to growth potential and performance variation in avian species.

5.7 Declarations

Ethics approval

The experimental protocol used in this study, including animal management, housing, and slaughter procedures were by the University of Adelaide Animal Ethics Committee (approval #S-2015-171) and the PIRSA Animal Ethics committee (approval #24/15).

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Authors' contributions

N-LW, PH, RF, RH and GN designed the study. N-LW and RF were involved in performing the experiment and laboratory analysis. RT performed RNA-seq data analysis. N-LW wrote the manuscript, PH, RF and RH revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare they have no competing interests.

Consent for publication

Not applicable

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CHAPTER SIX

Transcriptional analysis of liver from chickens with fast (meat bird), moderate (F1 layer x meat bird cross) and slow (layer bird) growth potential

Statement of Authorship

Title of Paper	Transcriptional analysis of liver from chickens with fast (meat bird), moderate (F1 layer x meat bird cross) and low (layer bird) growth potential
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	BMC Genomics

Principal Author

Name of Principal Author (Candidate)	Nicky-Lee Willson
Contribution to the Paper	Experimental design, conducted animal experiment, completed all laboratory work, data analyses and interpretation, wrote the manuscript
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.
Signature	Date 20 / 3 / 2017

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

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Transcriptional analysis of liver from chickens with fast (meat bird), moderate (F1 layer x meat bird cross) and low (layer bird) growth potential

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6.1 Abstract

Background: Divergent selection for meat and egg production in poultry has resulted in strains of birds differing widely in traits related to these products. Modern strains of meat birds can reach live weights of 3.0kg in 35 days, while layer strains are now capable of producing more than 300 eggs per annum but grow slowly with quite different body compositions to meat birds. This wide phenotypic divergence provides a powerful model for dissecting the underlying genetic basis of chicken meat and egg production. In this study, RNA-Seq was used to investigate differences in hepatic gene expression between three groups of birds with large differences in growth potential; meat bird, layer strain as well as an F1 meat bird x layer cross. The objective was to identify differentially expressed (DE) genes between all three strains to elucidate biological factors underpinning variations in growth performance.

Results: RNA-Seq analysis was carried out on total RNA extracted from the liver of meat bird (n=6), F1 layer x meat bird cross (n=6) and layer strain (n=6), males. Differential expression of genes was considered significant at $P < 0.05$, and a false discovery rate of < 0.05, with any fold change considered. A total of 6,278 genes were found to be DE with 5,832 DE between meat birds and layers (19%), 2,935 DE between meat birds and the cross (9.6%) and 493 DE between the cross and layers (1.6%). Comparisons between all three strains identified 155 significant DE genes. Gene ontology (GO) enrichment and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis of the 115 DE genes showed the FoxO signalling pathway was most enriched ($P = 0.001$), including genes related to cell cycle regulation and insulin signalling. Significant GO terms included 'positive regulation of glucose import' and 'cellular response to oxidative stress', which is also consistent with FoxOs regulation of glucose metabolism. There was a high correlation between FoxO pathway genes and bodyweight, as well as genes related to glycolysis and bodyweight.

Conclusions: This study demonstrated that there are large transcriptome differences between meat and layer birds. There was strong evidence that the FoxO signalling pathway is contributing to the growth differences seen between the three groups of birds as this signalling

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pathway was consistently identified in all comparisons between groups. Functional analysis of the 155 DE genes between all three bird groups also identified enrichment of the FoxO signalling pathway, particularly genes related to cell cycle regulation and insulin signalling. Functional analysis of the FoxO genes themselves is required to understand how they regulate growth and egg production.

Key words: RNS-seq, Meat bird, Layer, Liver, Functional analysis, FoxO

6.2   Background

Advancement in livestock production through selective breeding is perhaps best demonstrated in the poultry industry, where genetic selection, combined with advances in nutrition and improved management, have resulted in increases in meat bird growth in excess of 400% over the past 50 years (Zuidhof et al., 2014). Despite intense selection, there is still a significant amount of performance variation observed in commercial meat bird flocks, for both growth and feed efficiency (Emmerson, 1997, Tallentire et al., 2016). Feed costs account for ~70% of the variable costs of production in chicken meat enterprises (Aggrey et al., 2010), therefore optimising performance is of economic importance to the producer and industry alike. Despite historic production gains due to selection, increased growth has not been achieved without unfavourable consequences, with modern meat strains now predisposed to; excess fat deposition (Foud and El-Senousey, 2014), increased leg deformities and lameness (Bessei, 2006), metabolic disorders including pulmonary hypertension, ascites, and sudden death syndrome (Julian, 2005, Olkowski et al., 2007), as well as altered immune function (Cheema et al., 2003), especially when compared to slower growing lines such as layers and heritage line meat birds used in these studies.

Studies of different lines of chicken have explored physiological and/or anatomical growth constraints due to differential selection pressure. For example, comparison of heritage

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line meat birds unselected for growth, and commercial meat birds, demonstrates gross increases in breast muscle mass in modern meat birds (Schmidt et al., 2009). A major difference was identified at day 14 post hatch where breast muscle growth of the heritage line plateaued at ~9% of total bodyweight, while breast muscle continued to increase in the commercial strain (at 14% of total bodyweight at d14 to ~18% by d28). Conversely, organs such as the heart, lungs and digestive system (Havenstein et al., 2003b, Schmidt et al., 2009, Zuidhof et al., 2014) have been shown to decrease as a percentage of bodyweight compared to heritage strains.

Similarly, comparative studies of strains allow for identification of physiological constraints. Experimental models of meat birds identified differential fatty acid metabolism in birds selected for either high or low abdominal fat (Leclercq et al., 1980, Leclercq and Simon, 1982), or very low density lipoprotein (VLDL) plasma concentrations (Whitehead and Griffin, 1984). Comparisons of these lines, regardless of nutritional status, shows that total plasma lipids and lipoprotein levels are higher in the fat line, suggesting a higher rate of hepatic lipogenesis in fat-line birds (Hermier et al., 1984). Transcriptional analyses of genetically lean and fat chickens (Resnyk et al., 2013, Resnyk et al., 2015) as well as juvenile and mature laying hens (Li et al., 2015) also reveals differential expression (DE) and regulation of lipogenic genes. Additionally, fat-line birds have been shown to have significant activation of the early steps of insulin signalling at 9 weeks post hatch, which may partially account for the increased lipogenesis in the liver (Dupont et al., 1999). Comparisons of domestic meat birds with the ancestral red jungle fowl, identified an intestinal glucose uptake 'surge' by means of increased brush border glucose transporter activity in meat birds at 2 weeks of age, not seen in the red jungle fowl (Jackson and Diamond, 1996). The general finding was that the meat birds had decreased glucose transporter activity (with the exception of week 2), but had higher glucose transporter capacity, due to an overall increase in small intestinal mass. Furthermore, modern meat birds have been shown to be less immunologically responsive to immune challenges in comparison to heritage lines (Cheema et al., 2003) and more recent studies have associated gut

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microbes with improved feed conversion ratio (FCR; feed intake per unit of bodyweight gain) in meat birds (Stanley et al., 2012, Stanley et al., 2016). These examples are far from exhaustive, but highlight the value of comparing phenotypically different breeds and/or lines with different trait selection histories, to identify key biological pathways involved.

Meat and layer strain chickens have undergone differential genetic selection, with meat strains for high carcass yield and feed efficiency (reduced feed conversion ratio), and layers for high egg production and also reduced feed conversion ratio (Druyan, 2010), but also lower bodyweight. Selection pressure on different traits has resulted in meat and layer stains with vastly divergent growth potential, with the bodyweights of meat birds being five times that of layers by d42 post hatch (Zhao et al., 2004). This divergent growth rate makes meat birds and layers an excellent phenotypic model to study the underlying biological mechanisms contributing to growth and performance (i.e. FCR). However, the negative consequences of high growth rates of meat birds can complicate comparisons, particularly metabolic disturbances, which are in themselves associated with dramatic shifts in gene expression. In order to bridge the phenotypic gap and reveal dominant/recessive effects, we used an intermediate growth phenotype for comparison by crossing layer ISA Brown roosters with a line of commercial meat bird breeder hens, producing an F1 layer x meat bird cross. In chapter five, we compared fatty acid metabolism, parameters of innate immunity and indicators of cellular stress to identify the physiological parameters that contributed to the differing growth potential of these birds. All birds were raised on the same diet as not to confound the effects of dietary difference with potential effects of biological traits. The results provided additional evidence that genetic selection has altered metabolic processes between the strains of poultry, with increases in genes related to both fatty acid synthesis and β-oxidation in the meat birds compared to the cross and layer birds. There was no evidence of interactions between fatty acid metabolism, innate immunity or cellular stress as we hypothesised to be contributing to differential growth.

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Advances in DNA sequencing technology are broadening the knowledge of gene regulation and interaction. RNA-Sequencing (RNA-Seq) has recently been used to explore gene expression in livers of juvenile and laying hens to assess differences in the transcriptome at the different developmental stages (Li et al., 2015) and also to study differences in the transcriptome of abdominal fat between genetically lean and fat strains of meat birds (Resnyk et al., 2015). In the current study, we hypothesised that genes driving growth and performance variation in poultry could be discovered in genes DE between groups of birds with differing growth potentials. We utilised our previous differential growth phenotypes to compare the transcriptomes of meat birds, F1 layer x meat bird crosses and layer line males at d14 post hatch. The objective was to identify the genes and biological pathways contributing to growth and performance differences between strains.

6.3   Methods

6.3.1   Birds and Management

All procedures were approved by The University of Adelaide Animal Ethics committee (approval # S-2015-171) and the PIRSA Animal Ethics committee (approval # 24/15). In total, 150 newly hatched male chicks were obtained from the HiChick Breeding Company Pty Ltd, Bethel, South Australia; $n=50$ meat birds (commercial line), $n=50$ F1 layer (Isa Brown cockerels) x meat bird (commercial line) crosses and $n=50$ layers (Isa Brown). Chicks were placed in a 6 unit rearing pen ($n=25$ birds/pen), separated in breed groups ($n=2$ pens/breed) in a temperature controlled room at the SARDI PPPI Poultry Research Unit, Roseworthy Campus, The University of Adelaide. All birds were fed a standard commercial meat bird starter diet *ad libitum* with no added in-feed antimicrobials or coccidiostats, and had unrestricted access to water via nipple drinker lines. The three experimental groups of males were chosen for their growth potential: fast growing (meat bird), moderate (F1 layer x meat bird) and slow growing (layer strain). Feed conversion ratios were recorded weekly as was bodyweight and bodyweight

gain. On d14 post hatch, 36 birds (*n=12* birds/breed) were randomly selected and euthanised by cervical dislocation. Liver tissue samples were rapidly collected, snap-frozen in liquid nitrogen and stored at -80 °C for RNA extraction and RNA-sequencing.

6.3.2   RNA Extraction

Samples were randomly selected for total RNA extraction (*n=6*/strain) using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Approximately 80mg of frozen (-80°C) liver tissue was homogenised in 2 mL of Trizol reagent (Invitrogen, Carlsbad, CA). 1 mL aliquots of the Trizol homogenate were combined with 200 µL of chloroform and centrifuged for 15 mins at 4°C. The upper aqueous phase (350 µL) was transferred to a gDNA eliminator spin column and centrifuged at >8000 *g* (14,000 rpm) for 30s. The flow through (300 µL) was collected and mixed with an equal volume of 70% ethanol and transferred onto RNeasy columns. The remaining collection and wash steps were performed according to the manufacturer's instructions. RNA was eluted in 200 µL of RNA-free water. Purity and concentration was determined using UV spectrophotometry (Nanodrop 1000; Thermo Scienfic, Wilmington, DE).

6.3.4   RNA-Seq Library Construction and Sequencing

RNA-Seq was carried out by the ACRF Cancer Genomics Facility, Adelaide, SA. The sample quality was analysed on an Agilent Bio-analyser (minimum RIN requirement of 7) and sequencing libraries were made using 2 µL of total RNA. PolyA mRNA isolation was performed using oligo dT beads. Libraries were prepared using KAPA Library Quantification Kits for Illumina platforms (KAPABiosystems, Massachusetts, USA). 2x 100nt sequencing was carried out on an Illumin HiSeq 2500 Sequencing System to generate a minimum depth of 25 million reads.

6.3.5 RNA-Seq Analysis

Reads were returned in fastq format. FastQC and adaptor sequences were trimmed from the 3'

end of reads with Cutadapt. Hisat2 (Pertea et al., 2016) was used to map reads to the reference

genome Galgal5.0 (ftp://ftp.ncbi.nlm.nih.gov/genomes/Gallus_gallus). Duplicate reads were

then removed. Stringtie (Pertea et al., 2016) was used to define the transcripts from the read

mappings for each sample, and to merge the transcript definitions for all samples. Transcripts

were cleaned up using in-house scripts. The number of raw read counts were calculated for each

transcript and sample using the function featureCounts of the R package Rsubread (Liao et al.,

2013). Another R package, edgeR (Robinson et al., 2010) was used to analyse differential gene

expression using normalised counts per million transcripts (CPM) to correct for varying depth

of sequence among samples. Transcript data were aggregated by gene. Genes where the

maximum CPM was <1 were removed. Gross transcriptome relationships between the three

types of bird were analysed by multidimensional scaling of the CPMs.

6.3.6 Functional Annotation Analysis and Statistical Analysis

Functional enrichment of the DE genes between meat bird vs layer, meat bird vs cross and layer

vs cross and DE between all three groups was conducted for gene ontology (GO) terms and

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the web based tools in

DAVID (Huang et al., 2009b, Huang et al., 2009a). Only GO terms and KEGG pathways with

$P < 0.05$ were taken into account as significantly enriched among the DE genes. Phenotypic

data, including bodyweight, bodyweight gain and liver weights (normalised and actual), were

analysed by a one-way ANOVA using SPSS (IBM SPSS Statistics 22). Gene expression levels

were correlated with individual bodyweights (all three groups combined) using Pearson's

correlation in SPSS (IBM SPSS Statistics 22).

6.4   Results

6.4.1   Phenotypic Data

Bodyweight, bodyweight gain, and liver phenotypic data are presented in Figure 6.1. Starting bodyweights (mean ± SEM) at hatch were significantly different between meat bird (44.4 ± 0.4 g); cross (42.5 ± .04 g; $P < 0.008$) and layer birds (38.5 ± 0.4 g; $P < 0.001$). At d14 post hatch, the time of RNA-Seq analysis, bodyweight was significantly different ($P < 0.001$) between all three groups; meat bird (560 ± 8 g); cross (311 ± 8 g) and layer birds (159 ± 2 g). Bodyweight remained different ($P < 0.001$) between the three groups for the remainder of the growth period to d28, with final bodyweights (mean ± SEM) for meat birds (2102 ± 35 g); cross (1037 ± 31 g) and layers (403 ± 6) g.

Day 0 liver weights (mean ± SEM) did not differ between the meat birds (1.30 ± 0.04 g) and cross (1.19 ± 0.04 g; Figure 6.1c). The layer livers (0.99 ± 0.02 g) were however significantly lighter than the meat bird ($P < 0.001$) and cross ($P < 0.002$) livers. From d7 onwards, liver weights were significantly different (at $P < 0.001$) between all three groups for d7,-14 and -28. Normalised liver weights (liver weight/ bodyweight x 100; Figure 6.1d) reached maximum weight in layers and cross birds at d7 post hatch and declined thereafter. Meat birds had a higher relative ratio and reached maximum relative liver weight later at d14 post hatch, which was significantly different from cross ($P < 0.001$) and layer birds ($P < 0.001$). The meat birds had a more pronounced decline in relative liver weight compared to cross and layer birds between d14-d28. By d28, there was no difference in normalised liver weight between any of the groups ($P > 0.05$).
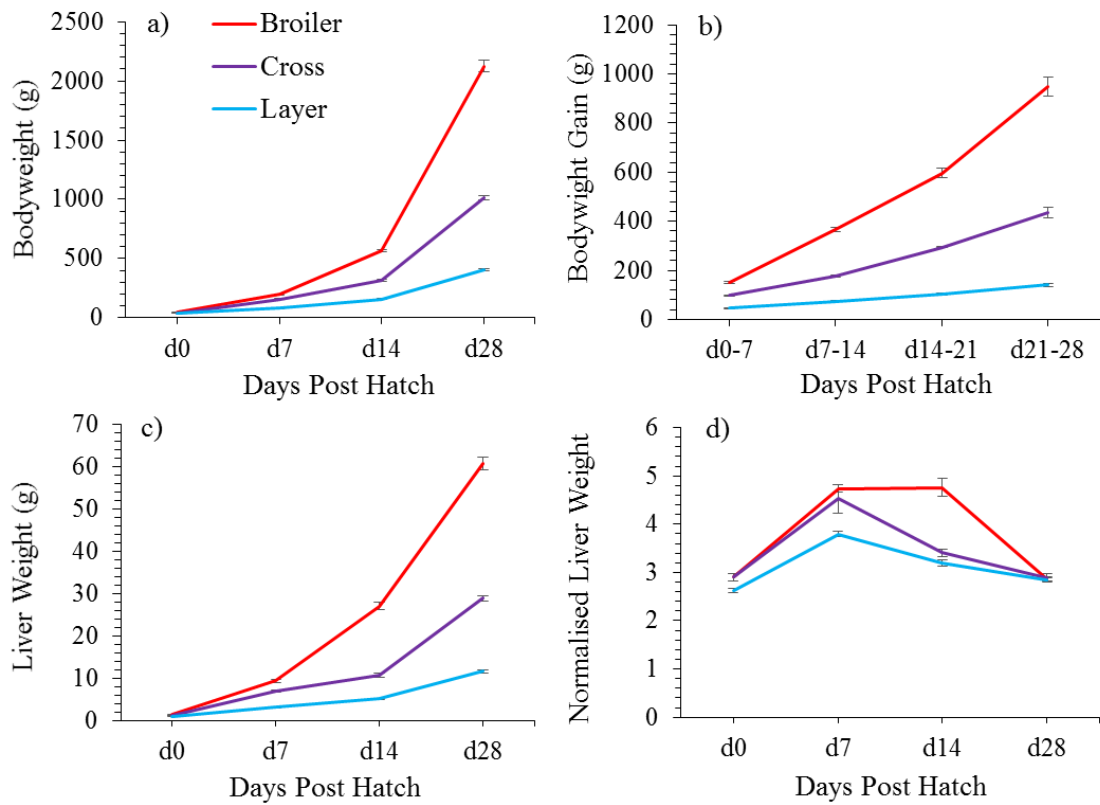
Figure 6.1 Growth of liver in meat bird, cross and layer strains; a) Bodyweight (g) versus days post hatch; b) Bodyweight gain (g) versus weekly intervals; c) Normalised liver weight (liver weight/total bodyweight) x 100 versus days post hatch; d) Liver weight versus days post hatch. Error bars are ± SEM.

6.4.2 Identification of Expressed Transcripts and Gross Transcriptional Relationships

RNA-Seq generated from 27,010,839 to 52,131,987 raw 2x 100 paired end reads per sample with the average number being: meat bird (44,346,591), cross (40,568.610) and layer (35,862,746). After filtering the low quality reads, the average number of clean reads and percent retained were; meat bird (43,887,348; 99.0%), cross (40,146,845; 99.0%) and layer (35,447,280; 98.8%). Reads were mapped to the reference genome Galgal5.0. A total of 30,586 genes were identified among the chicken liver libraries, both known and novel. After removal of genes with no or low counts in all samples (<1 CPM), 16,968 genes remained for analysis. Gross transcriptional analysis was undertaken using multidimensional scaling to determine how similar the transcriptomes were between the three strains. The results showed separate non-overlapping clusters of type; meat bird, cross and layer, indicating that each has a distinct transcriptome (Figure 6.2).

Sequence variants were called from the transcriptome data using GATK HaplotypeCaller (McKenna et al., 2010), outputting a gVCF file for each sample and then merging. Ratios of heterozygous to /homozygous loci were compared for the three strains, for genomic positions called across all 18 samples. Meat birds had a ratio of 1.07 indicating the population was relatively inbred. The layers were slightly higher with a ratio of 1.46, whilst the cross was higher again, as would be anticipated as a result of crossing the two strains, at 1.77.
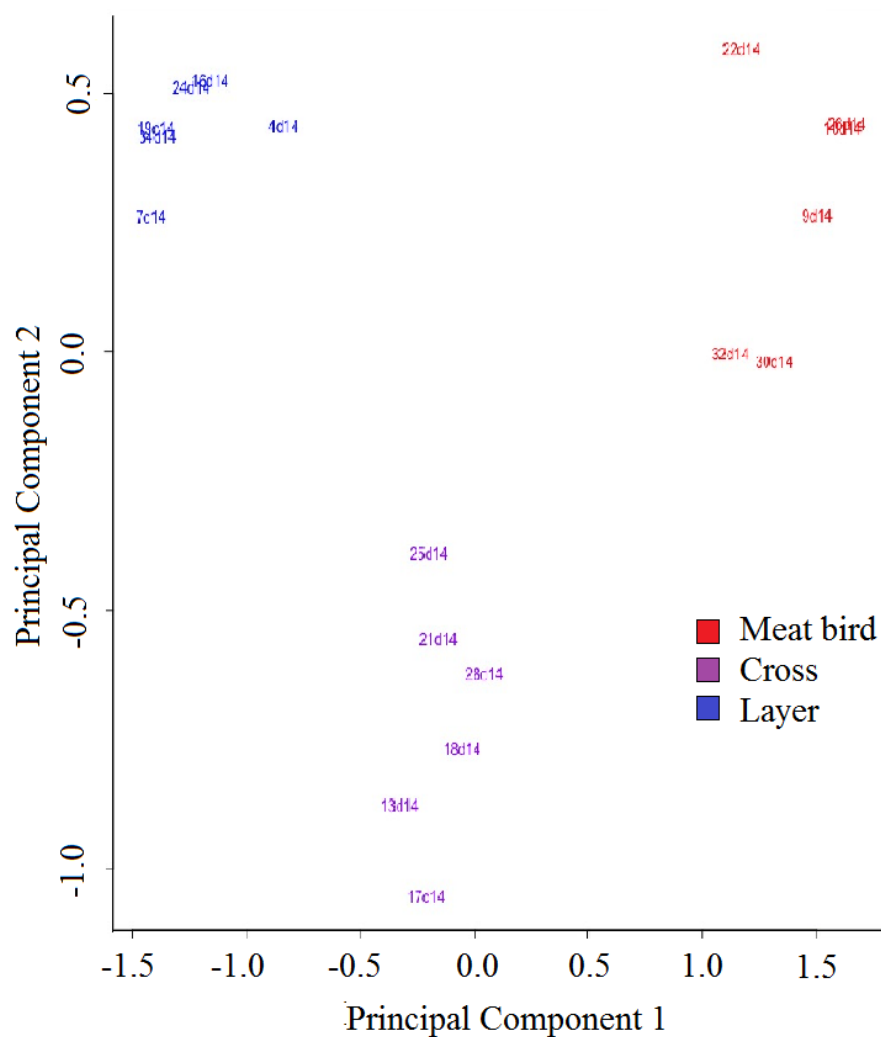
Figure 6.2 Principal component 1 vs principal component 2 analysis of meat bird (*n=6*), cross (*n=6*), and layer (*n=6*) transcriptomes. Clusters can be seen for meat birds, crosses, and layers indicating gross transcriptome differences between the three strains.

6.4.3 Identification of Differential Gene Expression

Of the 16,968 genes expressed in at least 1 sample, 6,278 genes were found to be DE for at least one of the comparisons of meat birds vs layers, meat birds vs crosses or layers vs crosses (Figure 6.3). Of these 6,278 genes identified as DE, 5,832 were DE between meat birds and layers (19%), 2,935 DE between meat birds and crosses (9.6%) and 493 DE between the layers and crosses (1.6%), highlighting that the transcriptome difference was greater in the meat birds than the layer or the cross. Percentages represent; the number of DE genes/ total genes (30,586), identified in the chicken libraries. Consideration of the transcriptome difference relative to body weight increases showed that a 1.8 fold increase in body weight from layer to cross was associated with a 1.6% transcriptome difference. The 2.0 fold increase in body weight between the cross and meat birds was associated with a 9.6% transcriptome difference, while the 3.5 fold bodyweight difference between meat birds and layers was associated with a 19% transcriptome difference. Comparisons between meat birds, crossed and layer birds identified 155 genes that are DE between all three groups. Of these 155 genes, 60% were found to be progressively upregulated in the direction meat bird > cross > layer, 38.1% down regulated meat bird < cross < layer, and 1.9% did not follow any directional pattern associated with growth rate.
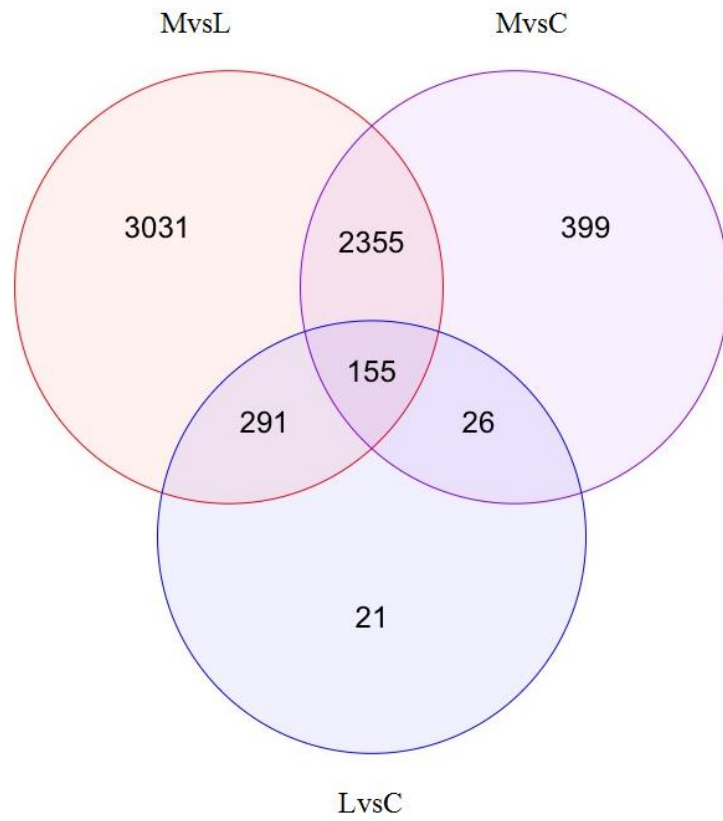
Figure 6.3 Venn diagram illustrating differential gene expression between meat birds (*n=6*) vs layers (*n=6*) (M v L); meat birds (*n=6*) vs cross (*n=6*) (M v C), and layers (*n=6*) v cross (*n= 6*) (L v C).

6.4.4 Characterisation of the 155 DE Genes

The 155 DE genes were characterised in terms of abundance and fold change. Additionally, correlations were tested between the 155 DE genes with individual bodyweight. The top 10 most abundantly expressed genes were; alpha 2-HS glycoprotein (*AHSG*), fibrinogen alpha chain (*FGA*), fibrinogen gamma chain (*FGB*), fibrinogen beta chain (*FGG*), ferratin heavy polypeptide 1 (*FTH1*), compliment C4 (*C4*), acetyl-CoA transferase 2 (*ACAA2*), Dihydrolipoamide S-acetyltransferase (*DLAT*), saccharopine dehydrogenase (*SCCPDH*) and one unknown (NA) (Table 6.1). Of these top 10 most abundantly expressed, 6 were down regulated in meat birds (*AHSG, FGA, FGG, FGB, FTH1* and *C4*; meat bird < cross < layer), and 4 upregulated (NA, *ACCA2, DLAT* and *SCCPDH*; meat bird > cross > layer).

Table 6.1 The top 10 most abundantly expressed genes (mean ± SEM) presented as counts per million for male; meat (*n=6*), cross (*n=6*) and layer (*n=6*) birds at d14 post hatch

Gene ID	refSeqID	Meat bird (*n=6*)	Cross (*n=6*)	Layer (*n=6*)	Regulation ↑↓
AHSG	424956	4433.1 ± 288.2	6102.8 ± 228.5	8048 ± 257.7	↓
FGA	396307	5401.6 ± 166.2	6529.3 ± 142.4	7963.3 ± 332.5	↓
FGB	373926	4040.9 ± 123.3	4833.8 ± 105.2	5975.5 ± 304.1	↓
FGG	395837	3914.1 ± 98.9	4593.6 ± 71.9	5650.2 ± 259.1	↓
NA	NA	1602.9 ± 83.3	491.3 ± 163.2	7.1 ± 0.9	↑
FTH1	395970	782.6 ± 19.7	912.1 ± 23.9	1129.1 ± 49.1	↓
C4	426611	430.7 ± 24.5	557.7 ± 34.7	926.4 ± 55.0	↓
ACAA2	426847	601.6 ± 32.2	472.5 ± 23.3	348.9 ± 10.7	↑
DLAT	419796	590.5 ± 14.7	449.1 ± 8.2	327.2 ± 16.1	↑
SCCPDH	421485	578.8 ± 31.3	470.1 ± 13.8	310.7 ± 11.0	↑

↓ Gene down regulated in meat birds (meat bird < cross < layer)

↑ Gene up regulated in meat birds (meat bird > cross > layer)

Fold changes were calculated using the mean CPMs for meat bird vs layer, meat bird vs cross and layer vs cross (Table 6.2). The largest fold change detected within the 155 DE genes was a 227-fold upregulation (CPM mean ± SEM) in meat birds (1602.90 ± 83.31) compared to layers (7.06 ± 0.89) for an uncharacterised gene (Un_24875). The second highest fold change was a 147.7 fold upregulation between the meat birds (1.12 ± 0.38) compared to layers (0.01 ± 0.01) for bacterial/permeability-increasing protein-like 3 (*BPIL3*). One individual with significantly increased expression in the meat birds influenced the magnitude of the BPIL3 fold change. Six of the top 10 highest fold change (all genes) were novel and uncharacterised, highlighting gaps within the chicken genome. Among the top 10 characterised genes were *BPIL3*, LOC107055086 and LOC107057467 genes which have both been characterised as sperm-associated antigen 4 protein-like, Histamine N-methyltransferase-like (LOC771456), cyclin dependant kinase inhibitor 2B (*CDKN2B*), platelet glycoprotein VI-like (LOC10087809, leucine protein zipper 2 (*LUZP2*), butyrophilin subfamily 3 member A2-like (LOC107049070), ubiquitin C-terminal hydrolase L1 (*UCHL1*) and prostaglandin D2 synthase (*PTGDS*) (see Table 6.2).

Table 6.2 Top 10 fold changes of the 155 DE genes between meat birds, crossed and layer birds

Gene name	Gene description	RefSeqID	Mean CPM (±SEM)			Direction[1]	Fold Change[2]		
			Meat bird ($n=6$)	Cross ($n=6$)	Layer ($n=6$)		M&L	M&C	L&C
Top 10 genes (all genes)									
NA	Uncharacterised	NA	1602.90 ± 83.31	491 ± 163.19	7.06 ± 0.89	↑	227.1	3.3	69.6
BPIL3	Bactericidal/permeability-increasing protein-like 3	419290	1.12 ± 0.38	0.25 ± 0.06	0.01 ± 0.01	↑	144.9	4.4	32.8
NA	Uncharacterised	NA	0.01 ± 0.01	0.29 ± 0.13	1.77 ± 0.32	↓	121.6	20.1	6.1
LOC107055086	Sperm-associated antigen 4 protein like	107055086	0.01 ± 0.01	0.29 ± 0.08	1.45 ± 0.39	↓	99.3	19.8	5.0
NA	Uncharacterised	NA	1.96 ± 0.51	0.43 ± 0.08	0.03 ± 0.01	↑	57.4	4.6	12.5
NA	Uncharacterised	NA	12.80 ± 4.15	1.70 ± 0.53	0.23 ± 0.10	↑	56.4	7.5	7.5
LOC107057467	Sperm-associated antigen 4 protein like	107057467	0.06 ± 0.04	0.69 ± 0.18	2.95 ± 0.54	↓	49.0	11.4	4.3
LOC771456	Histamine N-methyltransferase-like	771456	7.63 ± 1.30	1.08 ± 0.18	0.17 ± 0.08	↑	45.3	7.1	6.4
NA	Uncharacterised	NA	0.04 ± 0.02	0.47 ± 0.11	1.88 ± 0.35	↓	44.5	11.1	4.0
NA	Uncharacterised	NA	0.24 ± 0.10	1.68 ± 0.46	9.68 ± 2.24	↓	40.4	7.0	5.8
Top 10 characterised genes									
BPIL3	Bactericidal/permeability-increasing protein-like 3	419290	1.12 ± 0.38	0.25 ± 0.06	0.01 ± 0.01	↑	144.9	4.4	32.8
LOC107055086	Sperm-associated antigen 4 protein-like	107055086	0.01 ± 0.01	0.29 ± 0.08	1.45 ± 0.39	↓	99.3	19.8	5.0
LOC107057467	Sperm-associated antigen 4 protein-like	107057467	0.06 ± 0.10	0.69 ± 0.18	2.95 ± 0.54	↓	49.0	11.4	4.3
LOC771456	Histamine N-methyltransferase-like	771456	7.63 ± 1.30	1.08 ± 0.18	0.17 ± 0.08	↑	45.3	7.1	6.4
CDKN2B	Cyclin dependent kinase inhibitor 2B	395076	363.81 ± 55.77	115.67 ± 17.64	12.90 ± 3.62	↑	28.2	3.1	9.0
LOC100857809	Platelet glycoprotein VI-like	100857809	0.28 ± 0.06	2.85 ± 17.64	7.61 ± 1.44	↓	27.5	10.3	2.7
LUZP2	Leucine protein zipper 2	423001	0.94 ± 0.19	0.26 ± 0.08	0.04 ± 0.06	↓	26.7	3.5	7.5
LOC107049070	Butyrophilin subfamily 3 member A2-like	107049070	7.79 ± 0.90	2.31 ± 0.45	0.51 ± 0.05	↑	15.4	3.4	4.6
UCHL1	Ubiquitin C-terminal hydrolase L1	770302	77.02 ± 5.87	16.85 ± 3.89	5.07 ± 0.85	↑	15.2	4.6	3.3
PTGDS	Prostaglandin D2 synthase	374110	7.28 ± 1.01	21.80 ± 4.28	104.20 ± 25.95	↓	14.3	3.0	4.8

[1] Direction of regulation: ↑ Meat bird upregulated (meat bird > cross > layer); ↓ Meat bird downregulated (meat bird < cross < layer)

[2] Fold change comparisons: M&L = Meat bird and Layer; M&C = Meat bird and Cross; L&C = Layer and Cross

The 155 DE genes were correlated with individual bodyweight. Of the top ten correlated (Table 6.3), the highest correlation was between dihydrolipoamide S-acetyltransferase (*DLAT*), which is the E2 component on the pyruvate dehydrogenase complex, linking glycolysis to the citric acid cycle. DLAT was also among the top 10 most abundant of the 155 DE genes. Three of the top 10 genes correlated with bodyweight are novel and uncharacterised, e.g. LOC770248 and two unknown. Other genes highly correlated with bodyweight included quiescin Q6 sulfhydryl oxidase 1 (*QSOX1*), receptor accessory protein 5 (*REEP5*), myosin VI (*MYO6*), transmembrane protein 246 (*TMEM246*), cyclin G2 (*CCNG2*) and WW domain binding protein (*WBP2*).

Table 6.3 Top 10 genes with highest correlation with individual bodyweight

Chromosome	Gene ID	Gene Name	r[1]	r2
24	*DLAT*	Dihydrolipoamide S-acetyltransferase	.968**	0.937
1	NA1	N/A (Uncharacterised)	.956**	0.914
8	*QSOX1*	Quiescin Q6 sulfhydryl oxidase 1	.954**	0.910
Z	*REEP5*	Receptor accessory protein 5	.948**	0.899
3	*MYO6*	Myosin VI	.947**	0.897
1	NA3	N/A (Uncharacterised)	.947**	0.897
Z	*TMEM246*	Transmembrane protein 246	.946**	0.895
4	*CCNG2*	Cyclin G2	.945**	0.893
18	*WBP2*	WW domain binding protein	.944**	0.891
1	LOC770248	Uncharacterised	.943**	0.889

[1]Pearsons correlation coefficient; **Sig at $P < 0.01$

6.4.5 Functional Analysis of DE Genes

All 6,278 DE genes were analysed for GO terms and KEGG pathways using both edgeR and the web based tools in DAVID (Huang et al., 2009b, Huang et al., 2009a). There were 38 biological GO terms (GO: BP) identified for 5832 DE genes ($P < 0.05$) between meat bird and layer groups, 28 GO terms for 2935 DE genes between meat bird and the cross, and 19 GO terms for 493 DE genes between the layer and cross groups.

To understand the biological differences contributing to growth between the two strains and the cross, we focused on the 155 DE genes among the meat birds, crossed and layer birds. For these 155 DE genes, 27 GO terms were identified (Table 6.4). Many of the GO terms were found to be significant due to the expression levels of *FGA*, *FGB* and *FGG*, which were among the most abundantly expressed genes. These three genes dominated 20 of the 27 GO terms identified, ranging from fibrinolysis, blood clot formation, fibrin clot formation, plasminogen activation, positive regulation of exocytosis, response to calcium ion and platelet aggregation. However, despite their high abundance, these genes had lower correlations with bodyweight than other DE genes (mentioned above), although still significant at $P < 0.01$. Of the 155 DE genes, ranked in order of correlation strength with bodyweight, *FGA* was 89th ($r = -0.874$), *FGG* was 111th ($r = -0.085$) and *FGB* was 124th ($r = -0.836$). GO BP terms that were not largely dominated by *FGA, FGB* and *FGG* included positive regulation of glucose import, cellular response to oxidative stress and regulation of cell death. GO CC terms included chromatin and extracellular exosome. The extracellular exosome GO CC term (GO: 0070062) included 21 genes, 6 of which are in the top 10 most abundant (*AHSG*, *FGA*, *FGB*, *FGG*, *FTH1* and *ACCA2*), 2 in the top 10 fold changes (*PTGDS* and *UCHL1*), and 3 in the top 10 correlated with individual bodyweight (*QSOX*, *REEP5* and *MYO6*).

Table 6.4 Gene Ontology (GO) terms for the 155 DE genes.

GO ID	GO Function	P-value	Gene ID[1]
	GO Term BP		
GO:0042730	Fibrinolysis	7.20E-05	*FGA↓, FGB↓, FGG↓, CPB2↓*
GO:0034116	Positive regulation of heterotypic cell-cell adhesion	2.20E-04	*FGA↓, FGB↓, FGG↓*
GO: 0072378	Blood coagulation, fibrin clot formation	2.20E-04	*FGB↓, FGG↓, FBLN↓*
GO: 2000352	Negative regulation of endothelial cell apoptotic process	3.90E-04	*NFE2L2↓, FGA↓, FGB↓, FGG↓*
GO: 0051258	Protein polymerization	7.30E-04	*FGA↓, FGB↓, FGG↓*
GO: 0031639	Plasminogen activation	1.10E-03	*FGA↓, FGB↓, FGG↓*
GO: 0090277	Positive regulation of peptide hormone secretion	2.00E-03	*FGA↓, FGB↓, FGG↓*
GO: 0045921	Positive regulation of exocytosis	3.20E-03	*FGA↓, FGB↓, FGG↓*
GO: 0046326	Positive regulation of glucose import	6.30E-03	*INSR↓, NFE2L2↓, SLC1A2↑*
GO: 1902042	Negative regulation of extrinsic apoptotic signalling pathway via death domain receptors	7.20E-03	*FGA↓, FGB↓, FGG↓*
GO:0045907	Positive regulation of vasoconstriction	8.20E-03	*FGA↓, FGB↓, FGG↓*
GO:0050714	Positive regulation of protein secretion	1.00E-02	*FGA↓, FGB↓, FGG↓*
GO:0070527	Platelet aggregation	1.70E-02	*FGA↓, FGB↓, FGG↓*
GO:0051592	Response to calcium ion	1.80E-02	*FGA↓, FGB↓, FGG↓*
GO:0034599	Cellular response to oxidative stress	2.30E-02	*PARP1↑, SLC25A24↑, NFE2L2↓*
GO:0043152	Induction of bacterial agglutination	2.60E-02	*FGA↓, FGB↓*
GO:0010941	Regulation of cell death	3.40E-02	*JUN↑, SLC25A24↑*
GO:0070374	Positive regulation of ERK1 and ERK2 cascade	3.60E-02	*FGA↓, FGB↓, FGG↓, JUN↑*
GO:0007160	Cell-matrix adhesion	4.70E-02	*FGA↓, FGB↓, FGG↓*
	GO Term CC		
GO:0005577	Fibrinogen complex	1.30E-05	*FGA↓, FGB↓, FGG↓*
GO:0005938	Cell cortex	1.20E-03	*FAM110C↑, FGA, FGG, MYO6↑*
GO:0031091	Platelet alpha granule	3.30E-03	*FGA↓, FGB↓, FGG↓*
GO:0072562	Blood micro-particle	7.00E-03	*AHSG↓, FGA↓, FGB↓, FGG↓*
GO:0000785	Chromatin	8.90E-03	*FBXO18, MAU2, CCND2↑, NFE2L2↓*
GO:0070062	Extracellular exosome	2.30E-02	*ACAA2↑, AKR1A1↑, AHSG, ANXA13↑, CDHR2↑, CPB2↓, ECI1↓, FTH1↓, FGA↓, FGG↓, FGB↓, FBLNI↓, INSR↓, MRAS↓, MYO6↑, PFKL↑, PTGDS↓, QSOX1↑, REEP5↑, TSTA3↑, UCHL1↑*
	Go Term MF		
GO:0005198	Structural molecule activity	3.50E-02	*FGA↓, FGB↓, FGG↓*, NES↑
GO:0050662	Coenzyme binding	4.40E-02	*GCLC, TSTA3*

[1] Direction of regulation: ↑ Meat bird upregulated (meat bird > cross > layer); ↓Meat bird downregulated (meat bird < cross < layer)

KEGG analysis of the 5,832 DE genes between meat birds and layers revealed 13 pathways significantly enriched ($P < 0.05$). Pathways included; metabolic pathway (singular KEGG term), PPAR signalling pathway, biosynthesis of antibiotics, FoxO signalling pathway, cell cycle, drug metabolism, peroxisome, steroid biosynthesis, nicotinate and nicotinamide metabolism, glycine, serine and threonine metabolism, pentose phosphate pathway, glutathione metabolism and fatty acid metabolism. KEGG analysis of meat birds vs cross (2,935 DE genes) identified 15 significantly enriched pathways, 10 pathways overlapped with those significant for meat birds vs layers including; metabolic pathway, PPAR signalling pathway, biosynthesis of antibiotics, FoxO signalling pathway, cell cycle, peroxisome, steroid biosynthesis, glycine, serine and threonine metabolism and the pentose phosphate pathway. Three pathways were significantly enriched for the layers vs cross; metabolic pathway, folate biosynthesis and FoxO signalling pathway. The metabolic and FoxO signalling pathway were the only two common pathways between the three types of bird identified ($P < 0.05$).

KEGG pathway analysis of the 155 genes DE between all three types of birds identified two enriched pathways at $P < 0.05$ (Table 6.5). Three genes were enriched for fructose and mannose metabolism ($P = 0.024$); 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (*PFKFB2*), phosphofructokinase liver (*PFKL*), tissue specific transplantation antigen P35B (*TSTA3*). Five genes were associated with the FoxO signalling pathway ($P = 0.001$); cell cycle regulators; cyclin D2 (*CCND2*), cyclin G2 (*CCNG2*), cyclin-dependent kinase inhibitor 1B (*CDKN1B*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*) as well as insulin receptor (*INSR*). Just falling out of significance at $P = 0.053$ was the glycolysis/gluconeogenesis pathway, involving three genes: *PFKL* (overlapping with fructose/mannose metabolism), which is rate limiting in glycolysis, catalysing the transformation of fructose-6-phospate to fructose-1,6-diphosphate (Uyeda, 1979); glutamate transporter (*SLC1A2*), which was upregulated in the direction of meat birds (5.02 ± 0.70) crosses (2.16 ± 0.18) and layers (0.89 ± 0.14); and alcohol dehydrogenase (*AKR1A1* or *NADP$^+$*), which was upregulated in the direction of meat birds

(278.63 ± 12.84), crosses (230.01 ± 7.51) and layers (179.24 ± 6.45). *INSR* occurs in the FoxO pathway, and *SLC1A2* also overlaps with the GO term, GO: 0046326, positive regulation of glucose import.

Table 6.5 Pathways and associated genes identified as enriched by KEGG of the 155 DE genes between meat birds, crossed and layer birds.

| Gene name | Mean CPM (± SEM) | | | Direction[1] | Fold Change[2] | | |
	Meat bird (*n=6*)	Cross (*n=6*)	Layer (n=6)		M&L	M&C	C&L
FoxO signalling pathway							
CCND2	182.89 ± 13.38	126.40 ± 9.25	79.19 ± 3.43	↑	2.31	1.45	1.60
CCNG2	348.26 ± 16.00	192.62 ± 15.44	103.89 ± 16.22	↑	3.35	1.81	1.85
CCKN1B	51.95 ± 0.73	59.75 ± 1.73	73.09 ± 1.96	↓	1.41	1.15	1.22
CDKN2B	363.81 ± 55.77	115.67 ± 17.64	12.90 ± 3.62	↑	28.19	3.15	8.96
INSR	86.48 ± 1.45	105.98 ± 3.91	131.37 ± 4.33	↓	1.52	1.23	1.24
Fructose and mannose metabolism							
PFKFB2	24.60 ± 1.61	30.61 ± 1.14	38.70 ± 1.26	↓	1.57	1.24	1.26
PFKL	295.60 ± 18.22	235.25 ± 7.22	170.06 ± 12.64	↑	1.74	1.26	1.38
TSTA3	49.15 ± 4.38	30.87 ± 1.38	20.78 ± 1.62	↑	2.37	1.59	1.49

[1] Direction of regulation: ↑ Meat bird upregulated (meat bird > cross > layer); ↓ Meat bird downregulated (meat bird < cross < layer)

[2] Fold change comparisons: M&L = Meat bird and Layer; M&C = Meat bird and Cross; L&C = Layer and Cross

6.5   Discussion

Liver transcriptomes of males of meat birds, F1 layer x meat bird crosses and layer birds were compared to identify DE genes between all three groups. Selection of the groups were based on their fast, moderate and slow growth potential, respectively. Day 14 post hatch was selected as the primary sampling time due to the rapid increase in growth seen in meat birds from 2-3 weeks of age compared to other strains. By sampling at this time point, it was hoped to capture transcriptional changes at the beginning of rapid growth phase to further understand the biological factors associated with the high growth rates seen in meat birds.

The results of this study revealed that selection for growth or egg laying is associated with altered transcriptomes between meat and layer birds. Bodyweight at d14 post hatch was 1.8 fold higher for crosses vs layers, and also 2.0 fold higher for meat birds vs crosses (meat bird > cross). The difference in transcriptomes associated with birds of differing bodyweights was quite remarkable. Of the total genes analysed, 1.6% were DE between crosses and layers; 9.6% DE between meat birds and crosses; and 19% DE between meat birds and layers. The differences in gene expression observed between the meat birds, layers and their F1 cross are not all driving the increases in bird size, particularly given the confounding effect of the many metabolic disturbances modern meat birds exhibit. These include; excessive fat deposition (Foud and El-Senousey, 2014), increased skeletal defects (Bessei, 2006), pulmonary hypertension, sudden death syndrome (Julian, 2005, Olkowski et al., 2007) and altered immune function (Cheema et al., 2003). However, it is likely that the drivers of growth are represented in the DE genes, particularly those that differ between all three groups.

GO and KEGG analyses of DE genes for meat birds vs layers, meat birds vs crosses and layers vs crosses identified overlapping biological functions that were affected and may contribute to the differential growth between types of birds. Two affected KEGG pathways were identified between all three comparisons; metabolic pathway (singular KEGG term) and

the FoxO signalling pathway. The Forkhead box O (FoxO) genes central to this pathway are a family of transcription factors that regulate gene expression related to cell cycle regulation, cell survival, and metabolism, including glucose and lipid metabolism (Eijkelenboom and Burgering, 2013). KEGG analysis of the 155 genes DE between each types of birds again identified the FoxO signalling pathway, enriched at $P < 0.05$. The fructose and mannose metabolism pathway was also enriched, with an overlap of genes involved in glycolysis, as well as the GO term 'positive regulation of glucose import'. Among the functions of the FoxO signalling pathway is maintenance of homeostasis, particularly in response to stress (Eijkelenboom and Burgering, 2013).

FoxOs have previously been identified as potential candidate genes for growth in chickens. A genome-wide association study using a reciprocal cross between White Recessive Rock (WRR) and Xinghua (XH) chickens, identified a 1.5 Mb region on chromosome 1 containing 5 SNPs, including a SNP 8.9 kb upstream of *FoxO1* for bodyweight at 22-24d and 70d post hatch (Xie et al., 2012). *FoxO1* contained two SNPs in the intron region of the gene; however, these two SNPs were not significantly associated with growth traits. The authors questioned whether a regulatory mechanism was involved in the significant SNP effects associated with growth traits located up and downstream of *FoxO1*.The most significant SNP for average daily gain at d42 was in a region containing gene LOC770248, which is uncharacterised. Comparatively, LOC770248 was amongst the top 10 genes correlated (r = 0.934) with individual d14 bodyweight in the present study. The identification of LOC770248 as a potential regulator of growth traits suggests further investigation is warranted to characterise the function of the encoded protein. More recently, RNA-Seq of the breast muscle of WWR and XH chickens at 7 weeks post hatch identified *FoxO3* as a candidate gene (supported by siRNA analysis and association analysis) for further investigation into breast muscle growth in the chicken (Chen et al., 2015). The significant enrichment of the FoxO

signalling pathway in all comparisons in the current study strongly supports the contribution of this pathway to the growth differences between meat birds, layers and their F1 cross.

Of the 155 DE genes identified between the three types of bird, five genes associated with the FoxO signalling pathway were upregulated (meat birds > crosses > layers). These were insulin receptor (*INSR*), as well as genes essential for cell cycle regulation, cyclins *CCND2*, *CCNG2* and cyclin-dependent kinase inhibitors *CDKN2B*. Down regulation of *CDKN1B* was seen in meat birds compared with crossed and layer birds (meat birds < crosses < layers). Cyclins, such as *CCND2*, activate cyclin-dependent protein kinases (CDKs) which form complexes to transition the cell from one cell cycle state to another (Pines, 1995), for example; activation of cyclin-D dependent kinases initiates progression of the cell cycle through the G1 phase (Sherr, 1995). *CCND2* binds to several types of CDKs, with the main partners *CDK4* and *CDK6* (Pines, 1995). We did not find *CDK4* in this gene set (of the 30,586), however, found abundant levels of *CDK6*, although not DE expressed. CDKs are normally present in the cell in excess of their cyclin partner (Pines, 1995), which was the case at the RNA expression level of *CDK6*:*CCND2* for meat birds, crosses and layers, with ratios of 1.3, 3.0 and 100.3 respectively. Interestingly, we found high DE of *CDKN2B* between all three groups (meat birds > crosses > layers) which inhibits the activity of *CDK4* and *CDK6*. *CDKN2B* is known to weaken the binding of D-type cyclins and as well as interact with the catalytic domains of *CDK4* and *CDK6* as a potent inhibitor of kinase activity (Asghar et al., 2013). Meat birds were the only group that had higher levels of *CDKNB2* relative to either *CDK6* or *CCDN2*, and the ratios for *CDKNB2*:*CCND2* and *CDKNB*:*CDK6* decreased (meat birds > crosses > layers) in both instances. *CCKNB2* was also amongst the top genes categorised by fold difference, being 28 fold higher in meat birds compared with layers.

Cyclin *CCNG2* was also upregulated in meat birds compared to cross and layer birds. Unlike 'conventional' cyclins that promote cell cycle progression, *CCNG2* upregulation in

murine B cells is associated with cell cycle arrest or apoptosis in response to inhibitory stimuli, and conversley, *CCND2* is down regulated during G1 phase growth arrest (Bennin et al., 2002, Horne et al., 1997). There is limited information of *CCNG2* activity in birds or in the liver for comparison. One study however compared Arbor Acres meat birds divergently selected for lean and fat lines, and identified *CCNG2* with a 0.209 and 0.249 lean/fat fold change at 2 and 4 weeks respectively in liver tissue, which is similar to the fold change we observed between layers/meat birds (0.296) (He et al., 2014). These studies, together with *CCNG2* being amongst the top 10 DE genes correlated with bodyweight in the present study, supports the differences between meat birds, crosses and layers being a result of differential cell cycle progression between the three types of birds.

Here we report that the liver (as a percentage of total bodyweight) reaches maximum size at d14 post hatch in meat birds compared with the crossed and layer birds, where the ratio between liver and body size is lower, and the relative liver weight maximum is reached earlier, at d7 post hatch. By d28 there was no difference in relative liver weight (~3%) between any of the groups. In many plants and animals, organ scaling is controlled at the level of cell number (Orr-Weaver, 2015). However, for meat birds, although liver weight continued to increase at the same rate as the cross and layer birds from d14-d28, the expression studied, combined with the higher deceleration in relative liver weight from d14 onwards in meat birds suggests that either; a) the total cell cycle time is increased or b) there is a decrease in growth fraction. This has been defined as the number of cells remaining in the cell cycle vs the total organ cell number (Lui and Baron, 2011) . Therefore a decrease in growth fraction is likely due to fewer dividing cells as more remain in the G_0 cell phase (Lui and Baron, 2011),. This would be supported by increased expression of *CCGN2* and *CDKNB2*. Thus, increased growth in the meat birds compared to cross and layer birds, likely results from hypotrophy (increased cell size) rather than hypoplasia (increased cell number). Hypotrophy via cellular polyploidy in the liver is not uncommon, with polyploid cells appearing late in fetal development, coinciding with terminal

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differentiation (Gupta, 2000). Polyploidy is associated with rapid growth by facilitating an increase in cell volume without division, which may permit cells to be more metabolically active (Ho et al., 2009, Orr-Weaver, 2015). Without histological analysis on hepatocytes, increased polyploidy is speculative, however, there is evidence in this study to suggest the meat birds are more metabolically active.

The insulin receptor (*INSR*) was down regulated in broilers compared with cross and layer birds and was amongst the 155 genes with DE between the three groups. *INSR* was also enriched to the FoxO signalling pathway. The *INSR* pathway is conserved from flies to humans, and is a key sensor of nutrient availability, playing an important role in the control of cellular proliferation, cellular size and response to nutrient availability (Marr II et al., 2006, Puig and Tjian, 2005). Insulin regulates not only glucose metabolism, but also lipid homeostasis by increasing lipogenesis in the case of nutrient excess. In hepatocytes, activation of FoxO promotes the expression of key gluconeogenetic and glycogenolytic enzymes in the fasted state, resulting in increased hepatic glucose production (Kousteni, 2012). In the fed state, high insulin blocks FoxO activity through the PI3-kinase (*PI3K*)-Atk pathway (Kousteni, 2012). Atk phosphorylates the FoxO protein, retaining it in the cytoplasm in its inactive state (Kousteni, 2012, Puig and Tjian, 2005, Puig et al., 2003). This would favour glucose uptake and glycolysis. Furthermore, FoxOs have been shown to directly regulate the insulin signalling response to nutrients in C2C12 lines (Puig and Tjian, 2005). Upregulated insulin mRNA levels were associated with dephosphorylation of *FoxO1*, conversely down regulated insulin mRNA levels were associated with phosphorylation of *FoxO1* (Puig and Tjian, 2005). As phosphorylation of *FoxO1* results in decreased activation of *FoxO1*, it would be anticipated that this direct effect would result in decrease gluconeogenesis and increased glucose uptake and glycolysis. A major limitation in this study is that without functional analysis of the FoxO genes themselves, we cannot determine their activation status.

The lower expression levels of *INSR* in meat birds compared with crosses and layers however, is consistent with increased levels of phosphofuctokinase (*PFKL*; upregulated meat birds > crosses > layers), glutamate transporter *SLC1A2*, and *AK1A1,* which would be expected with increased levels of glycolysis, particularly as *PFKL* is a rate limiting enzyme in glycolysis. *PFKL* catalyses the transformation of fructose-6-phospate to fructose-1,6-diphosphate (Uyeda, 1979). Furthermore, the pyruvate dehydrogenase complex (PDC) links glycolysis to the citric acid cycle. Therefore it is significant that dihydrolipoamide S-acetyltransferase (*DLAT*) was in the top 10 most abundantly expressed genes, upregulated in meat birds, and showed the highest correlation with bodyweight (r = 0.968). *DLAT* is the E2 component of the PDC, catalysing the oxidative reaction of pyruvate (end product of glycolysis) to acetyl-CoA in the mitochondria. Interestingly, chickens have been shown not to accumulate pyruvate in the liver, so the increase in *DLAT* is also consistent with the conversion to, and utilisation of acetyl-CoA in the mitochondria as soon as pyruvate is formed (Ochs and Harris, 1978).

The mRNA expression of key genes regulating cell cycle progression, insulin signalling and increased glycolysis draws interesting parallels with other avian studies. For example, we mentioned that comparisons of domestic meat birds with the red jungle fowl, identified an intestinal 'surge' of brush border glucose transporter activity in meat birds only, at d14 post hatch (Jackson and Diamond, 1996). In the current study, two GO terms; 'cellular response to stress' and 'positive regulation of glucose import', were also enriched, which may also support the author's conclusions of increased glucose activity due to energy stress associated with rapid growth. He et al., (2014) speculated that the insulin signalling pathway and cell cycle pathway in the liver also had important effects on chicken lipid metabolism. In chapter five, we also identified increased fatty acid synthesis and fatty acid β-oxidation in meat birds at d14 post hatch compared to cross and layer birds. These studies, in conjunction with identification of FoxOs as growth candidate genes (Chen et al., 2015, Xie et al., 2012), and the mRNA

expression data presented in this study, provide a strong body of evidence that the FoxO signalling pathway warrants further functional investigation in chickens as a driver of growth.

6.6   Conclusion

In this study, we used RNA-Seq to show that the transcriptomes of meat birds, layers (and the F1 cross between them) are highly divergent, particularly between meat and layer type birds. Metabolic pathway (singular KEGG term) and the FoxO signalling pathway were identified as significantly enriched in comparisons between the three types of birds, with trends between meat, crossed and layer birds. Functional analysis of the 155 genes DE between all three strains also identified enrichment of the FoxO signalling pathway, particularly genes related to cell cycle regulation and the insulin receptor. These data suggest that differences in cell cycle regulation and glucose metabolism are associated with differences in growth rate, and provide evidence that meat birds have a higher rate of glycolysis. Functional analysis of the chicken hepatic FoxO genes and associated pathway targets warrants further investigation to determine the role of this pathway in regulating the growth of meat birds.

6.7   Declarations

Acknowledgements

Ethics approval

The experimental protocol used in this study, including animal management, housing, and slaughter procedures were by the University of Adelaide Animal Ethics Committee (approval #S-2015-171) and the PIRSA Animal Ethics committee (approval #24/15).

Availability of data and materials

The fastq files containing the raw RNA-Seq data for the 18 birds will be deposited in the NCBI SRA.

Authors' contributions

N-LW, PH, RF, RH and GN designed the study. N-LW and RF were involved in performing the experiment. N-LW conducted the laboratory analysis. RT performed RNA-seq data analysis. N-LW, PH, RF, GN, RT and JLW were involved in interpreting the results. N-LW performed GO, KEGG and data analysis and wrote the manuscript, PH, RF, RT, JLW, RH and GN revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare they have no competing interests.

Consent for publication

Not applicable

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CHAPTER SEVEN

General Discussion

The objective of this thesis was to elucidate biological mechanisms driving growth and performance variations in commercial poultry, with particular emphasis on meat birds. Feed conversion ratio (FCR) is a highly important economic trait, and the consequence of increased variations in FCR stretch beyond economic implications; including environmental and sustainability impacts related to production i.e. increased agricultural land use for crop production, as well as increased greenhouse gas emissions from fossil fuels used for crop production (Tallentire et al., 2016). This chapter discusses the experimental conclusions and outcomes derived from chapters three, five and six, in relation to identification of biological factors that may be attributed to performance variations, and critically examines experimental limitations encountered. Additional to the three experimental chapters, the thesis contains two literature reviews. A second review was necessary to accommodate the change in experimental focus at the conclusion of the chapter three. The directional change of the thesis will also be discussed in further detail.

The initial hypothesis for this thesis was that functional changes in intestinal barrier function and innate immunity may be contributing to performance variation in healthy (unchallenged) commercial broilers; performance being characterised phenotypically in this context by FCR. After reviewing literature surrounding multiple branches of avian intestinal innate immunity, it became clear that the majority of investigative studies were either pathological challenges or in response to dietary modulation. Whilst many of these studies demonstrated functional changes in innate immunity and bird performance in response to treatment factors, it was not clear whether functional differences in intestinal innate immunity were contributing to performance variations in unchallenged individuals. Furthermore, there did not appear to be a comprehensive characterisation of intestinal innate immunity in the avian intestine in unchallenged (or challenged for that matter) individuals.

To test the founding hypothesis, individual birds were selected as either high-performing (low FCR) or low-performing (high FCR) based on individual FCR values (chapter three). In total, 16 candidate genes related to avian intestinal innate immunity were selected from the literature. Genes were assayed using RT-PCR over 96 individual samples, collected from experimental work previously conducted by Stanley et al., (2012) and Crisol-Martinez et al., (2017). The purpose of extensive replication was to identify consistent and repeatable findings in differential gene expression, which was concluded not to be the case. On the contrary, there was very little variation in the expression levels of the majority of the 16 genes across all experiments. The results provided considerable evidence that the phenotypic expression of FCR was not being driven by functional changes in intestinal innate immunity in chapter three. We found the highest gene variation in the antimicrobials, avian β-defensin 1 (*AvBD1*) and avian β-defensin 2 (*AvBD2*), which was likely reflective of individual's intestinal microbial populations and aligned with the suggestion that individuals exhibit tailored antimicrobial responses (Cormican et al., 2009).

A major experimental limitation in chapter three was the FCR values of the high and low performing broilers. Rearing chickens in an experimental facility generally allows hygiene to be maintained at a higher standard than that of a commercial facility, based on the small size of the experimental facility and few birds reared compared to commercial size operations. Thus, although FCR values were significantly different, the differences produced in a controlled experimental environment were not likely reflective of the true variation seen in a commercial environment, as our "poor' performing broilers, would still be considered to be performing exceptionally well commercially. However, in order to amplify FCR differences experimentally, the introduction of treatment factor would be required; i.e. dietary or immunological. Therein lies the problem, as an experimental model for comparing unchallenged individuals is lost if a treatment factor is applied. An alternative approach would be to work in conjunction with a commercial producer and sample birds from a commercial

flock; however, this too would require an alternative phenotypic selection trait such as live weight, as individual FCR data would not be available.

Despite experimental limitations, the data indicated that designing a further experiment focused solely on intestinal innate immunity linked to unchallenged performance variation was likely futile, given how tight the majority of the gene expression was. What was of interest from this study however, was the third and fourth most variably expressed genes in these repeated experiments (behind *AvBD1* and *AvBD1*), the pathogen recognition receptor, Toll-like receptor 2 (*TLR2*) and membrane protein *CD36* also known as *FAT/CD36*. Both *TLR2* and *CD36* have been shown to interact with each other, and each have been linked to both fatty acid metabolism and innate immunity, as reviewed in chapter four. For example, *TLR2* and *CD36* are known to form complexes in lipid rafts (Hoebe et al., 2005), fatty acids have been linked to *TLR2* and *TLR4* activation (Lee and Hwang, 2006) and *CD36* has been described in facilitating *TLR2* signalling (Wolowczuk et al., 2008). Additionally, *CD36* has been demonstrated to promote the synthesis of triglycerides in adipocytes, clearance of chylomicrons from plasma, as well as mediate lipid metabolism and fatty acid transport (Silverstein and Febbraio, 2009, Drover et al., 2005).

Just as growth has been shown to be an immunological trade off (van der Most et al., 2011), excess fat deposition (particularly abdominal visceral fat) in modern meat birds has also manifested as an unfavourable consequence of genetic selection for growth, and increased fat deposition is negatively associated with decreased FCR (Gaya et al., 2006). Excessive fat deposition is thought to have peaked in the 1970s and somewhat reduced (Tallentire et al., 2016), however, total body fat/lipid content is still considered as the most variable body component in modern meat birds, accounting for 15-18% of the total bodyweight (Choct et al., 2000). Current literature often refers to the total body fat % estimate presented by Choct et al., (2000), however, in chapter five; we found broiler carcass fat percentage to be 11.3%, which

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could indicate improvements and reductions in total body fat. Alternatively, the age of the birds (d14 post hatch) used in chapter five may account for the reduction, as hyperplasia of adipocytes (particularly abdominal fat pad) continues until ~4 wks post hatch, followed by hypertrophic growth (Matsubara et al ., 2005). Thus, it is likely that the total carcass fat percentages presented in chapter five would increase with age.

The association between excess fat deposition and reduced feed efficiency has been well established in meat birds, particularly in experimental models of birds divergently selected either for abdominal fat pad weight (Leclercq et al., 1980, Leclercq and Simon, 1982) or plasma levels of very low density lipoproteins (Whitehead and Griffin, 1984). General findings between fat and lean broiler lines include increased rates of lipogenesis in fat lines (Hermier, 1997, Hermier et al., 1984), and an insulin/glucose imbalance between fat and lean lines has also been observed (Dupont et al., 1999). It must be noted however, in these instances, birds are specifically selected for fat traits over multiple generations. Therefore, selection itself could be a reflection of such differences in fat and lean lines, rather than direct cause of the fat deposition variations seen in birds unselected for fatness traits. The identification of variation in genes linking innate immunity and fatty acid metabolism in chapter three was of interest for two striking reasons. Firstly, the negative association of increased fat deposition with increased FCR, and secondly, given that obesity is now widely correlated with a chronic state of inflammation, and that many modern meat birds deposit excessive fat in comparison to wild or layer strains (Fresno et al., 2011, Lin et al., 1980). These considerations lead to the hypothesis that differences in fatty acid metabolism as well as interactions with parameters of innate immunity could be contributing to growth/performance variations. Lipogenesis primarily occurs in the liver in birds (Goodridge and Ball, 1967, Goodridge and Ball, 1966), thus the focus was shifted from the small intestine to the liver and an additional literature review conducted (chapter four) to determine how innate immunity and fatty acid metabolism may be contributing to variations in growth performance of meat birds.

Due to the previous limitations of producing high variation in FCR experimentally, it was decided to utilise an alternative phenotypic model of growth and efficiency to further explore performance variation in poultry. A collaboration was sought and established with the North Carolina State University to compare a heritage meat bird line (Athens Canadian Random Bred) with modern meat birds, as the unselected heritage line allows for direct comparison of change in meat birds due to selection for growth and efficiency. This model has been previously utilised in highly regarded studies by Havenstein and colleagues (Havenstein et al., 2003a, Havenstein et al., 2003b, Havenstein et al., 1994a, Havenstein et al., 1994b). Unfortunately, this particular collaboration did not come to fruition due to technical delays and therefore an alternative model utilising breeds of poultry available in Australia was established.

Meat and layer birds have been divergently selected for different production traits over the decades, meat birds for high growth and increased feed efficiency, and layers for increased commercial egg production (Druyan, 2010). If considered from a FCR perspective, the broiler is much more efficient at rapidly converting feed to mass. Therefore, in utilising the two strains as a phenotypic model of growth and efficiency, a meat bird could be considered high-performing and a layer bird low-performing in this context. As discussed briefly in chapter six, negative consequences resultant of selection for rapid growth in meat birds can somewhat confound such a comparative-model of growth and performance, as meat birds are now predisposed to a number of metabolic disturbances, which in themselves may be associated with dramatic shifts in gene expression. In order to bridge the phenotypic gap and reveal dominant/recessive effects, a genetically related intermediate growth phenotype was produced in conjunction with the HiChick Breeding Company Pty Ltd in South Australia, utilising their commercial breeding stock. An F1 cross was produced by crossing Isa Brown layer cockerels and commercial meat bird breeder hens. The cross proved to be an excellent intermediate growth phenotype, with a 1.8 fold increase in bodyweight compared to layers, and 2.0 fold increase in bodyweight between the cross and meat birds. The growth findings between layer

and meat birds in chapter five were comparable with other studies comparing meat and layer strains, with Zhao et al. (2004) reporting a 4-5 fold growth difference between the two strains. By d14 post hatch we observed a 3.5 fold bodyweight increase in broilers compared to layers in this model.

In chapter five, meat birds, layer birds and the F1 layer x meat bird crosses were used to test the hypothesis that differences in fatty acid metabolism, as well as interactions with parameters of innate immunity, could be contributing to variations in growth performance. Genes related to lipogenesis and fatty acid β-oxidation, as well as total carcass lipid %, and carcass and blood lipid composition were assessed. Immune linked genes *TLR2*, *TLR4,* and *CD36* as well as genes linked to cellular stress, including the x-box binding protein (*XBP1*) and inositol-requiring kinase 1 (*ERN1*) were also compared. Heterophil: lymphocyte whole blood counts were performed as an additional indicator to evaluate whether the immune status was differing between the three groups of birds. Several results were found that were not anticipated in this study. Firstly, comparisons of total carcass lipid content showed significant composition differences between the groups. For example, the meat birds had higher total saturated fatty acid carcass content, while the cross and layer birds had total higher omega-3 and omega-6 carcass content. This was surprising given that, in meat birds at least; it has been shown multiple times that the carcass lipid composition is reflective of dietary lipid intake (Frttsche et al., 1991, Newman et al., 2002). All birds in this study however were fed the same diet, and therefore differences in lipid carcass composition could not be attributed to diet, but likely, to altered lipid metabolism between strains, which was supported at the mRNA level. A reciprocal treatment where all birds were fed a commercial layer diet may have been useful for comparison, as it is likely the energy and protein content of the diet exceeded the requirements of the cross and layer birds. However, the decision to feed all birds the same diet was done so as to not confound effects of dietary differences with potential effects of biological traits. Secondly, total carcass fat percentage was also unexpected. Despite the 1.8 fold increase in

bodyweight difference between layer and cross birds (crosses > layers), there was no difference in total fat as a percentage of bodyweight. Given the crosses were twice the size of the layers it may be anticipated that crossing a meat bird and a layer would result in crosses with increased fat content, as increased fat deposition it regarded as a consequence of selection for growth. Furthermore, there was no indication of functional differences in the select parameters investigating links between innate immunity, or evidence suggestive of cellular stress. It was concluded that the meat birds not only had higher rates of lipogenesis, as is reported between genetically selected fat and lean line of meat birds, but also increased rates of fatty acid β-oxidation. Therefore, the increase in fat deposition and concurrent reduction in feed efficiency commonly seen in commercial meat birds does not appear to be solely due to increased lipogenic activity, but a result of other alterations in metabolic processes.

The results from the first two experimental chapters (three and five) highlighted that investigations into growth and performance variation by applying a candidate gene approach, although useful, was not the most efficient method to elucidate biological differences in growth and performance variations thoroughly. Therefore, a novel approach was adopted for the third experimental chapter, given the success of the growth model devised, to perform RNA-sequencing and analyse liver transcriptomes of six birds from each group. It was hypothesised that genes driving growth and performance variation could be discovered in genes differentially expressed (DE) between the three groups, which would allow us to determine gene and biological pathways contributing directly to performance variations.

The results of the RNA-seq transcriptome analysis between the three strains in chapter six demonstrated dramatically altered transcriptomes between meat birds and layers, with 19% of the total genes identified in the chicken genome library DE between the two. What was more surprising however was the relatively few genes DE between cross and layer birds. Despite the 1.8 fold increase in body weight between layer and cross birds, only 1.62% of the total genes

identified were DE between the two groups. Comparisons between all three groups identified 155 DE genes, accounting for < 0.5% of the total genes identified in the chicken liver libraries. KEGG analysis of the DE genes between meat birds vs layers, meat birds vs crosses and layers vs crosses all identified various biological pathways enriched in each comparison, however only two were consistently identified in all three comparisons, metabolic pathway (singular KEGG term) and the FoxO signalling pathway. Analysis of the 155 DE genes between all three strains again identified the FoxO signalling pathway with particular emphasis on genes essential for cell cycle regulation and insulin signalling. There was also strong evidence to support a higher rate of glycolysis in the meat birds, upregulated (meat bird > cross > layers) which would be influenced by insulin signalling and thus potentially also under the regulation of the FoxO signalling pathway. The results of chapter six would have been strengthened with histological analyses of the liver to determine the extent of hypotrophy and/or polyploidy of hepatocytes between bird types. Unfortunately, liver samples were not collected specifically for histology and this would be recommended in future investigations. Additionally, the FoxO genes were not identified within the 155 genes DE between all three groups. It is reasonable to speculate given the expression studied however, that their post-translational activation status may well be different, thus, functional analysis of the FoxOs central to the FoxO signalling pathway is also recommended. Biochemical analysis of circulating insulin and glucose levels as well as glucose clearance rates would have also been beneficial for comparison with the mRNA differences observed for genes related to glycolysis and the insulin receptor gene.

There were overlapping conclusions from the three experimental chapters presented in this thesis. The increased rate of glycolytic activity in meat birds compared to layers and cross birds identified in chapter six would support the conclusion of overall increased fatty acid metabolism in chapter five. An increase in lipogenesis could be sustained by an increase in glycolysis (and upregulation of the conversion of pyruvate to acetyl-CoA), as this would result in more available acetyl-CoA for hepatic fatty acid synthesis as well as inclusion into the TCA

cycle. Conversely, the increase in hepatic β-oxidation of fatty acids also found in meat birds would support an overall increase in metabolic activity concluded in chapter six. Although there were no apparent associations with innate immune function with performance detected in chapter three or chapter five, results from chapter six may offer some additional biological insight as to why heavier breeds of poultry are found to be less responsive to immune challenges (van der Most et al., 2011). Fibrinogen alpha chain (*FGA*), fibrinogen gamma chain (*FGB*) and fibrinogen beta chain (*FGG*), were amongst the top ten most abundantly expressed genes, present in the 155 DE genes between the three groups and directionally upregulated layers > cross > meat birds. These genes received little discussion in chapter six as their association with bodyweight and growth differences were not as significant, however, fibrinogen genes are expressed almost exclusively in human hepatocytes by a degree of co-regulation to maintain abundant mRNA levels (Fish and Neerman-Arbez, 2012). Additional to fibrinogen's role in clotting, functions include platelet cross-linking, contribution to blood viscosity, binding surface for proteins in vascular physiology and as an extracellular matrix component (Fish and Neerman-Arbez, 2012). Furthermore, fibrinogen has been significantly implicated in inflammation and immune responses (Davalos and Akassoglou, 2012). The basal differences in mRNA levels of *FGA, FGG* and *FGB* between the three groups of birds may therefore be of interest to researchers who compare immunological responses in heavier breeds of poultry, particularly those in breeds shown to be less responsive to immune challenges.

7.1   Final Conclusions

The results demonstrate that intestinal barrier and innate immune function parameters examined were not associated with individual FCR in meats birds across three separate experiments. Investigation into links between innate immunity and fatty acid metabolism comparing meat birds, layer birds and their F1 cross further supported the conclusion that there was no evidence of basal immune functional differences contributing to growth. Fatty acid metabolism was found to be altered between meat birds, layer birds and their F1 cross, with upregulation of

genes related to both fatty acid synthesis and fatty acid β-oxidation in the meat birds. This would suggest that, unlike fat line birds which commonly exhibit higher rates of lipogenesis than lean line birds, the meat birds studied herein had an overall increase in fatty acid metabolism. Therefore the increased growth and fat deposition were not solely due to increased fatty acid synthesis, but likely due to altered metabolic processes. Furthermore, carcass lipid and blood lipid compositions were different between three groups, despite being fed the same diet, and provided additional evidence of alterations in fatty acid metabolism (carcass composition usually reflects dietary lipid intake in poultry). RNA-sequencing highlighted interesting transcriptional differences, particularly between meat and layer birds. The most significant finding was the repeated enrichment of the FoxO signalling pathway, particularly genes related to cell cycle regulation and the insulin receptor. The results provided significant evidence implicating the FoxO signalling pathway (via cell cycle regulation and altered metabolism) as an active driver of growth variations in chicken. Further functional characterisation and analysis of the FoxO signalling pathway is recommended in future studies investigating variations in growth performance in poultry. Such investigations may uncover biological mechanisms driving performance variations in poultry, particularly meat birds, and enable continued production improvements to maintain poultry as a sustainable and cost effective source of protein.

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