Coccidiosis in modern broiler chickens: targeted nutritional modulations for consequences on bone quality

By

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Abstract

This thesis aimed to assess the consequences of coccidiosis on broiler long bone quality and to explore ameliorative nutritional strategies. The impact of coccidiosis on long bone mineralisation, as well as resistance and tolerance to coccidian infection, was similar in two broiler lines differing in their genetic growth rate (GR) potential. Penalties of infection on bone quality persisted after the impaired GR of infected birds attained a similar level as that of their non-infected counterparts [Chapter 2].

Offering 25-hydroxycholecalciferol (OHD) instead of cholecalciferol (D3) as the source of dietary vitamin D (VitD), and higher VitD levels (4000 vs 1000 IU/kg) improved bone mineralisation and performance of both infected and control birds [Chapter 3]. Although, the performance of infected birds was penalised to a higher degree when offering low VitD, offering OHD or high VitD increased parasite burden, suggesting that dietary VitD supply is crucial for broilers during coccidiosis.

Offering marginally deficient Ca/P diets reduced mineralisation of both control and infected birds [Chapter 4]. Offering OHD instead of D3 at high levels (4000 IU/kg) did not ameliorate effects of either coccidiosis or reduced Ca/P supply, but promoted higher mineralisation in birds offered adequate Ca/P diets. Parasite burden and performance was similar for 4000IU/kg OHD- and D3-fed broilers, suggesting that the benefits of OHD over D3 were limited at 4000 IU/kg.

Chapter 5 investigated the benefit of diet dilution-induced reduction in early GR on coccidiosis-impaired long bone mineralisation. Bone quality, especially femur strength, was improved by reducing GR whilst coccidiosis-impaired bone mineralisation was independent of GR.

Overall, the effects of coccidiosis on bone development were provided; they persisted at later stages of infection and differed amongst femur and tibia bones. Genetic and dietary-induced differences in GR and dietary level of VitD and Ca/P improved responses of both control and infected birds whilst infected birds additionally benefited from a higher VitD supply.

Declaration

This thesis contains my work and has not been submitted for any previous application for a degree. All sources of information have been individually acknowledged by means of referencing.

Idiegberanoise Oikeh

Dedication

I dedicate this thesis to my owner and sustainer, '**The Omniscient God**'
who graciously allowed me to start and complete this PhD study despite all odds

and

To my father, Evang. Merriman O. S. Oikeh (1941 – 2016)
who encouraged me to start this PhD study, but passed on before my graduation

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Publications and Conference abstracts

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Oikeh, I., Sakkas, P., Blake, D.P., Hill, T.R. and Kyriazakis, I. (2017). Effects of Vit D source and Ca and P adequacy in coccidia infected broilers, Proceedings of the 21st European Symposium on Poultry Nutrition, ESPN 2017, Salou/Bila-Seca, Spain. 8th – 11th May.

Sakkas, P., **Oikeh, I.**, Blake, D.P., Hill, T.R. and Kyriazakis, I. (2017). Effects of Vit D source and level of vitamin D supply in coccidia infected broilers, Proceedings of the 21st European Symposium on Poultry Nutrition, ESPN 2017, Salou/Bila-Seca, Spain. 8th – 11th May.

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List of Abbreviations

1,25D3	-	1,25-dihyroxycholecalciferol
A	-	Adequate
ADFI	-	Average Daily Feed Intake
ADG	-	Average Daily Gain
avP	-	Available Phosphorus
BS	-	Breaking Strength
BW	-	Body Weight
C	-	Control
Ca	-	Calcium
CD	-	Crypt Depth
CEAA	-	Conditionally Essential Amino Acid
D3	-	Cholecalciferol
DDB	-	Dry Defatted Bone
EAA	-	Essential Amino Acid
FCR	-	Feed Conversion Ratio
GLM	-	General Linear Model
GRAS	-	Generally Recognised as Safe
H	-	High
I	-	Infected
L	-	Low
M	-	Marginally Deficient
Mg	-	Magnesium
NEAA	-	Non-Essential Amino Acid
NO	-	Nitric Oxide
nPP	-	Non-Phytate Phosphorus
OHD	-	25-hydroxylcholecalciferol
OPG	-	Oocysts Per Gram
OUT	-	Operational Taxonomic Unit
P	-	Phosphorus
P	-	Probability
Pi	-	Post Infection
PP	-	Phytate Phosphorus
qPCR	-	Quantitative real-time Polymerase Chain Reaction

VC	-	Villi height to Crypt Depth Ratio
VH	-	Villi height
Vit	-	Vitamin
YN	-	Nucleotide-rich Yeast Extracts
Zn	-	Zinc
VDR	-	Vitamin D Receptor
BCO	-	Bacterial Chondronecrosis with Osteomyelitis

Chapter 1: Introduction

1.1 Coccidiosis: A perennial problem affecting poultry production

Globally, the poultry industry records a minimum financial loss of £2 billion annually due to coccidia-induced production losses combined with the cost of preventing and treating the infection (Dalloul and Lillehoj, 2006). Williams (1999) revealed that as of 1995 the United Kingdom poultry industry suffered a loss in excess of £38.6 million due to coccidiosis, and because poultry production tripled over the last 20 years (faostat.fao.org) with no definite cure for the disease, the current economic impact of coccidiosis in the United Kingdom alone can be very substantial. Coccidiosis is a parasitic disease caused by *Eimeria* species of the phylum Apicomplexa and family Eimeriidae (Jeurissen and Veldman, 2002). There are over 1200 species of *Eimeria* and chickens are susceptible to at least seven of these species; *Eimeria tenella, E. brunetti, E. necatrix, E. maxima, E. acervulina, E. praecox, E. mitis* (Chapman *et al.*, 2013), which constitute the most challenging problem for poultry production worldwide in terms of economic impact and animal welfare (Sharman *et al.*, 2010; Tewari and Maharana, 2011; Clark *et al.*, 2016). All seven *Eimeria* species are present in all the continents of the world with three additional genetic variants known as operational taxonomic units x, y and z; OTU-x, OTU-y and OTU-z (Morris *et al.*, 2007; Clark *et al.*, 2016). All of these species and OTUs reduce the profitability of commercial chicken farms and threatens the sustainability of the poultry business, especially in Africa (Fornace *et al.*, 2013).

Over the years, chemotherapy via the use of anticoccidial drugs and vaccination have been employed for the prevention and control of coccidiosis in chickens. However, there is growing evidence that these control methods are not sustainable due to a number of shortcomings. Resistance to anticoccidial drugs has emerged, making them ineffective to tackle the disease (Chapman, 1997; Thanner *et al.*, 2016; Lan *et al.*, 2017). The build-up of pathogen resistance, which has negative implications for human health, caused the European Union to place a ban on in-feed antibiotics as growth promoters for farm animals since January 2006, and extended the ban to include anticoccidial ionophores since 2007 (Castanon, 2007; Lillehoj and Lee, 2012; Seal *et al.*, 2013). The United States of America, USA, has also ordered a total removal of all medically important antibiotics from livestock feed since January 2017 (Robinson *et al.*, 2018). Moreover, the possibility of drug toxicity to birds (Abdelrahman, 2014) and public health concerns regarding the accumulation of chemical residues in chicken meat, eggs and by-products has triggered some legislative restrictions on the addition of drugs in chicken feed (McEvoy, 2001; Blake and Tomley, 2014).

On the other hand, using live vaccines to enhance natural immunity of chickens which involves recycling low doses of coccidial oocysts (Chapman *et al.*, 2002) has been relatively successful in some parts of the world (Shirley *et al.*, 2005; Sharman *et al.*, 2010). However, an issue with *Eimeria* vaccines is the lack of cross-protection among different species, and for this reason, most vaccines usually contain at least three *Eimeria* species (Dalloul and Lillehoj, 2006). The inclusion of several species in one vaccine may negatively affect early growth rate, feed conversion and possibly lead to vaccination failure (Chapman *et al.*, 2002; Dalloul and Lillehoj, 2006). Yet another limiting factor is that whilst vaccines help to stimulate immunity, they also have the capacity to induce lesions that predisposes birds to increased intestinal colonisation of bacterial pathogens like *Clostridium perfringens* which causes necrotic enteritis, and *Salmonella enterica* serovars Typhimurium and Enteritidis: notable culprits in zoonotic food-borne diseases (Qin *et al.*, 1996; Blake and Tomley, 2014).

Furthermore, Shaw *et al.* (2012) compared vaccination with the use of in-feed coccidiostat as control methods for coccidiosis and observed significantly impaired body weight (BW) and feed conversion ratio (FCR) for the vaccinated than the in-feed coccidiostat administered broilers over a more extended period, i.e. 0-11d vs 0-20d of age. Hence, there is the need for more effective and sustainable coccidiosis-control strategies; genomics, nutritional modulations and administration of herbal extracts are the focus of current investigations.

1.1.1 Mode of infection and life cycle of coccidia

Coccidia infection in poultry follows a faecal-oral route (illustrated in Figure 1.1) with environmentally resistant *Eimeria* oocysts shed in the excreta of infected birds as the infectious agents (Jeurissen and Veldman, 2002). These oocysts undergo a process of sporogony to form sporozoites contained within sporocysts. When the sporozoite is fully mature, the oocysts are referred to as sporulated and they at this point become infective to chickens. The sporulated oocysts are ingested by uninfected chickens, undergo a process of excystation during which the grinding process of the gizzard breaks the oocyst wall and then the action of pancreatic enzymes and bile salts causes the release of the infective sporozoites. The motile sporozoites then penetrate the intestinal epithelium at specific sites of invasion that are thought to be unique to individual *Eimeria* species (Jeurissen *et al.*, 1996). Furthermore, sporozoites are transformed into trophozoites which undergo merogony to form merozoites. The merozoites develop into male microgametocytes and female macrogametocytes. The macrogametocytes are fertilised by the microgametocytes to form zygotes which form the environmentally resistant oocysts that are shed in the excreta. This process from ingestion to excretion takes 4 to 7 days (Jeurissen and Veldman, 2002).

Although chickens are susceptible to at least seven *Eimeria* species, upon post-mortem examination of dead birds, it was observed that the three dominant *Eimeria* species affecting chickens are *E. tenella*, *E. maxima and E. acervulina* (Cervantes, 2002). *E. acervulina* lesions are usually most prevalent, confined to the upper small intestine and presented in the form of internal white patches or transverse white lines that may be visible from outside the gut. *E. maxima* lesions comprise multiple pin-point size haemorrhages often seen from the outside of the mid-gut area, accompanied by segmental enlargement of the mid-gut with the presence of orange-tainted mucous. *E. tenella* lesions are confined to the caeca and consists of external or internal haemorrhages on the caeca wall, free blood within the caeca, a thickening of caeca wall or the presence of a substantial core of cellular debris and blood (Johnson and Reid, 1970; Cervantes, 2002).

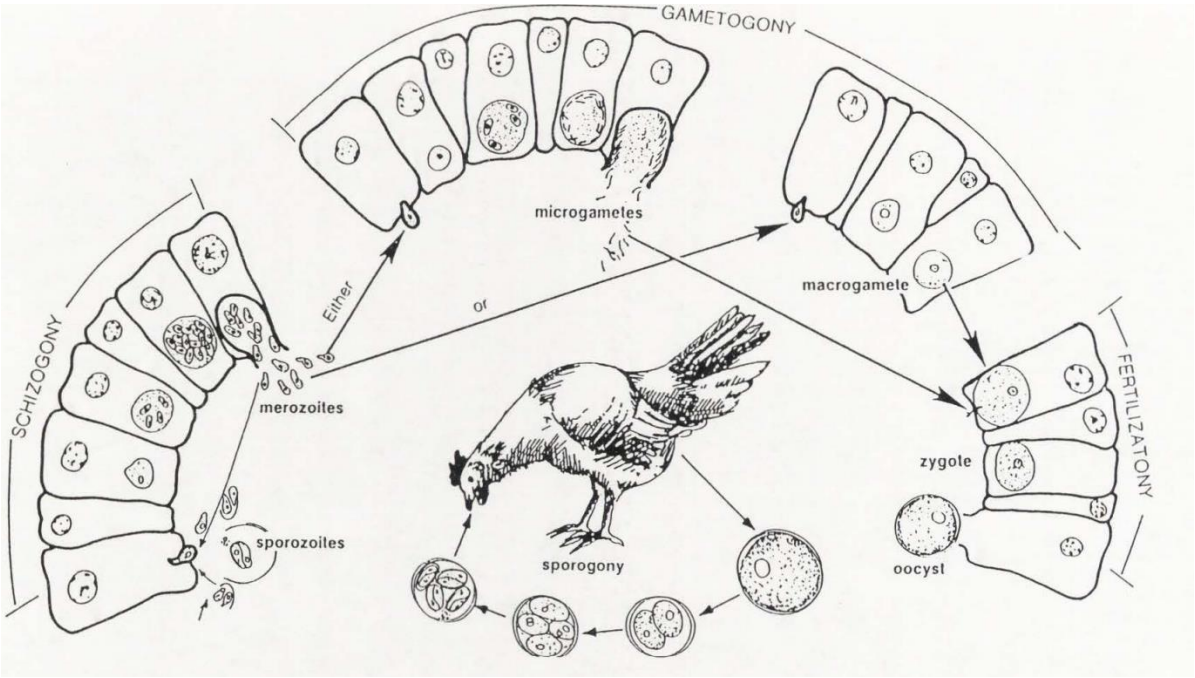

Figure 1. 1 Lifecycle of coccidia (Adapted from Wilson (1995))

1.1.2 Effects on growth performance of broilers

The hallmark of broiler coccidiosis is reduced weight gain (Tyzzer, 1929) which is caused by anorexia and reduced efficiency of feed utilization due to poor digestion, malabsorption of nutrients, changes in metabolism (Adams *et al.*, 1996a) and repartitioning absorbed nutrients away from growth processes towards the functioning of the immune system (Coop and Kyriazakis, 1999; Colditz, 2002). Anorexia has been defined as a reduction in voluntary food

intake, which usually occurs during pathogen infection in animals and humans (Kyriazakis, 2010; Kyriazakis, 2014). It is thought to be mediated by cytokine production in the presence of infection and reduces the amount of nutrients available to the host for vital bodily functions, including the immune response (Kyriazakis, 2014).

Coccidiosis-induced reduction in the weight gain of broilers is *Eimeria* species and dose-dependent (Adams *et al.*, 1996a; Sandberg *et al.*, 2007). A recent meta-analysis on the growth performance of broilers challenged with either *E. acervulina, E. tenella, E. maxima or* a pool of *Eimeria* species suggested that the degree to which performance of broilers is penalised differs amongst challenges with coccidia species and dosage (Kipper *et al.*, 2013). At the same level of feed intake, *E. maxima*-infected broilers recorded the most significant reduction in weight gain and the worst feed conversion ratio followed by *E. tenella* and then *E. acervulina* infected broilers (Kipper *et al.*, 2013). The infection dose of oocysts required to stimulate immune response was also smallest for *E. maxima* compared with *E. acervulina and E. tenella*. It has also been observed that within species, particular strains are more pathogenic than others judging from performance, parasitological and haematological parameters including body weight, feed conversion ratio, lesion scores, oocysts count, haemoglobin content, total erythrocyte count and packed cell volume (Abu-Akkada and Awad, 2012).

Poor absorption of nutrients during coccidian infections impacts negatively on the efficiency of feed utilisation (Preston-Mafham and Sykes, 1967; Preston-Mafham and Sykes, 1970; Jenkins *et al.*, 2008). Although malabsorption occurs in parasitised regions of the small intestine, it has been argued that compensatory absorption from the uninfected areas should help to attain a normal overall absorption of nutrient in the long run (Preston-Mafham and Sykes, 1970; Turk, 1978; Noblet and Turk, 1979). However, broilers challenged with either *Eimeria maxima, E. acervulina, E. brunetti or E. mivati* experienced significantly less nutrient absorption in the intestinal regions of maximum infection, whilst compensatory absorption from the uninfected areas occurred only in *E. acervulina* infected broilers (Ruff and Wilkins, 1980).

Reduced digestibility and utilisation of ingested nutrients including fat and carbohydrate (Preston-Mafham and Sykes, 1970; Turk, 1970), amino acids (Turk, 1972; Ruff, 1974), calcium, zinc and total minerals (Turk and Stephens, 1966; Turk, 1973), vitamin A, vitamin E and xanthophylls (Erasmus *et al.*, 1960; Ruff and Fuller, 1975) are well reported in coccidia-infected broilers. For this reason, dietary modulations of specific nutrients as a means of ameliorating coccidiosis has been explored in several studies: it also forms the central focus

of this thesis. Nutrient malabsorption during coccidia infections is thought to arise from increased digesta viscosity (Waldenstedt *et al.*, 2000), gastrointestinal tract villous atrophy causing reduction in the ratio of villous height to mucosal thickness (Pout, 1967; Preston-Mafham and Sykes, 1967), and reduced activities of digestive enzymes (Adams *et al.*, 1996a). It also arises from increased intestinal acidity which probably lengthens the gut passage time, intestinal leakage of plasma proteins which explains the higher concentration of uric acid in excreta of infected birds (Adams *et al.*, 1996a; Waldenstedt *et al.*, 2000; Williams, 2005b), and also from downregulation of genes encoding amino acid transporters in the intestine of infected birds (Fetterer *et al.*, 2014).

Set aside reduced nutrient uptake resulting from reduced absorption and consumption, nutrient requirements of broilers are altered by activation of the immune system (Adams *et al.*, 1996a; Adams *et al.*, 1996b), which could impact negatively on performance. Lymphocyte proliferation, gene expression and production of proteinaceous molecules such as acute phase proteins (Chamanza *et al.*, 1999), antibodies, cytokines and other cytotoxic substances increase amino acid requirements during coccidia infections (Yun *et al.*, 2000; Li *et al.*, 2007). Similarly, alteration in vitamin requirements including vitamins A, D, E, C, the B-vitamins, and minerals (i.e. trace metals) such as Zu, Mu, Cu and Se suggest that increased supplementation will improve immunity (Kidd, 2004), and consequently performance. Also, *E. acervulina* infection has been shown to cause depletion in ascorbic acid concentration in blood plasma, small intestine, liver and the adrenal glands of parasitised birds (Kechik and Sykes, 1979).

The significant reduction in feed intake and weight gain of coccidiosis-infected birds, which is accompanied by severe mucosa damage and the shedding of oocysts, may also be induced by free radicals or their products (Allen, 1997b; Costantini and Møller, 2009). Oxidative stress arising from an imbalance (favouring production) between the production and elimination of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion and nitric oxide in cells, tissues and physiological fluids, has been associated with coccidia infections in birds (Sepp *et al.*, 2012). It was observed that the levels of plasma and mucosa NO metabolites increased reaching peak values on day six post-infection whilst the activities of NADPH oxidase also increased. Furthermore, the high susceptibility of carotenoids to oxidation by free radicals has made plasma carotenoids concentration (mainly lutein and zeaxanthin) a useful marker for both oxidative stress (Allen *et al.*, 1996b) and nutrient absorption (Allen, 1997b) during coccidia infections. All the effects

highlighted above jointly contribute to the impairment of growth performance in coccidiosis-infected broilers.

1.1.3 Effects on bone quality of broilers

Generally, bone-related consequences associated with broiler coccidiosis have not been well researched. Given that coccidiosis penalises digestion, absorption or utilisation of vital bone minerals (Turk and Stephens, 1966; Turk, 1973), penalties on bone mineralisation are to be expected. Nevertheless, there is a scarcity of studies in the scientific literature with a detailed examination of the bone quality of coccidiosis-infected broilers measured across time points. A few studies approached the subject of bone quality indirectly, e.g. as a support marker for other research interests such as the effect of phytase when offering mineral-deficient diets to coccidiosis-infected broilers (Watson *et al.*, 2005; Walk *et al.*, 2011; Shaw *et al.*, 2012). Therefore, this thesis incorporated a more direct approach to provide specific details including the point at which bone strength and mineralisation are affected post-coccidia infection in modern broilers. Related studies available in the bone literature suggested that for day-old broilers, mild infections with *E. acervulina, E. maxima, E. mivatti* and *E. tenella* live oocysts may not affect tibia ash percentage (Lehman, 2011; Walk *et al.*, 2011), but significantly penalise tibia breaking strength (Shaw *et al.*, 2011) at d21 of age. Another study (Giraldo *et al.*, 1987) reported that coccidiosis might cause a reduction in bone Ca and an increase in bone P concentrations when offering to broilers a high dietary Mg from oxide or sulfate source. Nevertheless, these studies are not devoid of sources of experimental error, which in this context is the fact that the diets offered could influence bone quality.

1.1.4 Immune response to coccidia infection

The immune response to coccidia infection in chickens is species-specific and most potent at the intestinal site of infection of coccidia species (Cornelissen *et al.*, 2009). The gut associated lymphoid tissues (GALT) comprising the bursa of Fabricius, cecal tonsils, Peyer's patch, Meckel's diverticulum and lymphocyte aggregates scattered along the intra-epithelium and lamina propria of the gastrointestinal tract play the role of processing and presentation of antigens, production of intestinal antibodies and the activation of cell mediated immunity (Lillehoj and Trout, 1996; Yun *et al.*, 2000).

A wide range of immune effector mechanisms have been described for several *Eimeria* species primary infections, which mediate protection to a variable degree, including upregulation of cytokine and chemokine production, heterophil infiltration in the gut, NK cell activation, B cell proliferation and antibody production, macrophage and T cell activation

(Lillehoj, 1998; Yun *et al.*, 2000; Dalloul *et al.*, 2007; Wallach, 2010). The profile of the effector mechanisms employed depends on the stage of coccidia development, prior exposure to coccidia, the dose of infecting oocysts, the nutrient status of infected birds, and their genetic make-up (Lillehoj, 1986; Yun *et al.*, 2000).

Overall, the three most common *Eimeria* species induce both $CD4^+$ and $CD8^+$ T-cells, as well as macrophage, and a wide range of cytokine responses (Lillehoj and Li, 2004). Cytokine mediated T-cell immunity (CMI) in particular is crucial for *Eimeria* control, with $CD4^+$ T-helper and $CD8^+$ cytotoxic T lymphocytes being involved in host responses (Hong *et al.*, 2006a; Chapman *et al.*, 2013). The exact mechanisms mediated by various T lymphocyte subpopulations in host protection against avian coccidiosis remain to be determined. However, there is a consensus that T-cells are essential mediators of host immunity, both in primary and challenge coccidian infections (Lillehoj and Lee, 2012), whilst antibody mediated immunity plays a role which is of far less significance in comparison to CMI (Lillehoj, 1986; Rose, 1987). It is generally believed that $CD4^+$ T cells initiate an immune response, and $CD8^+$ T cells are known to bring about effector responses in coccidian infections (Shivaramaiah *et al.*, 2014).

Immunological response of infected host classically follows two pathways. T-helper 1 (Th1) immune response mediates protective cellular immunity against intracellular infection such as coccidiosis, whilst the Th2 humoral immune responses control extracellular pathogens (Min *et al.*, 2013). Th1 / Th2 balance is critical for protective host response against intracellular infections such as coccidiosis (Cornelissen *et al.*, 2009; Haritova and Stanilova, 2012). The cytokine environment drives the differentiation of these subsets with IFN-γ promoting Th1 and inhibiting Th2 cell production, and IL-4 and IL-10 inhibiting Th1 cell production and IL-4 driving Th2 cell production (Min *et al.*, 2013). The profile of produced cytokines, as well as the ratio of CD4+ and CD8+ T-cells, differs among coccidian species in their respective site of infection (Cornelissen *et al.*, 2009). *E. acervulina* and *E. tenella* are shown to induce Th1 (IL-2, IL-8 and IFN-γ) and Th2 (IL-4, IL-10) responses in the duodenum and caecum respectively, whereas *E. maxima* induced a strong Th1 biased duodenal and jejunal response (Cornelissen *et al.*, 2009). Given that the changes in T cell subpopulations are species and host-breed specific, local changes in T cell subpopulation induced by *Eimeria* infection may reveal the level of susceptibility to coccidiosis in different strains of chickens (Lillehoj, 1994; Bessay *et al.*, 1996).

Cellular immune response to parasite invasion is associated with the production of free radical species such as nitric oxide (NO) (Allen, 1997b). Activated macrophages produce NO during *Eimeria* infection, and the level of production can be estimated by analysing its stable metabolites (NO_2^- + NO_3^-) in the plasma and intestinal mucosa (Allen and Lillehoj, 1998). Although the virulence of an *Eimeria* strain is unrelated to NO production (Allen, 1997b), it has been shown that resistant broiler lines produce higher levels of plasma NO_2^- + NO_3^- in response to primary *Eimeria* infections compared to susceptible lines (Allen and Lillehoj, 1998). However, it is yet unknown whether the immune response to coccidiosis is affected by selection for growth potentials, which constitutes a gap in the scientific literature.

1.2 Nutritional interventions relevant to broiler coccidiosis

The possibility of nutritional immunomodulation in broilers has increased the potentials of dietary interventions as a candidate for sustainable coccidiosis control given the earlier stated problems associated with reliance on in-feed drugs or live vaccines. In a recent review, Robinson *et al.* (2018) highlighted nutritional modulations involving butyrate- and VitD-enhanced host defence peptides (HDPs; also called antimicrobial peptides (AMPs)), synthesis as a possible alternative to the use of in-feed antibiotics in livestock systems (Robinson *et al.*, 2018). This followed a previous study on broilers (Wen and He, 2012) and a review on swine (Xiao *et al.*, 2015), which suggested a link between enhanced growth and gut health, and HDP-supplemented diets especially over the course of a disease. Butyrate, a short-chain fatty acid (SCFA), and fat-soluble VitD were identified as the most efficacious compounds with the ability to promote HDP synthesis (Robinson *et al.*, 2018), whilst the immunomodulatory, antimicrobial and barrier-protective activities of HDPs (Xiao *et al.*, 2015) were implicated as the cause of the enhanced growth and gut health (Robinson *et al.*, 2018). Also, the ability of medium-chain fatty acids (MCFA), caprylic and nonanoic, to enhance intestinal immunological barrier functions through the combined effects of HDP upregulation and histone deacetylase (HDAC) inhibition was recently suggested (Wang *et al.*, 2018).

The nutritional requirements for immunity differ from those for growth or skeletal tissue accretion (Kidd, 2004). Hence, several nutritional interventions expected to boost immunity and ameliorate the consequences of coccidia infections have been investigated (Jeurissen and Veldman, 2002; Williams, 2005b; Abdelrahman, 2014). The manipulation of dietary inclusion levels of protein and amino acids, fatty acids, dietary fibre, vitamins and minerals as well as the inclusion of enzymes, probiotics, plant extracts and other additives have been shown to attenuate some effects of *Eimeria* infections to a variable degree (Jeurissen and Veldman,

2002; Williams, 2005b; Abdelrahman, 2014). The subsections below touch briefly on nutrients that can be supplemented to improve coccidiosis-impaired performance and bone quality, as well as resistance, tolerance, or both during coccidiosis in birds.

1.2.1 Proteins and Amino acids (AA)

Wu (2013b) classified amino acid for poultry into three; 1) essential (EAA), e.g. arginine, cysteine, glycine, histidine, proline, isoleucine, leucine, methionine lysine, phenylalanine, threonine, tryptophan, tyrosine and valine; 2) non essential (NEAA), e.g. alanine, asparagine, aspartate, and serine, and 3) conditionally essential (CEAA), e.g. glutamate, glutamine and taurine. This classification was based on species, developmental state, physiological status, intestinal lumen microbiota, environmental factors and pathological state. Furthermore, the growing recognition that arginine, cysteine, glutamine, leucine, proline and tryptophan regulate vital metabolic pathways relevant to growth, maintenance, reproduction and immunity, has earned them the name, functional AA (Wu, 2009).

Maintenance of body protein involving the reversal of pathophysiological effects of parasitism on the host has been shown to gain priority over body functions like growth and reproduction in the allocation of nutrient resources (Coop and Kyriazakis, 1999). Sandberg *et al.* (2007) suggested that there is a significant, but variable increase in requirements for protein and specific amino acids during pathogenic infection which is caused by innate immune functions, repair of damaged tissue and expression of acquired immunity. Lee *et al.* (2011) reported that increased protein concentration in starter diets led to an improvement in body weight, feed conversion and overall performance of broilers during a vaccination program to prevent coccidiosis. Indeed, combining anticoccidial vaccines with an appropriate starter diet containing increased protein levels may result in a similar performance for broilers receiving either immunoprophylaxis or coccidiostat as control measures for coccidiosis (Lee *et al.*, 2009).

In two separate studies, increasing the concentration of dietary supplementation levels of arginine (Tan *et al.*, 2014) and threonine (Wils-Plotz *et al.*, 2013) in coccidia-infected broilers helped to attenuate intestinal mucosal disruption and altered the immune response. Arginine plays an essential role in the proper development of lymphoid organs especially the thymus and spleen (Kwak *et al.*, 1999); increased concentration of arginine may, therefore, help to optimise immune response or disease resistance in coccidia-infected broilers (Jahanian, 2009). Furthermore, it was proposed that increasing dietary arginine probably attenuate intestinal mucosal disruption by decreasing Toll-like receptor 4 (TLR4) mRNA expression and

activating mechanistic target of rapamycin (mTOR) complex 1 pathways (Tan *et al.*, 2014). Serine, cysteine and especially threonine are vital components of mucin, an essential constituent of the mucus layer (Montagne *et al.*, 2004). Dietary supplementation of threonine to alter the dynamics of mucin and hence influence the integrity of the mucous layer and nutrient absorption (Horn *et al.*, 2009) can be explored to improve the health of coccidia-infected birds. Wils-Plotz *et al.* (2013) reported an improved intestinal immune response at higher levels of dietary threonine supply, which also helped to sustain normal growth in coccidia-infected broilers (Wils-Plotz *et al.*, 2013).

However, information on the dietary modulation of other amino acids including asparagine, glycine, lysine, histidine, proline, tryptophan and tyrosine during coccidian infections is scarce. These AAs may play vital roles in immunity; for instance, proline and cysteine are known to have pathogen killing and antioxidant functions (Wu, 2013a), which can be explored during coccidian infections. Increased levels of sulfur amino acids (methionine and cysteine) (Takahashi *et al.*, 1997; Swain and Johri, 2000), arginine (Tan *et al.*, 2014) and threonine (Wils-Plotz *et al.*, 2013) in broiler diets were also reported to improve cellular immune response and ameliorate the consequences of pathogen infections including coccidian infections (Wils-Plotz *et al.*, 2013; Tan *et al.*, 2014).

Bortoluzzi *et al.* (2017) in their review of recent studies, confirmed the potentials of increased dietary AAs to reduce mucosa atrophy, maintain microbiota balance and stimulate local immune response during coccidia infections. The review also highlighted the role played by threonine, arginine and glutamine towards mucin production, immune function and epithelial proliferation respectively, and towards the overall proper functioning of the intestinal tract (Bortoluzzi *et al.*, 2017). However, it remained unclear whether administering AAs to improve gut-healing capacity or to reprogramme intestinal mucosa before infection, which would be more effective in the context of coccidiosis.

1.2.2 Fatty acids

A major distinguishing factor between the lymphoid cell and other tissues is the high level of fatty acids and sterols in the membranes of lymphoid cells (Kigoshi and Ito, 1973). It is therefore perceived that metabolism of lipid and fatty acid in lymphoid cells may influence physiological activities of the cells, which may have an impact on the immune status of animals (Gross and Newberne, 1980). Unlike saturated and monounsaturated fatty acids (Gross and Newberne, 1980), polyunsaturated fatty acids (PUFAs) such as arachidonic acid (C20:4 n-6), eicosapentaenoic acid (C20:5 n-3) and docosahexaenoic acid (C22:6 n-3) are

known to have immunomodulating effects. This is associated with mechanisms involving the reduced synthesis of pro-inflammatory cytokines and decreased antigen-presenting cell activity with a concomitant increase in feed intake and weight gain (Selvaraj and Cherian, 2004). Diets rich in n-3 PUFAs increase growth performance and antibody mediated responses, while n-6 PUFAs increase cell mediated responses (Selvaraj and Cherian, 2004). However, a proper balance between n-6 and n-3 PUFAs is required, as a high n-6: n-3 ratio will result in cytokine production (Simopoulos, 2002) leading to a reduction in food intake (Klasing, 1998; Ferket and Gernat, 2006).

In coccidia-infected broilers, dietary n-3 PUFAs supplementation has been shown to reduce lesion scores from acute *E. tenella* infections (cecal or haemorrhagic coccidiosis) but this was not the case with *E. maxima* infections (intestinal or malabsorptive coccidiosis) (Allen *et al.*, 1996a; Allen *et al.*, 1997; Allen and Danforth, 1998). Although some researchers found no impact of dietary PUFAs supplementation on immunity and resistance to infections (Puthpongsiriporn and Scheideler, 2005), there is sufficient evidence to support the ability of fatty acids to modulate intestinal immune responses and cytokine secretion (Fritsche *et al.*, 1991; Yang and Guo, 2006; Robinson *et al.*, 2018). For example, butyrate supplemented in broiler diets as sodium butyrate (1 g/kg) or butyrate glycerides (4 g/kg) improved performance while suppressing IL-1β, IL-6, IL-10 and IFN-γ cytokines induction by lipopolysaccharides (Zhang *et al.*, 2011; Zhou *et al.*, 2014). Also, 0.2% butyric acid supplementation maintained performance and carcass quality of vaccinated broilers challenged with coccidiosis (Leeson *et al.*, 2005). Furthermore, a recent study reported the ability of 750 mg/kg dietary supplementation with coated sodium butyrate (30% sodium butyrate, coated with palm stearin) to balance the shift in caecal microbial composition, i.e. increase in firmicutes and proteobacteria, and decrease in bacteroidetes, caused by *E. tenella* infection in broilers (Zhou *et al.*, 2017). Indeed, the above findings suggest that fatty acids nutrition holds excellent potentials for coccidiosis-infected broilers.

1.2.3 Vitamins: Focus on VitD

The antioxidant, membrane stabilising and immunomodulating properties of some vitamins can be explored to boost resistance or tolerance to coccidian infections, or both, in broilers (Wunderlich *et al.*, 2014). Research has shown that deficiency of fat-soluble vitamins A (Dalloul *et al.*, 2002), D (Aslam *et al.*, 1998) and E (Erf *et al.*, 1998) in chicken diets resulted in depressed cellular, humoral and innate immunity. Although dietary supplementation of fat-soluble vitamins may boost resistance to coccidian infection (Colnago *et al.*, 1984), there are some noteworthy antagonistic tendencies. For instance, diets with excess Vit A may reduce

Vit E efficacy (Kidd, 2004), and antagonisms between Vit A and Vit D are known to exist (Mora *et al.*, 2008). Vitamins A (Dalloul *et al.*, 2002), E (Allen and Fetterer, 2002b) and C (Kechik and Sykes, 1979) have been supplemented at varying levels to ameliorate the consequences of *Eimeria* infections in chickens. Also, dietary supplementation with β-carotene, a major constituent of Vit A, may induce antioxidant functions that help to maintain mucosal integrity as well as functions in both humoral and cell-mediated immune response: its deficiency increased susceptibility to *E. acervulina* infection via impaired immune response and increased oocysts production (Chew, 1995). Dalloul *et al.* (2002) reported that changes in the duodenal intraepithelial lymphocytes (IEL) subpopulations of Vit A-deficient broilers compromised local immune defence during *E. acervulina* infection and caused increased oocyst shedding.

Several studies supplemented Vit E based on its antioxidant functions protecting against free radical oxidative processes in cellular membranes and also its ability to function as an immunomodulator in chickens (Erf *et al.*, 1998; Allen and Fetterer, 2002b). On the other hand, there are conflicting results from studies manipulating supplementation levels of α-tocopherol, the most active compound in Vit E, which may be dependent on factors related to vitamin dose, and age and genetics of birds (Khan *et al.*, 2012). At 316 mg/kg inclusion level, dietary Vit E supplementation decreased intestinal lesion, oocysts output, and improved cellular defence system without affecting plasma antioxidant status in *E. tenella*-infected broilers (Jafari *et al.*, 2012b). However, further increments had adverse effects on plasma antioxidant status and also recorded higher oocysts output (Jafari *et al.*, 2012b). Furthermore, dietary supplementation with Vit E plus Selenium (Se), a component of glutathione peroxidase aiding antioxidant protection of cells, reduced cecal lesions and mortality during *E. tenella* infection in chickens (Colnago *et al.*, 1984). In broilers infected with *E. maxima*, dietary supplementation with DL-α-tocopherol acetate caused a moderate reduction in lesion score severity and plasma carotenoid concentration, and a slight increase in plasma NO metabolites concentrations. However, there was no effect on weight gain whilst it increased oocyst output at higher inclusion levels (Allen and Fetterer, 2002b). About Vit C, Kechik and Sykes (1979) found that ascorbic acid concentration in blood plasma and intestinal tissue of *E. acervulina* infected birds was depleted and dietary supplementation of 1000 mg/kg prevented this depletion. Dietary ascorbic acid supplementation also had minimal effects on cellular immunity of broilers (Murray *et al.*, 1988).

The role of VitD as a potent regulator of immune responses has gained wider publicity in the last decade (Baeke *et al.*, 2010; Rodriguez-Lecompte *et al.*, 2016; Robinson *et al.*, 2018). No

previous research has explored the benefits of VitD immunomodulatory potentials for malabsorptive coccidiosis-infected broilers in relation to bone quality. Fat-soluble VitD is a steroid hormone essential for the regulation of systemic calcium, phosphorus and bone metabolism (DeLuca, 2008; Bikle, 2011). It occurs either as cholecalciferol (D3) or ergocalciferol (D2) but the latter is not effectively utilised by broilers (Chen and Bosmann, 1964; Hay and Watson, 1977). Hence, D3 or its metabolite, 25-hydroxycholecalciferol (OHD) is supplied in broiler diets. Other D3 metabolites such as 1,25-dihydroxycholecalciferol (1,25D3) (Rennie *et al.*, 1993), and those with hydroxyl group in 1-C position (Honma *et al.*, 1983; Edwards, 1990), do have higher capacity to mediate VitD activities compared to D3 and OHD (Edwards, 1989; Morris *et al.*, 2015). However, caution is applied in supplementing these in broiler diets for risk of toxicity if given in excess (Jones, 2008). A review of recent studies suggested that a dietary VitD supply level of 3000 IU/kg meets the requirement for healthy bone development, maximum mineral digestibility, egg shell quality, immunity and performance indices of the modern-day high-producing chickens (Swiatkiewicz *et al.*, 2017).

Like other sterols, VitD3 absorption is fat dependent. The process is optimised by prior solubilisation as a micelle containing bile salt (Thompson, 1971; Maislos *et al.*, 1981; Vaziri *et al.*, 1983), uptake from the intestinal lumen and transported from the gut wall mainly to the mesenteric lymph (Schachter *et al.*, 1964). Contrariwise, absorption of D3 metabolites, OHD or 1,25D3, is less fat dependent (Maislos *et al.*, 1981). In rats, OHD and 1,25D3 absorption rates were reported to be approximately 1.5 and 3 times faster than D3 respectively (Maislos *et al.*, 1981), which could suggest that they are the preferred form of VitD under malabsorptive conditions like coccidiosis in broilers. The fat independent absorption of OHD metabolite has been scientifically proven as oral administration of OHD was successfully employed to treat osteomalacia in humans suffering from steatorrhea (Wagonfeld *et al.*, 1976; Compston and Thompson, 1977; Reed *et al.*, 1980).

VitD metabolism is such that absorbed D3 undergoes further hydroxylation into 25-hydroxycholecalciferol in the liver and then to 1,25-dihydroxycholecalciferol mainly in the kidney, to become biologically active (White and Cooke, 2000; Lips, 2006; DeLuca, 2008; Haussler *et al.*, 2013). Hydroxylation of OHD to the active 1,25D3 also occurs in the small intestine, breast and thigh muscles, bone, and immune cells (Shanmugasundaram and Selvaraj, 2012). Renal 1,25D3 production is stimulated by parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and decreased by calcium, phosphate and 1,25D3 (Bikle, 2011), whilst cytokines such as IFN-γ and TNF-α may stimulate extrarenal 1,25D3 production, and depending on tissue, less affected by Ca, P and 1,25D3 (Lips, 2006; Bikle,

2009). Key functions of 1,25D3 include regulation of calcium (Ca) and phosphorus (P) homeostasis (Bienaimé *et al.*, 2011), stimulating osteoclast differentiation and calcium reabsorption from the bone, and promoting mineralisation of the bone matrix (Holick, 2004; St-Arnaud, 2008; Bikle, 2012; Haussler *et al.*, 2013). VitD also improves performance (Fritts and Waldroup, 2003; Whitehead *et al.*, 2004), integrity of intestinal mucosa barrier (Kong *et al.*, 2008), small intestine morphology in chicks (Ding *et al.*, 2011), and helps recovery during mucosal injury (Zhao *et al.*, 2012) in broilers.

VitD effects associated with growth and disease prevention are generally under-studied in poultry and other livestock. Specific physiological functions of VitD in humans suggested its immunomodulatory potentials in chickens. VitD exerts anti-inflammatory actions by causing a reduction in pro-inflammatory cytokine, i.e. IL-1β, IL-6, IFN-γ, IL-8 and TNF-α expression post-pathogenic challenge in humans (Di Rosa *et al.*, 2012; Zhang *et al.*, 2012). Also, in humans, VitD promoted macrophage activation and phagocytosis, whilst facilitating monocyte differentiation towards macrophages instead of dendritic cells (Zhu *et al.*, 2002; Griffin *et al.*, 2003). Recent studies utilising broiler chickens reported a VitD dose-dependent CATH-1 and CATH-1β induction in the spleen that was further enhanced by a deficiency in dietary Ca and P levels, whilst CATH-3 was suppressed (Rodriguez-Lecompte *et al.*, 2016). VitD3-enhanced cathelicidins were not observed in peripheral blood mononuclear cells (PBMCs, e.g. monocytes, lymphocytes and macrophages) at 2760 or 9800 IU/kg dietary inclusion levels. However, at 9800 IU/kg VitD3 supplementation, only CATH-1 was significantly induced in the bursa of Fabricius in broilers (Rodriguez-Lecompte *et al.*, 2016).

Furthermore, *in-vitro* studies using chicken embryo intestinal epithelial cells (CEIEPCs) and PBMCs (Zhang *et al.*, 2016), and macrophages (Shojadoost *et al.*, 2015) reported the capability of 1,25D3 to induce host innate immunity in chickens. 1,25D3 induced Avian β-defensin (AvBD) expression (Zhang *et al.*, 2016), as well as increased in macrophages the capacity to respond to stimuli and produce NO (Shojadoost *et al.*, 2015). However, the immunological effects of 1,25D3 in broilers can also be influenced by dietary Ca and P adequacy (Rodriguez-Lecompte *et al.*, 2016). High dietary VitD3, supplied at 9800 IU/kg, combined with low dietary Ca and P led to a higher Th1 (IL-12, IL-18 and IFN-γ) and Th2 (IL-4, IL-10 and IL-13) cytokines expressions in the spleen and bursa of Fabricius, as well as a higher CxCLi2 (formerly called IL-8) chemokine in the spleen (Rodriguez-Lecompte *et al.*, 2016). Rodriguez-Lecompte *et al.* (2016) suggested that although cytokine expression was enhanced in the spleen, the IL-12: IL-10 ratio favoured an anti-inflammatory status. They concluded that D3 and OHD both have robust immunomodulatory potentials with a more

favourable Th2 response, and the ability to enhance Th2 cytokine responses under both adequate and marginally deficient Ca and P inclusion levels in the diets of broiler chickens (Rodriguez-Lecompte *et al.*, 2016).

1.3 Interactions between broiler genetics and coccidiosis

The application of genetic control strategies for reducing the risk and severity of diseases in livestock populations has gained considerable attention in recent years (Doeschl-Wilson *et al.*, 2012), with emphasis on host resistance or host tolerance, or both (Swaggerty *et al.*, 2015). Host resistance is the mechanisms that restrict the entry of pathogens or prevent the replication of pathogens within the host or both. Tolerance is the ability of the host to limit the detrimental effect of pathogens on its performance without necessarily affecting parasite burden (Doeschl-Wilson and Kyriazakis, 2012). However, it is unclear whether resistance and tolerance have a similar impact on individual host fitness and performance as well as on population performance and disease risk and severity (Doeschl-Wilson *et al.*, 2012).

Genetic background, among other factors, is thought to influence susceptibility to coccidia infections in broilers (Long, 1968; Johnson and Edgar, 1986; Lillehoj, 1986). According to the resource allocation theory, when an organism is offered a finite resource such as nutrients during pathogen infections, selection for improved growth performance may cause an increase in allocation of the resource towards growth-related traits at the expense of immune functions and ultimately penalise its resistance and tolerance to disease (Rauw *et al.*, 1998; Coop and Kyriazakis, 1999). Indeed, the unprecedented selection for high production parameters over the years, which lead to approximately 300% increase in daily weight gain between broilers in the 1950s and modern broilers (Knowles *et al.*, 2008), may have resulted in penalised immune functions and increased susceptibility to infectious diseases by reducing resistance (Yunis *et al.*, 2000; Havenstein *et al.*, 2003).

Concerning coccidia-infected broilers, a recent meta-analysis demonstrated that pure broilers lines are less tolerant to coccidia infections when compared with cross-bred broilers (Kipper *et al.*, 2013), which may relate to the higher growth rates of the former in comparison to the later. Previous studies show that coccidia infection in broilers cause a reduction in the level of plasma carotenoids which may be breed dependent: breeds selected for resistance to diseases compared to susceptible breeds experience lesser plasma carotenoid reductions during coccidiosis (Lillehoj and Ruff, 1987). However, no study has examined the degree to which selection for performance or growth potentials penalises the ability of broilers to cope with coccidian infections; dissecting the impact on various aspects of performance. An experiment

involving different infection doses and genotypes differing only in their growth rate will be required to achieve this.

1.4 Skeletal development in modern broilers

Chicken long bone formation starts *in-ovo* with the prechondral or membranous process, which involves the laying down of a hyaline cartilage model of the appendicular skeleton (Gilbert, 1997). After that, the endochondral ossification process, which is responsible for converting the hyaline cartilage model to bone begins *in-ovo* but essentially occurs post-hatch (Bain and Watkins, 1993; Dibner *et al.*, 2007). Long bones grow in length by replacing new hyaline cartilage formed at the epiphyseal plate during the endochondral ossification process (Marks and Popoff, 1988). Bone mineralisation is restricted to the epiphyseal plate, and significant pathology such as osteopetrosis or osteoporosis may occur in the event of excessive and insufficient mineralisation (Dibner *et al.*, 2007). In modern broilers, bone formation and mineralisation are at their peak between 4 – 18 and 4 – 11 days of age, respectively (Williams *et al.*, 2000). The process of long bone formation is illustrated in Figure 1.2.

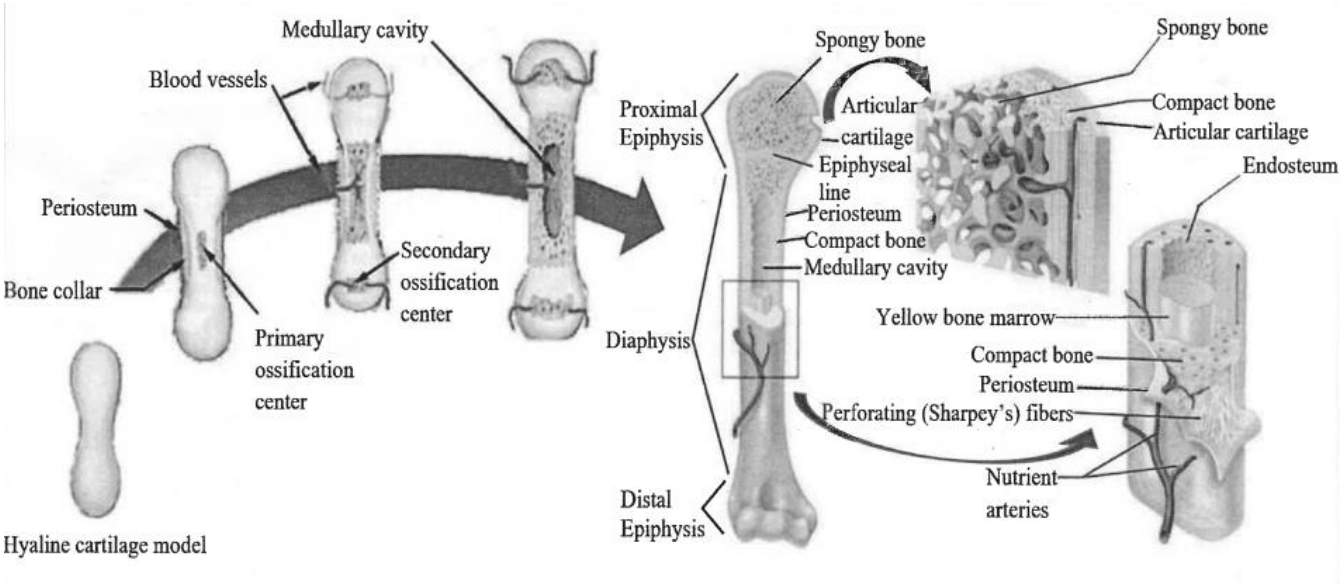

Figure 1. 2 Long bone growth process from hyaline cartilage model to bone (produced from 2 internet images https://s3.amazonaws.com/static.wd7.us/c/c0/Illu_bone_growth.jpg and http://www.knowyourbody.net/wp-content/uploads/2017/09/Endosteum-Image.png)

Generally, bone formation in all vertebrates is such that successive layers of bone are laid down to form dense, compact bone covered outwardly by the cellular periosteum. Long bones are hollow and filled with an extension of the air sacs, and bone marrow, whilst compact bone is modified to a concentric structure called the Haversian system. It contains Haversian canals that run parallel to the axis and carries blood vessels and nerve fibres. Plates of bone surround the Haversian canal and small spaces, lacunae, abound between the plates carrying bone cell called osteocyte. Nutrients from blood vessels reach the bone via the Haversian canal and small canals that are known as canaliculi (Maggiano *et al.*, 2016).

In modern fast-growing commercial broilers, it is not unlikely that closure of the epiphyseal plate may not occur before they are sold off having attained the required market weight (Rath *et al.*, 2000). This means that the axial bone could remain under development throughout its lifetime, never reaching the state of homeostasis and remodelling as seen in other adult vertebrates (Rath *et al.*, 2000; Roberson *et al.*, 2005). The implication is that healthy bone growth in modern broilers can be affected at any point by infectious conditions (Mireles *et al.*, 2005), as well as nutritional, environmental and developmental amongst other conditions (Dibner *et al.*, 2007). Figure 1.3, adapted from the recent study of Kierończyk *et al.* (2017), highlights selected factors that may affect leg bone development resulting in lameness of broilers. The rapid growth rate of modern broilers is responsible for rapid periosteal bone deposition, impaired mineralisation, increased cortical bone porosity and altered biomechanical properties (Williams *et al.*, 2004; Knowles *et al.*, 2008). The frequently affected areas in fast-growing broilers include the joints, tendons, structural bone and the connective tissues of the feet and leg (Dibner *et al.*, 2007).

1.4.1 Long bone mineralisation and coccidiosis in modern broilers

Broiler long bones are mainly the leg bones, i.e. femur, tibia and fibular, and then their corresponding arm bones, i.e. humerus, radius and ulna. Generally, bones are approximately 70% mineral, 20% organic and 10% water (Rath *et al.*, 2000). The mineral content which gives stiffness and compressional strength to the bone are basically Ca and P in the form of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (Turek, 1984). The organic content is collagen, which gives tensile strength (Knott and Bailey, 1998), and the collagen crosslink content is indicative of bone maturity (Boskey *et al.*, 1999). The bone is more or less the primary reservoir for Ca and P as it stores approximately 98% and 80% respectively of these minerals in the body (Rath *et al.*, 2000). Other essential minerals in bone structure that can be drawn upon during inadequate supplies include sodium, potassium and magnesium (Reid and New, 1997).

Dietary Ca and P inclusion levels, as well as ratio, is crucial for their retention and availability for utilisation in chickens (Rao *et al.*, 2006; Delezie *et al.*, 2012). Aviagen (2014a) set 0.96 and 0.48% for d0 – 10 of age, and 0.88 and 0.44% for d11 – 24 of age respectively as the dietary inclusion levels of Ca and nonphytate P (nPP or avP) required to optimise bone mineralisation in their popular fast-growing broiler breed, Ross 308. In like manner, narrow Ca:P ratios between 1.8 to 2 : 1 were recommended for optimum performance and bone development (Liu *et al.*, 2017), as well as for reduced occurrence of tibial dyschondroplasia (Edwards and Veltmann, 1983), and reduced faecal P (Rao *et al.*, 2006) in broilers. There is also an inter-relationship between Ca and P metabolism such that either excess or deficiency of one interferes with utilisation and metabolism of the other (Kebreab and Vitti, 2005).

VitD plays a critical role in facilitating Ca and P metabolism and has been used to augment deficiencies in dietary Ca and P intake in broilers. (Rao *et al.*, 2006; Delezie *et al.*, 2012). Availability of P from conventional feedstuff like cereals and oilseed by-products is usually impaired because it occurs mainly in phytate form, which requires further breaking down (Dilger *et al.*, 2004). Both dietary phytase and VitD supplementations can optimise the availability and utilisation of P from dietary sources. They also do have immunomodulatory potential that may be explored over the course of coccidiosis. Phytase supplementation in broiler diets increased levels of intestinal IgA, and blood T-helper and cytotoxic T cells (Liu *et al.*, 2008), which may have implications for resistance to *Eimeria* spp, whilst the robust immune-related potentials of VitD were described in section 1.2.3 above. Indeed besides the few studies highlighted in section 1.3.1 above, very little is known about the effect of coccidiosis on long bone mineralisation.

Another issue with modern fast-growing broilers is that their low activity levels (Bailie and O'Connell, 2015) and rapid growth rate (Williams *et al.*, 2004) may already impact negatively on long bone mineralisation. Hence, the poor absorption and utilisation of vital bone minerals associated with coccidiosis (Turk and Stephens, 1966; Turk, 1973) cause a further reduction in bone mineralisation. However, no study has looked into the effects of increasing the activity levels or reducing the growth rate of modern broilers to improve long bone mineralisation during coccidiosis.

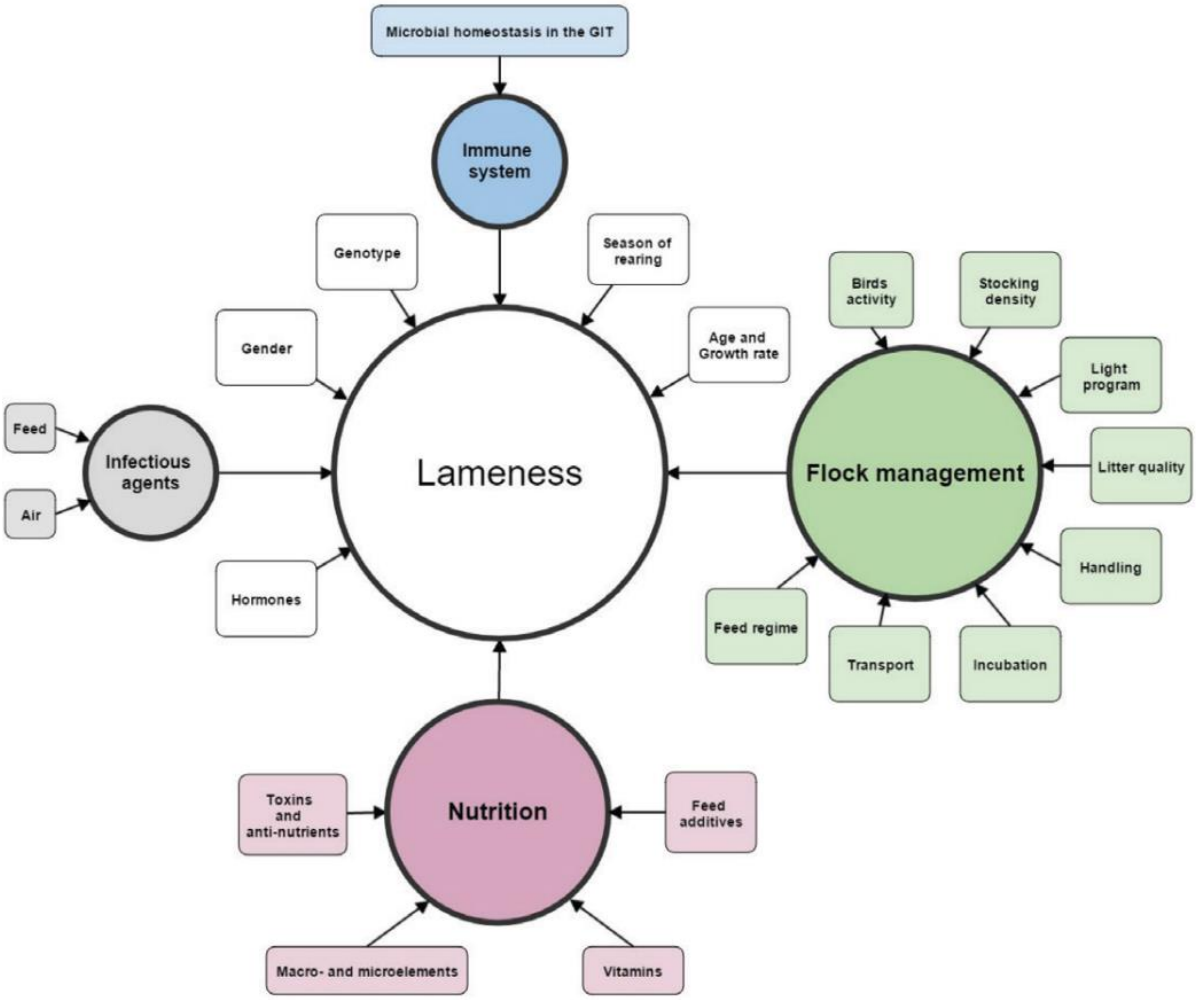

Figure 1. 3 Factors affecting leg bone development that may result in lameness and economic loss. (Adapted from Kierończyk *et al.* (2017))

1.5 Thesis Aims

The primary aim of the thesis was to assess the consequences of coccidiosis on the long bone quality of modern fast-growing broilers and to explore relevant nutritional intervention strategies that are capable of ameliorating these consequences alongside other effects of the disease. The significant effects of VitD on healthy bone development and mineralisation, growth performance of broilers, as well as its immunomodulatory potentials made VitD the preferred candidate in this thesis. Also, *E. maxima* was used to induce coccidiosis because of its higher pathogenicity and wider spread across the sections of the small intestine, compared to the other malabsorptive coccidia species. The specific aims of the thesis chapters were:

1) To investigate the degree to which selection for growth potential in modern broilers penalises resistance and tolerance to a primary coccidian infection [chapter 2]. For this

purpose, two modern broiler genotypes differing more than 25% in their growth rate were used.

2) To investigate the effects of increasing dietary VitD supplementation level (1000 vs 4000 IU/kg), and supplementing with a more efficiently absorbed metabolite (OHD) of D3 during coccidia infection [chapter 3].

3) To investigate whether offering OHD instead of VitD at commercially supplemented levels (4000 IU/kg) would result in improved bone mineralisation for coccidiosis – infected broilers receiving marginally deficient Ca:P diets [chapter 4].

4) To investigate whether an artificial reduction of early GR via diet dilution would result in improved bone mineralisation for coccidiosis – infected modern fast-growing broilers [chapter 5].

Chapter 2: Does selection for growth rate in broilers reduce resistance and tolerance to *Eimeria maxima* infection?

2.1 Summary

A total of 288 male broiler chickens of fast (F) or slow (S) growing lines (line effect) were raised from hatch in two consecutive batches to test the hypothesis that broilers selected for faster growth rate will show inferior resistance and tolerance to a coccidian challenge. Broilers from F and S lines were experimentally infected with different doses (dose effect) of 0 (control; C), 2500 (low-dose; L), or 7,000 (high-dose; H) sporulated *E. maxima* oocysts at day 13 of age in both rounds. To evaluate growth performance, feed conversion ratio (FCR), as well as average daily feed intake (ADFI), weight gain (ADG), and their values relative to body weight (BW) at infection (ADFI/BW and ADG/BW) were calculated over 13 days post-infection (pi). The performance was also assessed for three phases of infection; pre-patent (days 1 – 4 pi), acute (d5 – 8 pi) and recovery (d9 – 12 pi). Levels of plasma carotenoids and vitamins (Vit) E and A were measured at d6 and d13 pi. Long bone mineralisation and linear growth, and histological measurements were also assessed at the acute and recovery phases of infection using samples obtained at d6 and 13 pi, respectively. Furthermore, levels of nitric oxide metabolites, small intestine lesions, and the number of parasite genome copies (parasite replication) in the jejunum at d6 pi were measured. The F birds grew 1.42 times faster than the S birds in absolute terms whilst there was no significant interaction between line and parasite dose for ADG/BW and ADFI/BW, or FCR ($P > 0.05$). Amongst the plasma metabolites measured, line and dose interacted ($P = 0.05$) only for Vit E at d13 pi. C birds of the S line had a significantly higher concentration than L and H dose birds of line S and H birds of line F, whilst C, L and H birds of the F line had statistically similar values ($P > 0.05$). Similarly, only Vit A concentration was affected by genetic line ($P < 0.001$; F < S) at d13 pi. Infection significantly decreased levels of all metabolites, apart from NO, which increased ($P < 0.001$) in response to infection on d6 pi. The reduced concentration of metabolites was accompanied by changes in histomorphometric features, as well as the presence of *E. maxima* genome copies in infected birds; effects persisting at d13 pi. Line affected small intestine villi height (VH/BW) and crypt depth (CD/BW) expressed in proportion to body weight at sampling. Both variables were significantly decreased ($P < 0.001$) in duodenum and jejunum of the F than the S line at d6 and 13 pi. Furthermore, infection penalised tibia and femur linear growth, robusticity and mineralisation; the effects were more pronounced at d13 pi. Bone quality was generally higher for the S than the F broilers. Although femur percentage ash was

similar for both lines, femur strength in relation to body weight was higher for the S than the F line at d13 pi. Reductions in the measured variables were mostly independent of dose size, as was the level of parasite replication, and lesions inflicted on all three sections of the small intestine. The experimental factors had statistically similar effects ($P > 0.05$) on the measured variables in both rounds. In conclusion, the results herein suggest that a more robust selection scheme encompassing resistance to infection bears a higher significance than differences in growth rate per se.

2.2 Introduction

Improving productivity traits is the main focus for genetic selection in most livestock production systems (Dekkers and Hospital, 2002), but other vital traits can be compromised in the process (Van der Most *et al.*, 2011). For instance, selection in modern broiler chickens for increased daily gain or lower feed conversion ratio may have compromised tolerance to metabolic and skeletal disorders (Julian, 1998; Dawkins and Layton, 2012) and infectious pathogens (Yunis *et al.*, 2000; Leshchinsky and Klasing, 2001; Cheema *et al.*, 2003) as a consequence. Therefore, selection for improved performance traits may affect immune response and increase susceptibility to infections especially during a resource limitation situation (Rauw *et al.*, 1998; Van der Most *et al.*, 2011). The hypothesis is that birds from lines selected for productivity will continue to direct nutrient resources to productive traits at the expense of functional traits, such as the ability to limit or cope with a disease (Coop and Kyriazakis, 1999).

An excellent model to test the above hypothesis is a controlled coccidia infection using *E. maxima* which is the most pathogenic malabsorptive, and one of three predominant, coccidia species affecting chickens (Blake and Tomley, 2014). Through anorexia and severe inflammation of the small intestine, *E. maxima* infection impairs nutrient absorption and nutrient utilisation (Cervantes, 2002; Dalloul and Lillehoj, 2006) leading to reduced availability of nutrient resources as is the case with most health challenges. Susceptibility to *E. maxima* infections in broilers is here defined in terms of resistance; the ability to prevent the entry of oocysts, limit their proliferation within the intestinal tract, or both, and tolerance; the ability to limit the detrimental effects on performance (Doeschl-Wilson *et al.*, 2012). Broilers infected with *E. maxima* experience reduced weight gain due to anorexia and reduced efficiency of feed utilization arising from poor digestion, malabsorption of nutrients (Shirley *et al.*, 2007), changes in metabolism (Adams *et al.*, 1996a) and repartitioning of absorbed

nutrients away from growth processes towards the functioning of the immune system (Van der Most *et al.*, 2011).

Therefore, this study aimed to investigate the degree to which genetic selection for growth potentials in modern-day broilers penalises their resistance and tolerance to a primary coccidian infection. To assess tolerance, performance was measured using ADG, ADFI and FCR over the course of infection. Also, levels of plasma carotenoids and vitamins E and A, small intestine lesions and histological measurements to assess the level of damage to the intestinal mucosa, as well as markers of long bone mineralisation and physical development at the acute and recovery phases of infection, were examined. The level of nitric oxide metabolites (NO) during the acute stage of infection was also measured. In assessing the differences in resistance of the treatment groups, an estimation of the number of parasite genome copies in the jejunum, which is the primary site of *E. maxima* colonisation and replication, was done at peak of parasite replication, i.e. d6 pi (Blake and Tomley, 2014) and by proxy accounting for any possible underlying immune responses. It was further hypothesised that the effects of infection on performance would be more pronounced at higher *E. maxima* doses.

2.3 Materials and Methods

2.3.1 Birds, Husbandry and Diets

All procedures were conducted under the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU for animal experiments after obtaining the Home Office authorisation (P441ADF04). The experiment was conducted over two rounds with a 6-week interval between them. For each round, 72 male day-old chicks of a fast-growing line (Ross 308, F) and 72 male day-old chicks from a slow-growing line (Ross Ranger Classic, S) were obtained from the same breeding hatchery. The birds were from the same flocks of origin and whose parents have been subjected to similar husbandry. The parent stock used were from the same flock for each of the two lines, which ranged between 37 and 43 weeks of age for rounds A and B, respectively. The growth potential of these broiler lines differs by 25% according to the performance objectives of the breeding company. The lines F and S originated from the same paternal lines but different maternal lines in that the maternal lines of the S were not selected for growth rate.

The birds in each round were housed in 24 round pens each with a diameter of 1.2 m (1.13 m^2) situated in a windowless, thermostatically controlled room at the Newcastle University Cockle Park farm. The pens were arranged such that the treatments were allocated uniformly to represent

the different sides of the room. The floor of the pens was covered with wood shavings to a depth of approximately 5cm, and each pen was equipped with tube feeders and bell-drinkers. These birds had *ad libitum* access to feed and water throughout the trial. The temperature at pen level was monitored daily and maintained to meet Aviagen recommendations for spot brooding (Aviagen, 2014b), starting at 34°C at chick placement and gradually reduced to 20 °C by 25 days of age. Light (L) intensity at pen level ranged from 180-220 lux, whilst a lighting schedule of 23L:1 dark (D) was applied for the first seven days of age and then switched to 18L:6D for the remaining days of the trial.

Starter (d0-10) and grower (d11-26) diets were manufactured according to Aviagen nutrition specifications (Aviagen, 2014a), and offered to both lines (Table 2.1). The starter and the grower diets were offered in crumb and pelleted forms, respectively.

2.3.2 Experimental design and inoculations

The experiment was conducted according to a 3 × 2 factorial design with 3 coccidian doses representing infections and 2 lines as the independent variables, while the experimental round was treated as a blocking factor. Upon arrival, day-old chicks of F and S lines were randomly assigned to treatment groups at one of three levels of infection, a non-infected control group (C), a low level of infection (L), and a high level of infection (H). Each treatment group consisted of 8 pens as replicates, and the initial stocking density was six birds per pen. Broilers were orally inoculated at 13 days of age (d0 pi) with a single 0.5 ml oral dose of water (C) or doses of either 2500 (L) or 7000 (H) sporulated *E. maxima* oocysts of the Weybridge laboratory reference strain using 1-ml syringes. The inocula were prepared using a previously described method (Pastor-Fernández *et al*., 2018). The infection doses were chosen based on previous studies (Allen and Fetterer, 2002b) to maintain pathogenicity at a sub-clinical level.

2.3.3 Sampling

Bird individual weight and pen feed intake were measured at 1 and 13 days of age (pre-infection), and then daily from d1 to 13 pi when the trial was terminated. On d6 and 13 pi, a randomly selected bird from each pen was weighed, bled from wing vein, and then euthanised with a lethal injection of sodium pentobarbitone (Euthatal®, Merial, Harlow, United Kingdom). Blood was placed in 5 ml sodium heparin plasma tubes (BD Vacutainer, SST II Advance Plus Blood Collection Tubes - BD, Plymouth, United-Kingdom). The blood samples were immediately placed on ice and then centrifuged for 10 min at 1500 g at 4°C within 1.5 h after collection. Aliquoted plasma samples were stored at -80 °C pending analyses.

During necropsy, the small intestine was removed, and the duodenum, jejunum and ileum were scored for any lesions according to the method described by Johnson and Reid (Johnson and Reid, 1970). Following lesion scoring, 5 cm of intestinal tissue from the immediate region of Meckel's diverticulum, the main site of infection by *E. maxima* (Long *et al.*, 1976), was excised, opened longitudinally, the digesta carefully removed, and the tissue was submerged in 5 ml bizous filled with RNAlater® (Life Technologies; Carlsbad, CA, USA). These samples were immediately stored at -80 °C pending further analyses. Also, two 1 cm segments, one from the duodenal loop and one from the jejunum positioned 2.5 cm from Meckel's diverticulum, were sampled from birds dissected on d6 and 13 pi and were fixed in 10% phosphate buffered formalin, maintained at a pH of 7.0, for morphometric analysis. Finally, the right femur and tibia were dissected, much of the adhering soft tissues removed using scalpels and they were stored in airtight individually labelled polythene bags at -20 °C pending evaluation.

2.3.4 Sample analysis

Morphometric analysis of gut

Formalin-fixed intestinal sections from the duodenum and jejunum were dehydrated through a series of graded ethanol baths followed by xylene in a Shandon™ Excelsior™ ES Tissue Processor (Thermo Fisher Scientific Inc., Waltham, Massachusetts), before being embedded in paraffin wax, sectioned at 4 µm and stained with hematoxylin/eosin. Histological sections were examined under a Zeiss Primostar light microscope and images were captured using ZEN imaging software (Zeiss Germany, Oberkochen, Germany). Images were viewed to measure morphometric features at 10× magnification. The villus height (VH) and the crypt depths (CD) were determined from sections using ImageJ (NIH) software (Schneider *et al.*, 2012). The villus height was estimated by measuring the vertical distance from the villus tip to the villus-crypt junction level for ten villi per section, and the crypt depth by measuring the vertical distance from the villus-crypt junction to the lower limit of the crypt for ten corresponding crypts per section.

Parasite replication

Quantitative real-time PCR (qPCR). Using predicted genome sizes of 46.2 Mbp for *E. maxima* (Blake *et al.*, 2011) and 1.2 Gbp for *G. domesticus* (Furlong, 2005), and the method of Blake *et al*. (Blake *et al.*, 2008) to extract total genomic DNA (gDNA) from sporulated *E. maxima* oocysts and uninfected chicken intestinal tissue, ten-fold DNA dilution series were created using previously described methods (Blake *et al.*, 2006; Nolan *et al.*, 2015). For quantifying *E. maxima* genome copy number, the primers Ema_qPCRf (forward: 5′-TCG

TTG CAT TCG ACA GAT TC-3′) and Ema_qPCRr (reverse: 5′-TAG CGA CTG CTC AAG GGT TT-3′), targeting 138 base pairs of the Microneme Protein 1 (MIC1) gene, were used (Blake *et al.*, 2006). For normalization, the primers actbF (forward: 5′-GAG AAA TTG TGC GTG ACA TCA-3′) and actbR (reverse: 5′- CCT GAA CCT CTC ATT GCC A -3′), which amplify 152 base pairs of the chicken cytoplasmic beta-actin (*actb*) gene were used according to a previously employed protocol (Nolan *et al.*, 2015). Total gDNA was extracted from excised jejunal tissue using a DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. In brief, RNAlater® was removed from the defrosted jejunal tissue, which was then weighed and immersed in an equal w/v of Qiagen tissue lysis buffer. Each sample was homogenised with a Qiagen TissueRuptor, and the equivalent of ≤ 25 mg of the homogenate added to a sterile 1.5 ml microcentrifuge tube. Genomic DNA was then extracted according to the manufacturer's instructions and stored at -20 °C until analysis.

Quantitative real-time PCR was performed with a CFX96 Touch® Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). Amplification of each sample was performed in triplicate in a 20 µl volume containing 1 µl of total gDNA, 300 nM of each primer, 10 µl of SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories), and 8.9 µl of DNase/RNase free water (Gibco™, Life Technologies, Karlsruhe, Germany). Cycling qPCR conditions were 95 °C/2 m (enzyme activation/initial denaturation), followed by 40 cycles of 95 °C/15 s (denaturation), 60 °C/30 s (annealing/ extension), followed by melt analysis of 65–95 °C at increments of 0.5 °C/0.5 s. Assays were performed in white hard-shell® 96-well PCR plates (Bio-Rad Laboratories) sealed with Thermo Scientific adhesive sealing sheets and included the respective gDNA dilution series (standards) and no template controls (NTC). Calculation of copy number of each qPCR target was performed with the software CFC Manager v.3.1 (Bio-Rad Laboratories) according to the slope and intercept of the corresponding reference dilution series. A normalisation of the predicted parasite genome copy number was performed by comparison to the estimated host genome copy number. Parasite genome copy number was calculated based on the normalised parasite copy number/µl. All other procedures and calculations were as described previously (Nolan *et al.*, 2015).

Blood metabolites

Carotenoids, vitamins A and E. Plasma samples were analysed for lutein and zeaxanthin, which are the major carotenoids in cereal grains (Humphries and Khachik, 2003). Fat-soluble vitamins retinol (Vit A) and α-tocopherol (Vit E) levels in the blood plasma were also

measured. Both carotenoids and fat-soluble vitamin concentration in the blood plasma were used as indicators of the gut absorptive capacity during coccidiosis or coccidiosis-induced damage to intestinal epithelial (Singh and Donovan, 1973; Allen and Fetterer, 2002a; Allen *et al.*, 2004). Retinyl acetate and echinenone were used as internal standards. Retinoid (> 95% all-trans isomers) and α-tocopherol standards were purchased from Sigma-Aldrich while carotenoid standards were purchased from CaroteNature GmbH (Ostermundigen, Switzerland). HPLC-grade acetonitrile, ethanol, methanol, chloroform, hexane and triethylamine were purchased from Fisher Scientific (Loughborough, UK). Butylated hydroxytoluene (BHT) was obtained from Sigma-Aldrich. All procedures were undertaken under orange lighting to avoid analyte degradation. For the preparation of stock solutions, retinol and Vit E were dissolved in ethanol with 0.1% BHT, while lutein and zeaxanthin were dissolved in chloroform with 0.1% BHT. The concentrations of individual calibration standard solutions were confirmed by measuring the absorption in ethanol with a UV spectrophotometer. Internal standards were prepared in ethanol containing 0.01% BHT. 100 µl of each plasma sample was diluted in 100 µl of water to which 200 µl of the internal standard in ethanol was added. 2 ml of hexane was added to each sample and samples were vortexed in an orbital shaker for 10 min and then centrifuged at 1500× *g* for 5 min. Following centrifugation, the upper hexane phase was transferred to clean glass tubes, and samples were re-extracted with a further 2 ml of hexane. Hexane was evaporated under a nitrogen stream, and residues were dissolved in 100 µl of ethanol and transferred to amber glass vials with inserts (Fisher Scientific). Ten µl of sample extract was injected for the analysis using a Shimadzu HPLC System *via* photodiode array (PDA) detection, according to previously described methodology (Liu *et al.*, 2011). The concentrations of Vit E, lutein zeaxanthin and echinenone were quantified at 450 nm, while Vit A and retinyl acetate (IS2) were measured at 325 nm.

Nitric oxide metabolites. Plasma concentrations of nitric oxide metabolites ($NO2^-$ and $NO3^-$), which constitute a maker of the severity of coccidian infections (Allen, 1997b; Allen, 1997a), were analysed using methods described by Qadir *et al.* (2013). Spiking solution was prepared from 5 mM Sodium nitrate-^{15}N and 0.05 mM Sodium nitrite-^{15}N (Cambridge Isotope Laboratories, Inc., USA) and used as an internal standard. 100µL of plasma sample, 100 µL of spiking solution, 20µL of 2,3,4,5,6 - pentafluorobenzyl bromide (Sigma-Aldrich, Germany) and 800 µL of acetone (VWR, Lutterworth, Leicestershire, UK) were pipetted into Falcon™ round-bottom polystyrene tubes and placed in a heating block at 50 °C for 120 mins for incubation. Following incubation, acetone was evaporated under a nitrogen stream for 10mins. Samples were allowed to cool before

2ml of toluene (Fischer Scientific, UK) was pipetted into each tube and the tubes vortexed for 15 secs. Subsequently, 1ml of distilled water was pipetted into each tube and samples were re-vortexed twice for 15 secs with a rest of 15 secs in between. Using glass Pasteur pipettes, the top layer was transferred into amber glass vials and stored in at -20 °C pending GCMS analysis. Other variables such as column type and ionisation temperatures, were as described previously (Tsikas, 2000).

Bone evaluation

Frozen femur and tibia were thawed at 4ºC in a fridge overnight, and then placed at room temperature for 1 hour before further defleshing with scalpels. Tibia and femur length and diameter at the centre of the diaphysis were measured using digital callipers and recorded. Each bone was weighed on an analytical balance to obtain the weight before being subjected to a 3-point break test using an Instron testing machine (Instron 3340 Series, Single Column-Bluehill). The testing support consisted of an adjustable 2-point block jig, spaced at 30 mm for both tibia and femur bones. The crosshead descended at 5 mm/min until a break was determined by measuring a reduction in force of at least 5%. Following breaking strength evaluation, bones were split in two, and the bone marrow was manually removed. Subsequently, bones were soaked in petroleum ether for 48 hours for lipid removal and then placed in an oven at 105 °C for 24 hrs, and the dry defatted bone weight (DDB, g) was recorded. Samples were then placed in a furnace at 600 °C for 24 hrs for the determination of ash weight (g) and ash content (%) for tibia and femur. Hence, data deriving from femur and tibia of dissected birds include length and width (mm), ash weight (g), breaking strength (BS, N), DDB(g) and ash percentage (AP, %). Also, bone robusticity index (RI) (Riesenfeld, 1972; Mutuş *et al.*, 2006), as well as weight/length (Seedor) index (Seedor *et al.*, 1991), were derived using the following prescribed formulae.

$$Robusticity\ index = \frac{\text{length (mm)}}{3\sqrt{\text{weight (mg)}}}$$

$$Seedor\ index = \frac{\text{weight (mg)}}{\text{length (mm)}}$$

2.3.5 Calculations and Statistics

All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 22 (Armonk, NY: IBM Corp). For all statistical assessments, the pen was considered as the experimental unit. The ADFI (g/d), ADG (g/d) and FCR of birds were calculated over the pi period (d1 – 13 pi) and were analysed with dose, line and round as fixed factors and the interaction between line and dose, using the general linear model procedure (GLM) (Table 2.2). Furthermore, ADFI and ADG data pi were expressed as a proportion of BW at d0 pi (ADG/BW and ADFI/BW in g/g/d) to account for the *a priori* differences in performance between the broiler lines.

The ADG/BW and ADFI/BW calculated over the pi period were analysed with the repeated measurements mixed procedure. The model included dose, line, day and round as fixed factors, the 2-way interactions between dose and line, dose and day and the 3-way interaction between dose, day and line. Based on the day pi that a reduction of ADG and ADFI was observed (Figure 2.1), the pi period was divided into pre-patent (d1 – 4 pi), acute (d5 – 8 pi), and recovery (d9 – 12 pi) phases and ADFI, ADG, and FCR were calculated over each of the phases. The performance data for each phase was analysed using GLM with line, dose, round and the interaction between line and dose as the factors (Table 2.3).

Single time point data included plasma concentrations of zeaxanthin, lutein, vitamins E and A, histological and bone measurements deriving from one bird per pen dissected on d6 or d13pi, as well as *E. maxima* genome copy numbers and nitric oxide metabolites (NO) obtained at d6pi. These were analysed with GLM with dose, line, and round as fixed factors and the interaction between dose and line. Histological and bone measurements, apart from AP and villi height/crypt depth ratio (VCR), were expressed as a proportion of BW of dissected birds to account for the size difference between S and F growing birds, and between control and infected birds. Absolute values for histological measurements (i.e. not expressed relative to BW) were also analysed. Expressing bone variables as a proportion of BW has been previously used in studies comparing broiler genotypes with different growth potential (Shim *et al*., 2012). For *E. maxima* genome copy (GC) numbers, control birds were excluded from the model as their value was effectively 0. Hence, GC was analysed using GLM with the two lines (S and F), two doses (L and H), two rounds as the factors and the interaction between line and dose.

For all statistical analysis, the normality of the residuals was assessed with the Shapiro-Wilk test, and non-normalized data like predicted *E. maxima* genome copy numbers were log-

transformed before analysis. When significant differences were detected, treatment means were separated and compared by the Tukey's multiple comparison tests. Significance was determined at $P < 0.05$. All values are expressed as model-predicted least square means with the SEM.

Intestinal lesion scoring was done on a scale of 0 (no lesion) to 4 (very severe); 1, 2 and 3 representing mild, moderate and severe respectively. Data generated were analysed using ordinal logistics regression with line, dose and round as fixed factors and the interaction between line and dose. Control birds were excluded from the analysis, as there were no intestinal lesions found (Score 0). Significance was determined at $P < 0.05$.

Table 2. 1 Ingredient and analysed chemical composition of the Starter (d0 – 10) and Grower (d11 – 26) diets offered to broiler chickens.

Item	Starter	Grower
Ingredient (%)		
Wheat	47.8	51.5
Soybean meal (48% CP)	32.0	25.2
Corn	10.0	10.0
Soybean full fat	4.0	7.0
Dicalcium phosphate	1.89	1.66
Soy crude oil	1.84	2.32
Limestone	0.64	0.59
Vitamin and mineral premix	0.40	0.40
DL methionine	0.33	0.30
L-Lysine	0.27	0.24
Sodium bicarbonate (27%)	0.21	0.19
Sodium chloride (39%)	0.19	0.20
L-Threonine	0.14	0.12
Choline chloride (60%)	0.05	0.05
L-Valine	0.03	0.02
Xylanase[1]	0.02	0.02
Nutrient composition (%)		
ME (kcal/kg)	3000	3100
Crude Protein	23.5	21.7
Crude Fat	4.37	5.41
Calcium	0.96	0.87
Phosphorus	0.76	0.70
Available phosphorus	0.48	0.44
Ash	5.23	4.78

The nutrient composition was in accordance with Aviagen nutrient specification (Aviagen, 2014a).

[1]Ronozyme ® WX, DSM Nutritional Products Ltd.

2.4 Results

The main effect of round and the interaction between round and other experimental factors are not presented in the results as they were not statistically significant (*P > 0.05*).

2.4.1 Bird health and performance variables

No bird was euthanised due to health-related disorders, and coccidiosis caused anorexia and reduced weight gain according to expectations. The main effects of line, parasite dose and their interaction on ADG, ADFI, and FCR are presented in Table 2.2.

Post-infection period

Line and dose did not significantly interact for any of the performance parameters (*P > 0.1*). Parasite dose significantly affected BW (*P < 0.001*) at the end of the trial period (d13 pi), as well as ADG, ADFI, and FCR over the period, with H and L dosed birds showing inferior values in comparison to C birds, and similar to each other for these parameters. Line significantly affected (*P < 0.001*) all performance parameters over the pi period with F line birds showing higher BW at inoculation (d0 pi) and at the end of the trial (d13 pi).

Repeated measurements on daily ADFI/BW and ADG/BW

The repeated measurement analysis on ADFI/BW and ADG/BW for d1 to 13 pi is illustrated in Figure 2.1. There were no significant interactions (*P* > 0.1) between line and dose for ADG/BW or ADFI/BW (g/d/g). Even when expressing values as a proportion of BW at infection, the F line birds maintained higher (*P < 0.01*) ADG (0.194 *vs* 0.176; SEM = 0.0010) and ADFI (0.257 vs 0.252; SEM = 0.0010) than the S line birds. Dose affected relative ADG and ADFI (*P < 0.001*); in comparison to C birds, L and H dosed birds showed significantly lower ADG (C = 0.200 *vs* L = 0.177 *vs* H = 0.177; SEM = 0.0200) and ADFI (0.268 *vs* 0.247 *vs* 0.249; SEM = 0.0200 for C, L, and H birds, respectively). ADG/BW and ADFI/BW were affected by the interaction between dose and day (*P < 0.001*); birds on H and L dose showed significantly lower ADG and ADFI between d4 and d8 pi compared to the controls (Fig. 2.1). For this reason, the experimental period was divided into three equal periods that roughly equated to the pre-patent (d1 – 4 pi), acute (d5 – 8 pi), and recovery (d9 – 12 pi) periods of infection.

Performance during the pre-patent, acute, and recovery periods

The main effects of line, period and their interaction on relative ADG, ADFI, and FCR are presented in Table 2.3. Line and dose did not interact for performance variables during any period

pi (*P > 0.01*). The F line birds had significantly higher ADG/BW, ADFI/BW and smaller FCR than S line birds during all periods apart from the acute period when they had similar ADFI/BW with S line birds. Parasite dose significantly affected ADG/BW and FCR during the pre-patent and acute periods, as well as ADFI/BW during the acute period (*P < 0.05*); C broilers had significantly higher relative ADG and ADFI and smaller FCR than L and H broilers in all cases.

2.4.2 Plasma Carotenoids, Vitamins A, E, and Nitric oxide metabolites

The main effects of dose, line and their interaction on plasma concentrations of carotenoids, vitamins A, E, and NO are presented in Table 2.4. There were no interactions between line and parasite dose for plasma concentration of lutein, zeaxanthin, vitamins E and A, and NO metabolites on days 6 or 13 pi, apart from Vit E at d13 pi. Line and dose interacted for plasma Vit E (*P = 0.050*): control birds of the S line had significantly higher values than those of the L and H dose birds of line S and H dose birds of line F, whilst C, L and H birds had statistically similar values amongst F line broilers (Table 2.4).

Parasite dose significantly affected the concentration ($P < 0.001$) of lutein, zeaxanthin, and Vitamins A and E at d6pi. Values were significantly higher in control birds compared to L and H dose birds. Similar effects were observed at d13pi for lutein ($P < 0.001$), zeaxanthin (*P < 0.01*), and Vit E ($P < 0.05$). In contrast, dose induced the opposite effect on NO at d6pi ($P < 0.001$). Bird line did not affect the concentration of any of plasma metabolites measured at d6 and d13pi ($P > 0.1$), apart from Vit A ($P < 0.001$), which was higher for S than for F line birds at d13 pi (Table 2.4).

2.4.3 Parasite replication and histology

The main effects of dose, line and their interaction on the number of *E. maxima* genome copies derived from qPCR, and histological measurements obtained on d6 and 13 pi are presented in Tables 2.5 and 2.6, respectively. Parasite genomes were not detected in samples collected from control birds at d6 pi. Parasite replication in S and F line birds was not affected (*P > 0.05*) by any of the independent variables or the interaction at d6 pi. Dose significantly affected (*P < 0.05*) all digestive tract morphological parameters at d6 pi; expressed in absolute terms and relative to the BW at dissection. Infected H and L birds had shorter villi and enlarged crypts and a lower villi height/crypt depth ratio (VCR) in comparison to C birds, whilst being similar to each other. At d13 pi, duodenal and jejunal VH were not affected by dose, but they were significantly affected when expressed relative to their BW at dissection; being significantly higher (*P < 0.05*) in H and L dose birds in comparison to C birds. CD of

intestinal sections, both in absolute and relative terms were significantly affected by dose (*P < 0.001*) being higher for H and L dose birds compared to C birds. On the other hand, VCR was significantly affected by infection (*P < 0.05*), being significantly lower for H dose birds in the duodenum and both and H and L birds in the jejunum than C birds.

Although F birds had significantly longer villi in the duodenum at d6 (*P < 0.01*) and greater CD (*P = 0.05*) at d13pi, the opposite was the case for both VH and CD at all intestinal sites at both days pi when they were expressed relative to BW. Line and dose interacted for CD (*P < 0.05*) in the jejunum at d6 pi and in the duodenum at d13 pi. The F birds receiving the H dose displaying greater CD than all other line and dose treatment groups. When expressed as a proportion of BW at dissection, the interaction between line and dose for jejunal CD at d6 pi was maintained but with S birds receiving the H and L doses displaying significantly (*P < 0.05*) higher CD than F line L dosed birds (Table 2.5).

2.4.4 Intestinal lesion score

There were no intestinal lesions (score 0) detected in uninfected birds of either the S or the F line at d6 and 13 pi. Hence, these were excluded from the regression model. Also, the intestinal lesions observed amongst infected L and H birds at d6 pi had recovered by d13 pi. Small intestinal lesion score deriving from an average score of the three individual sections at d6 pi revealed no effect (*P > 0.1*) of line (S = 1.35 *vs* F = 1.60; SEM = 0.181), dose (L = 1.31 *vs* H = 1.65; SEM = 0.181), or the interaction between line and dose on small intestine lesions. Irrespective of line or dose, the cumulative score showed that 50% of infected birds had moderate lesions (score 2) while 34.4% and 15.6% had mild (score 1) and severe (score 3) lesions respectively. Amongst the three segments of the small intestine, the jejunum had the only occurrence of very severe lesions (score 4; 3.1%) as well as the highest occurrence (25%) of the severe lesion (score 3) compared to the duodenum (6.5%) and the ileum (9.4%).

2.4.5 Bone variables

The effects of line, dose and their interactions on femur and tibia measurements at d6 and d13 pi are presented in Tables 2.7 and 2.8, respectively. There were no significant interactions between line and dose on tibia and femur parameters at d6 and 13pi.

Bone linear growth relative to body weight at dissection

Line affected (*P < 001*) tibia and femur length/BW: values were higher for the S than the F line birds at d6 and 13 pi. Tibia and femur width/BW were affected (*P < 0.001*) by line with values

higher for the S than the F line at d6 and 13 pi. As expected, infection affected ($P < 0.05$) length/BW and width/BW of tibia and femur at d6 pi. Values were lower in uninfected (C) birds compared to infected (L and H) birds. Whilst at d13 pi, infection significantly increased ($P < 0.05$) tibia and femur length/BW and femur width/BW, but tibia width/BW was not affected (see Tables 2.7 and 2.8).

Relative bone breaking strength (BS) and dry defatted bone weight (DDB)

Broiler line did not affect ($P > 0.05$) femur or tibia BS/BW and DDB/BW at d6 pi. However, at d13 pi, line significantly affected ($P < 0.05$) femur BS/BW, as well as tibia and femur DDB/BW; values were consistently higher in the S compared to the F line broilers. Parasite dose significantly affected ($P < 0.05$) BS/BW of femur and tibia at d6 and 13 pi, respectively; the infected compared to C birds had reduced BS/BW. There were no other significant effects of parasite dose on tibia or femur BS/BW and DDB/BW at d6 and 13 pi.

Ash content relative to body weight and ash percentage in dry defatted bone

Parasite dose reduced ($P < 0.05$) femur AP for L and H in comparison to C birds at d6 pi, as well as tibia and femur ash/BW for H compared with C birds at d13 pi; values for L birds were intermediate and not significantly different from other two groups. Also, infection significantly reduced ($P < 0.05$) tibia and femur AP for L and H compared to C birds at d13 pi. On the other hand, Tibia ash/BW was significantly higher ($P < 0.05$) for the S than the F line on both d6 and 13 pi, but femur ash/BW only on d13 pi ($P < 0.001$). Tibia or femur AP did not differ ($P > 0.05$) between F and S lines at both time points.

Weight/length (Seedor) and robusticity indices

Line significantly affected ($P < 0.05$) seedor and robusticity indices for tibia and femur at d6 and 13 pi. Seedor index was lower, and robusticity index higher for the S than the F line birds. Dose significantly reduced ($P < 0.05$) seedor index for femur and tibia at d6 and 13 pi respectively: values were lowest in H birds and highest in C birds for the S and the F lines. Dose also significantly increased ($P < 0.05$) femur robusticity index for the infected (L and H) compared to C birds at d13 pi (Tables 2.7 and 2.8).

Table 2. 2 Effects of line, dose and their interaction on performance parameters in broiler chickens of either fast or slow-growing lines, inoculated with 0 (Control), 2500 (Low) or 7000 (High) sporulated *E. maxima* oocysts on d13 of age, over the period post-infection (d0 – 13 pi).

		BW d0 pi	BW d13 pi	ADG	ADFI	FCR
Line						
	Slow	371	1220	65.3	93.4	1.43
	Fast	479	1676	92.1	123	1.34
	SEM	3.4	14.4	1.01	1.17	0.009
Dose						
	Control	427	1541[a]	85.7[a]	114[a]	1.34[a]
	Low	422	1401[b]	75.3[b]	104[b]	1.39[b]
	High	427	1402[b]	75.0[b]	106[b]	1.42[b]
	SEM	4.2	17.6	1.24	1.4	0.012
Line × Dose						
Slow	Control	373	1296	71.0	98.6	1.39
	Low	369	1182	62.6	90.2	1.44
	High	373	1181	62.1	91.5	1.47
Fast	Control	481	1786	100	130	1.29
	Low	475	1619	88.1	118	1.34
	High	481	1624	87.9	120	1.37
	SEM	5.9	24.9	1.80	2.03	0.016
Source				Probabilities		
Line		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Dose		0.599	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Line x Dose		0.972	0.522	0.476	0.716	0.981

[a, b]Means within a sub-column that do not share a common superscript are significantly different ($P < 0.05$).
Abbreviations: BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio
The period from d0 to 13 pi equates to d13 to 26 of age.

Table 2. 3 Effects of line, dose and their interaction on ADG/BW (g/d/g) and ADFI/BW (g/d/g) and FCR, during the pre-patent (d1-4 pi), acute (d5-8 pi), or recovery (d8-12 pi) periods of infection in broiler chickens of either fast or slow-growing lines, inoculated with 0 (Control), 2500 (Low) or 7000 (High) sporulated *E. maxima* oocysts on d13 of age.

		Pre-patent			Acute			Recovery		
		ADFI/BW	ADG/BW	FCR	ADFI/BW	ADG/BW	FCR	ADFI/BW	ADG/BW	FCR
Line										
	Slow	0.194	0.149	1.30	0.217	0.144	1.57	0.326	0.226	1.44
	Fast	0.199	0.163	1.22	0.217	0.160	1.39	0.338	0.251	1.34
	SEM	0.0015	0.0018	0.001	0.0033	0.0035	0.029	0.0039	0.0030	0.009
Dose										
	Control	0.199	0.162[a]	1.23[a]	0.255[a]	0.197[a]	1.30[a]	0.335	0.239	1.40
	Low	0.195	0.153[b]	1.28[b]	0.200[b]	0.131[b]	1.54[b]	0.325	0.235	1.38
	High	0.195	0.153[b]	1.28[b]	0.196[b]	0.127[b]	1.60[b]	0.336	0.241	1.40
	SEM	0.0019	0.0022	0.014	0.0040	0.0042	0.005	0.0048	0.0037	0.012
Line × Dose										
Slow	Control	0.197	0.156	1.26	0.253	0.190	1.33	0.331	0.226	1.47
	Low	0.195	0.145	1.32	0.206	0.126	1.65	0.318	0.225	1.41
	High	0.191	0.147	1.32	0.193	0.115	1.72	0.329	0.227	1.45
Fast	Control	0.201	0.168	1.19	0.257	0.204	1.27	0.339	0.253	1.34
	Low	0.199	0.161	1.24	0.199	0.137	1.44	0.332	0.255	1.35
	High	0.195	0.159	1.23	0.195	0.139	1.48	0.342	0.246	1.34
	SEM	0.0025	0.0031	0.019	0.0057	0.0060	0.051	0.0068	0.0053	0.017
Source		Probabilities								
Line		**0.045**	**<0.001**	**<0.001**	0.972	**0.003**	**<0.001**	**0.045**	**<0.001**	**<0.001**
Dose		0.269	**0.008**	**0.020**	**<0.001**	**<0.001**	**<0.001**	0.210	0.546	0.479
Line x Dose		0.314	0.707	0.801	0.295	0.522	0.222	0.892	0.769	0.154

[a, b]Means within a sub-column that do not share a common superscript are significantly different ($P < 0.05$).

Abbreviations: BW, body weight; ADG/BW, average daily gain in proportion to BW; ADFI/BW, average daily feed intake in proportion to BW; FCR, feed conversion ratio

The period from d0 to 13 pi equates to d13 to 26 of age.

Table 2. 4 Effects of line, dose and their interaction on plasma metabolites concentration (µm/ml) in broiler chickens of either fast or slow-growing lines on d6 and 13 post-infection (pi). Broilers inoculated with 0 (Control), 2500 (Low) or 7000 (High) sporulated *E. maxima* oocysts on d13 of age.

		D6 post-infection					D13 post-infection			
		Lutein	Zeaxanthin	Vit E	Vit A	NO	Lutein	Zeaxanthin	Vit E	Vit A
Line										
	Slow	1.12	0.269	42.8	3.16	48.6	1.93	0.412	69.9	4.63
	Fast	1.19	0.285	41.9	2.95	42.9	1.86	0.402	69.2	3.84
	SEM	0.052	0.0117	1.91	0.117	3.10	0.064	0.0149	2.56	0.110
Dose										
	Control	2.27[a]	0.515[a]	93.0[a]	4.19[a]	28.7[a]	2.16[a]	0.456[a]	76.1	3.97
	Low	0.65[b]	0.169[b]	17.8[b]	2.65[b]	54.2[b]	1.77[b]	0.387[b]	68.4	4.34
	High	0.55[b]	0.146[b]	16.3[b]	2.32[b]	54.4[b]	1.75[b]	0.377[b]	64.1	4.39
	SEM	0.064	0.0140	2.34	0.143	3.79	0.079	0.0182	3.14	0.134
Line × Dose										
	Control	2.25	0.505	97.0	4.23	31.9	2.27	0.476	82.3[a]	4.46
Slow	Low	0.64	0.173	16.9	2.78	62.1	1.70	0.372	63.3[b]	4.64
	High	0.47	0.130	14.6	2.47	51.8	1.82	0.389	64.1[b]	4.79
	Control	2.30	0.526	88.9	4.15	25.5	2.05	0.436	70.0[ab]	3.49
Fast	Low	0.66	0.166	18.7	2.52	46.2	1.85	0.402	73.5[ab]	4.03
	High	0.62	0.163	18.0	2.18	57.0	1.68	0.366	64.1[b]	4.00
	SEM	0.090	0.0203	3.31	0.203	5.36	0.113	0.0258	4.44	0.190
Source						Probabilities				
Line		0.326	0.342	0.735	0.224	0.204	0.417	0.621	0.838	**<0.001**
Dose		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.007**	**0.031**	0.066
Line × Dose		0.758	0.601	0.184	0.865	0.164	0.225	0.370	**0.050**	0.622

[a, b]Means within a sub-column that do not share a common superscript are significantly different ($P < 0.05$).

Abbreviations: NO, Nitric oxide metabolites; Vit, vitamin

D6 and 13 post-infection equates to d19 and 26 of age respectively

Table 2. 5 Effects of line, dose and their interaction on intestinal morphology and *E. maxima* genome copy number (GC) at **d6 post-inoculation** in broiler chickens of either fast or slow-growing lines, inoculated with 0 (Control), 2500 (Low) or 7000 (High) sporulated *E. maxima* oocysts on d13 of age.

		Duodenum					Jejunum					
		VH (µm)	CD (µm)	VH/BW (µm/kg)	CD/BW (µm/kg)	VCR	VH (µm)	CD (µm)	VH/BW (µm/kg)	CD/BW (µm/kg)	VCR	*E. maxima* GC[1]
Line												
	Slow	1176	269	1699	396	5.45	528	197	761	293	3.71	5.87
	Fast	1338	263	1428	289	6.00	538	208	574	231	3.63	5.89
	SEM	36.5	12.1	46.9	16.8	0.260	27.1	11.1	35.8	14.7	0.282	0.120
Dose												
	Control	1594[a]	181[b]	1861[a]	212[b]	9.49[a]	647[a]	107	757[a]	125	6.45[a]	*NA*
	Low	1132[b]	289[a]	1467[b]	380[a]	4.26[b]	498[b]	233	646[b]	311	2.48[b]	5.89
	High	1045[b]	329[a]	1362[b]	436[a]	3.42[b]	454[b]	268	599[b]	349	2.07[b]	5.87
	SEM	44.7	14.8	57.4	20.6	0.320	33.2	13.6	43.8	18.0	0.340	0.120
Line × Dose												
	Control	1442	167	1957	227	9.28	624	100[c]	836	136[c]	6.63	*NA*
Slow	Low	1112	295	1669	444	3.98	488	255[b]	732	385[a]	2.19	5.91
	High	973	343	1471	517	3.10	472	237[b]	714	359[a]	2.29	5.87
	Control	1746	194	1765	196	9.71	669	113[c]	677	114[c]	6.27	*NA*
Fast	Low	1151	282	1265	317	4.54	509	211[b]	561	238[b]	2.78	5.88
	High	1118	314	1254	354	3.74	436	299[a]	483	340[ab]	1.85	5.87
	SEM	63.2	20.9	81.2	29.1	0.451	46.9	19.3	61.9	25.5	0.487	0.170
Source							Probabilities					
Line		**0.003**	0.759	**<0.001**	**<0.001**	0.146	0.789	0.515	**0.001**	**0.005**	0.858	0.940
Dose		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.049**	**<0.001**	**<0.001**	0.880
Line × Dose		0.122	0.381	0.370	0.073	0.973	0.682	**0.030**	0.825	**0.024**	0.498	0.927

[a-c]Means within a sub-column that do not share a common superscript are significantly different ($P < 0.05$).

Abbreviations: VH, villus height; VH/BW, villus height relative to BW; CD, crypt depth; CD/BW, crypt depth relative to BW; VCR, villi height:crypt depth ratio; BW, body weight at dissection; GC, genome copy numbers

[1]log-transformed values; NA, not applicable

D6 pi equates to d19 of age

Table 2. 6 Effects of line, dose and their interaction on intestinal morphology at **d13 post-inoculation** in broiler chickens of either fast or slow-growing lines, inoculated with 0 (Control), 2500 (Low) or 7000 (High) sporulated *E. maxima* oocysts on d13 of age.

		Duodenum					Jejunum				
		VH (µm)	CD (µm)	VH/BW (µm/kg)	CD/BW (µm/kg)	VCR	VH (µm)	CD (µm)	VH/BW (µm/kg)	CD/BW (µm/kg)	VCR
Line											
	Slow	1510	155	1241	128	10.15	702	117	578	97	6.33
	Fast	1591	171	956	104	9.77	775	132	467	80	6.28
	SEM	31.5	3.6	24.7	2.9	0.324	29.4	4.3	22.1	3.0	0.239
Dose											
	Control	1548	147	1026^{b}	98.0^{b}	10.8^{a}	680	104^{b}	448^{b}	68.0^{b}	7.05^{a}
	Low	1531	163	1118^{a}	120^{a}	9.76^{ab}	758	133^{a}	552^{a}	97.0^{a}	6.02^{b}
	High	1572	179	1151^{a}	130^{a}	9.30^{b}	777	137^{a}	566^{a}	100^{a}	5.84^{b}
	SEM	38.6	4.4	30.2	3.60	0.397	35.9	5.3	27.0	3.70	0.293
Line × Dose											
	Control	1510	142^{b}	1164	110	11.0	647	97.0	498	74.0	6.92
	Low	1513	162^{b}	1280	137	9.63	723	126	610	107	6.20
Slow	High	1507	160^{b}	1279	136	9.80	735	128	625	109	5.87
	Control	1586	153^{b}	888	86.0	10.6	713	111	399	62.0	7.19
	Low	1548	164^{b}	956	102	9.88	793	141	494	88.0	5.84
Fast	High	1638	197^{a}	1023	124	8.79	820	146	508	90.0	5.82
	SEM	54.6	6.2	42.8	5.10	0.561	50.8	7.50	38.2	5.20	0.413
Source		Probabilities									
Line		0.077	**0.002**	**<0.001**	**<0.001**	0.406	0.085	**0.016**	**0.001**	**<0.001**	0.887
Dose		0.750	**<0.001**	**0.016**	**<0.001**	**0.029**	0.148	**<0.001**	**0.008**	**<0.001**	**0.013**
Line × Dose		0.690	**0.021**	0.726	0.083	0.541	0.978	0.978	0.964	0.744	0.756

[a, b]Means within a sub-column that do not share a common superscript are significantly different ($P < 0.05$).

Abbreviations: VH, villus length; VH/BW, villus height relative to BW; CD, crypt depth; CD/BW, crypt depth relative to BW; VCR, villi height: crypt depth ratio; BW, body weight at dissection.

D13 pi equates to d26 of age

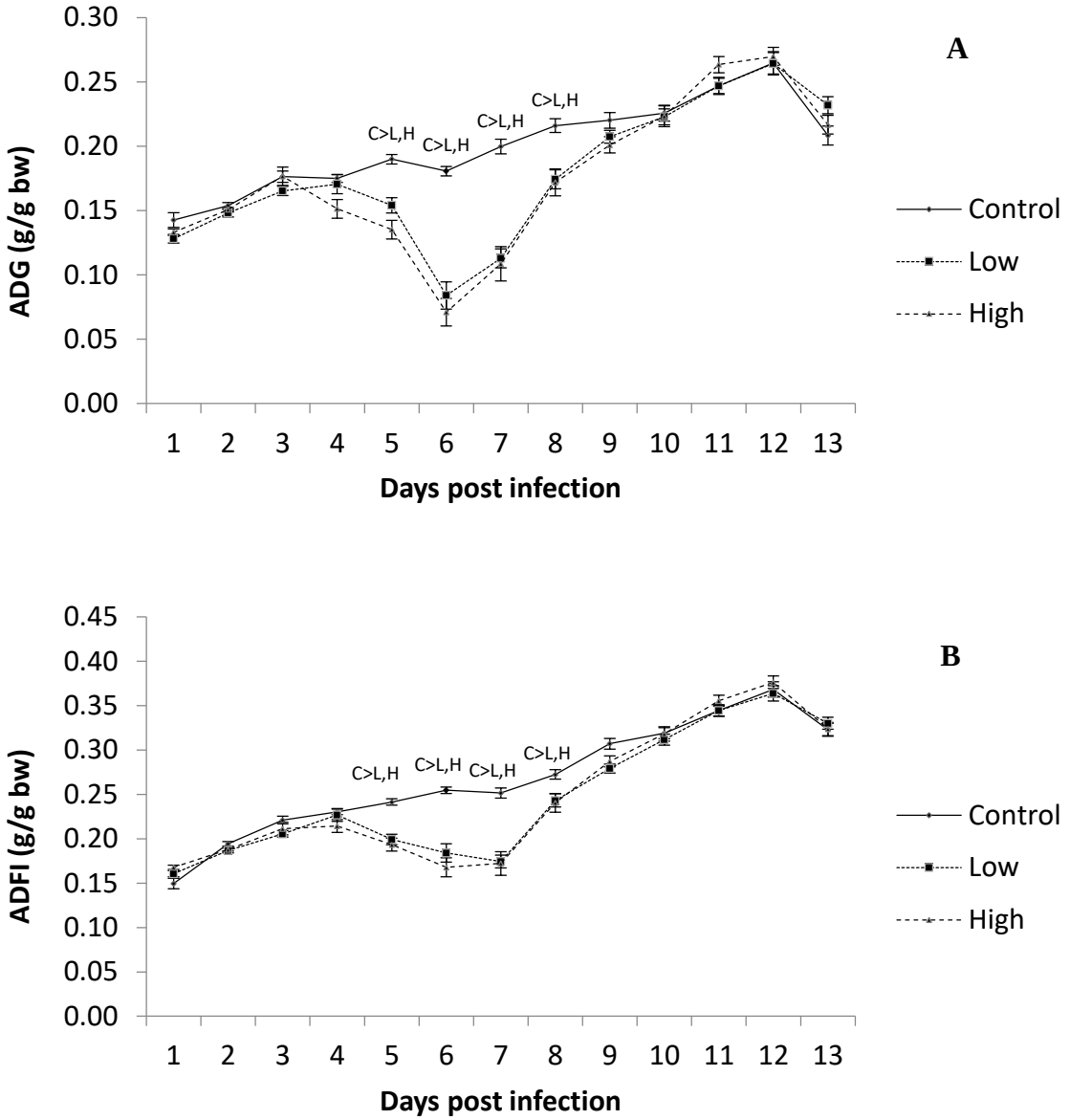

Figure 2. 1 Main effect of parasite dose on ADG (A) and ADFI (B) from d1 to 13 post-infection (pi) for broiler chickens, orally inoculated with 0 (Control), 2500 (Low) or 7000 (High) sporulated oocysts of *E. maxima* on d13 of age. Values were expressed in proportion to body weight (BW) at inoculation and analysed using repeated measurements mixed procedure. C > L,H represents a significant difference ($P < 0.05$) between control and infected (L and H) birds at the corresponding day pi.

Table 2. 7 Effects of line and infection dose and their interaction on tibia and femur length, width, breaking strength, dry defatted bone and ash, expressed relative to body weight (BW), and ash percentage, weight/length (seedor) index and robusticity index of broiler chickens of either a fast or a slow-growing line, **at d6 post-infection** (pi) with *E. maxima*. Broilers were orally inoculated with 0 (Control) 2500 (Low) or 7000 (High) sporulated oocysts of *E. maxima* on d13 of age.

		Tibia								Femur							
		Length/ BW (cm/g)	Width/ BW (cm/g)	Breaking strength/ BW (N/g)	DDB/ BW (g/g)	Ash/BW (g/g)	AP (%)	SI (mg/mm)	RI (mm/mg)	Length/ BW (cm/g)	Width/ BW (cm/g)	Breaking strength/ BW (N/g)	DDB/ BW (g/g)	Ash/BW (g/g)	AP (%)	SI (mg/mm)	RI (mm/mg)
Line																	
	Slow	105	7.22	212	2.65	1.13	42.6	72.5	4.18	78.9	8.04	194	1.82	0.805	44.3	69.2	3.50
	Fast	82.2	5.89	197	2.53	1.07	42.2	90.2	4.03	62.5	6.28	181	1.75	0.778	44.4	83.8	3.43
	SEM	0.92	0.143	10.0	0.045	0.018	0.45	1.27	0.019	0.72	0.125	6.2	0.029	0.0151	0.42	1.06	0.016
Dose																	
	Control	88.0^{b}	6.14^{b}	213	2.56	1.09	42.8	83.4	4.09	66.2^{b}	6.66^{b}	204^{a}	1.78	0.809	45.4^{a}	79.9^{a}	3.42^{b}
	Low	96.1^{a}	6.81^{a}	196	2.62	1.11	42.5	80.5	4.11	72.7^{a}	7.40^{a}	172^{b}	1.80	0.786	43.6^{b}	75.4^{b}	3.48^{a}
	High	96.4^{a}	6.71^{ab}	204	2.59	1.09	41.8	80.0	4.11	73.1^{a}	7.42^{a}	186^{ab}	1.77	0.779	43.9^{ab}	74.1^{b}	3.49^{a}
	SEM	1.12	0.176	12.2	0.055	0.022	0.55	1.56	0.024	0.89	0.153	7.6	0.036	0.0185	0.51	1.30	0.019
Line × Dose																	
	Control	98.2	6.58	209	2.59	1.12	43.3	74.5	4.15	73.5	7.35	209	1.79	0.813	45.4	71.6	3.46
Slow	Low	108	7.66	205	2.69	1.15	43.0	71.9	4.18	81.2	8.36	180	1.85	0.812	43.9	68.2	3.51
	High	108	7.42	222	2.67	1.10	41.4	71.1	4.19	82.0	8.4	191	1.81	0.788	43.6	67.7	3.53
	Control	77.8	5.69	218	2.52	1.07	42.3	92.4	4.03	58.9	5.98	173	1.77	0.805	45.5	88.2	3.39
Fast	Low	84.3	5.96	188	2.55	1.07	42.1	89.2	4.03	64.3	6.43	163	1.75	0.759	43.4	82.7	3.45
	High	84.5	6.00	185	2.51	1.07	42.2	88.9	4.02	64.3	6.43	181	1.74	0.769	44.2	80.5	3.45
	SEM	1.59	0.248	17.1	0.078	0.033	0.77	2.20	0.034	1.26	0.216	10.8	0.050	0.0262	0.71	1.83	0.028
Source		Probabilities															
Line		**<0.001**	**<0.001**	0.296	0.064	**0.030**	0.540	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.147	0.122	0.217	0.889	**<0.001**	**0.003**
Dose		**<0.001**	**0.020**	0.629	0.732	0.711	0.391	0.256	0.827	**<0.001**	**0.001**	**0.019**	0.885	0.477	**0.038**	**0.008**	**0.047**
Line x Dose		0.469	0.265	0.422	0.844	0.761	0.446	0.989	0.806	0.469	0.308	0.937	0.706	0.672	0.727	0.597	0.977

[a, b]Means within a sub-column that do not share a common superscript are significantly different ($P < 0.05$).

Abbreviations: SI, Seedor index; RI, Robusticity index; BW, body weight at dissection; DDB, dry defatted bone weight; AP, ash percentage.
D6 pi equates to d19 of age

Table 2. 8 Effects of line and infection dose and their interaction on tibia and femur length, width, breaking strength, dry defatted bone and ash, expressed relative to body weight (BW), and ash percentage, weight/length (seedor) index and robusticity index of broiler chickens of either a fast or a slow-growing line, **at d13 post-infection** (pi) with *E. maxima*. Broilers were orally inoculated with 0 (Control) 2500 (Low) or 7000 (High) sporulated oocysts of *E. maxima* on d13 of age.

		Tibia								Femur							
		Length/ BW (cm/g)	Width/ BW (cm/g)	Breaking strength/ BW (N/g)	DDB/ BW (g/g)	Ash/BW (g/g)	AP (%)	SI (mg/mm)	RI (mm/mg)	Length /BW (cm/g)	Width/ BW (cm/g)	Breaking strength/ BW (N/g)	DDB/ BW (g/g)	Ash/BW (g/g)	AP (%)	SI (mg/mm)	RI (mm/mg)
Line																	
	Slow	71.2	4.83	191	2.55	1.15	44.9	105	4.16	52.9	8.04	175	1.88	0.821	43.8	99.6	3.47
	Fast	52.6	3.83	194	2.40	1.08	45.1	123	3.98	39.8	6.25	152	1.72	0.750	43.7	119	3.34
	SEM	0.58	0.079	7.2	0.042	0.019	0.21	2.4	0.028	0.50	0.152	6.4	0.033	0.0118	0.42	2.02	0.022
Dose																	
	Control	59.6^{b}	4.24	215^{a}	2.52	1.15^{a}	45.8^{a}	121^{a}	4.05	43.9^{b}	6.70^{b}	168	1.80	0.809^{a}	45.1^{a}	113	3.39
	Low	63.2^{a}	4.44	189^{ab}	2.53	1.13^{ab}	44.7^{b}	115^{ab}	4.03	47.3^{a}	7.42^{a}	162	1.86	0.794^{ab}	42.7^{b}	107	3.40
	High	62.9^{a}	4.31	174^{b}	2.37	1.05^{b}	44.4^{b}	106^{b}	4.12	47.9^{a}	7.33^{ab}	160	1.74	0.754^{b}	43.4^{b}	107	3.42
	SEM	0.71	0.097	8.8	0.051	0.024	0.26	3.0	0.034	0.62	0.186	7.8	0.041	0.1450	0.51	2.5	0.027
Line × Dose																	
	Control	68.4	4.72	210	2.62	1.19	45.4	111	4.14	49.9	7.59	180	1.89	0.858	45.5	103	3.44
Slow	Low	72.9	5.06	200	2.64	1.19	45.2	106	4.12	54.1	8.46	186	1.98	0.846	42.9	99.8	3.45
	High	72.4	4.72	164	2.38	1.05	44.1	97.4	4.22	54.6	8.08	160	1.78	0.760	42.9	95.8	3.52
	Control	50.8	3.77	221	2.41	1.18	46.2	131	3.97	38	5.81	157	1.70	0.760	44.8	124	3.34
Fast	Low	53.6	3.82	179	2.42	1.07	44.3	124	3.94	40.5	6.39	139	1.76	0.743	42.4	115	3.35
	High	53.4	3.90	183	2.36	1.05	44.7	115	4.02	40.8	6.55	161	1.71	0.749	43.9	118	3.33
	SEM	1.00	0.137	12.4	0.072	0.032	0.37	4.2	0.059	0.87	0.263	10.9	0.058	0.0205	0.72	3.5	0.038
Source								Probabilities									
Line		**<0.001**	**<0.001**	0.764	**0.014**	**0.021**	0.593	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.014**	**0.001**	**<0.001**	0.925	**<0.001**	**<0.001**
Dose		**0.001**	0.351	**0.007**	0.062	**0.011**	**0.001**	**0.003**	0.165	**<0.001**	**0.017**	0.748	0.109	**0.030**	**0.006**	0.115	0.608
Line x Dose		0.703	0.281	0.237	0.309	0.182	0.052	0.945	0.950	0.504	0.586	0.104	0.398	0.052	0.437	0.557	0.396

[a, b]Means within a sub-column that do not share a common superscript are significantly different ($P < 0.05$).

Abbreviations: SI, Seedor index; RI, Robusticity index; BW, body weight at dissection; DDB, dry defatted bone weight; AP, ash percentage.
D13 pi equates to d26 of age

2.5 Discussion

The hypothesis of the present study that resistance and tolerance to *Eimeria maxima* infection would be affected by genetic selection for faster growth rate in modern broiler lines was based on the prioritised allocation of nutrient resources during periods of scarcity (Kyriazakis and Houdijk, 2006). Normally, broilers exposed to parasites like *Eimeria species* for the first time experience anorexia (Kyriazakis, 2014) which limit nutrient availability (Preston-Mafham and Sykes, 1970), and are expected to prioritise the acquisition of immunity over growth functions in allocating scarce nutrient resources. However, genetic selection for growth rate may cause the diversion of scarce resources towards growth functions at the expense of immunity and other vital functions (Kyriazakis and Houdijk, 2006) for selected birds. To that end, two broiler lines differing more than 25% in their growth rates were experimentally infected with *E. maxima* to limit availability (Blake and Tomley, 2014) and utilisation (Allen and Fetterer, 2002b) of nutrients. Under the hypothesis test herein, the fast-growing line would be expected to divert fewer nutrient resources towards health functions and suffer more the detrimental effects of infection as a consequence only if it exhibits a higher level of anorexia in comparison to the slow-growing line. In which case, increasing pathogen doses would lead to a higher degree of resource limitation for the fast than the slow line broilers.

In actual sense, it is doubtful that monospecific coccidian infections would occur in broiler production systems. Nevertheless, *E. maxima* infection was used in the present study as a model to test the above hypothesis because it is one of the commonly encountered coccidia spp, has widespread effects across the intestinal tract and is the most pathogenic malabsorptive coccidia spp limiting nutrient absorption (Cervantes, 2002). The magnitude of its effects was judged by the degree of tissue damage and inflammation (Lillehoj and Trout, 1996; Williams, 2005a). Reductions in growth performance typically occur around the period of maximum parasite schizogony and gametogony (Hein, 1968), which coincides with shortening of the villi and enlargement of crypts with a decrease in villi height to crypt depth ratio (VCR) (Kettunen *et al.*, 2001).

In the present study, variation in parasite replication due to differential resistance between F and S lines was assessed using quantitative real-time PCR to measure parasite genome copy number in tissues surrounding Meckel's diverticulum. The method supported higher throughput analysis and allowed the broilers to be maintained under conditions similar to that of commercial broiler farms throughout the study. The approach also minimised the impact of variation related to the temporal manner of oocyst excretion (Blake *et al.*, 2006). Quantitative

real-time PCR has previously been used to define variation in parasite replication in chicken lines with a known polymorphism in their resistance to *E. maxima*, revealing the most significant differences five and six days after infection. In this study, predicted parasite copy number at d6pi in line F and S birds was similar, disproving the hypothesis that a fast-growing line may show reduced resistance. Moreover, there were no significant differences observed amongst birds from lines S and F that received the L or H doses: likely illustrating the crowding effect on parasite replication (i.e. parasite fecundity reduces after a 'threshold' has been reached) (Williams, 2001).

Williams (2001) confirmed the possibility that crowding effects may occur during *E. maxima* infection in broilers. A basic generic model had predicted the occurrence of crowding effect during *E. maxima* infection for broiler chickens dosed at 1000 to 10000 sporulated oocysts (Johnston *et al.*, 2001). The model simulated the reduced availability of host cells as the cause of the crowding effect. However, other factors such as immune activities i.e. primary immune response (Rose *et al.*, 1984; Elaine Rose *et al.*, 1985; Rose *et al.*, 1992) and parasite-derived inhibitory toxins have been implicated as causes of reduced fecundity with a resultant crowding effect during *Eimeria* infections (Brackett and Bliznick, 1952; Smith and Hayday, 2000).

In the current study, infection reduced the growth performance of broilers from both F and S lines, but the focus was on whether the degree of reduction was higher for the faster-growing broiler line. During the pre-patent and recovery periods of infection, the degree of effect on growth performance for control and infected birds was similar in each line. This implies that there was no capacity for compensatory growth for infected birds within the duration of this study and agrees with previous findings (Gabriel *et al.*, 2006). However, during the acute phase (d4 - 8 pi), ADG and ADFI were significantly reduced for infected than control birds, though the degree did not differ in magnitude between lines. The FCR of infected birds was generally impaired, but the values implied a better feed utilisation for the F than the S line during the acute phase of infection. The observed reductions in performance during the acute stage of infection coupled with damage to the gastrointestinal mucosa of the duodenum and jejunum at d6 pi may account for the poor FCR over the acute period of infection. Indeed, reduction of absorptive area and costs of repair of damaged tissues reduce the efficiency of feed digestion and its utilisation by the host (Persia *et al.*, 2006; Sandberg *et al.*, 2007). The pathological effects of infection persisted at d13 pi as birds displayed increased crypt depth and reduced VCR at both intestinal sites.

On the aspect of intestinal lesions, severity was the same for the infected fast- and slow-growing broilers in this study based on the lesion score analysis. However, contrary to a general notion that *E. maxima* infects the jejunum (Cervantes, 2002), there was a spread of lesions to the duodenum and ileum sections of the small intestine in this study although scores for the jejunum were highest. This uncovers the potentials for a wider sphere of intestinal damage, which could as well mean a more significant effect on the absorptive capacity for young broilers infected with *E. maxima*. Also, although it is argued that evaluating gross lesions employed herein can underestimate the magnitude of *E. maxima* infection in broilers (Idris *et al.*, 1997a), the results presented herein are consistent with the observed levels of parasite replication showing no difference between the fast and the slow-growing broilers irrespective of dose.

A marked reduction in plasma carotenoids level was observed during the acute phase of infection, which is characteristic of coccidian infections affecting the proximal intestine (Allen, 1992; Zhu *et al.*, 2000; Hernández-Velasco *et al.*, 2014). Similar reductions were observed for Vit E in previous studies (Allen and Fetterer, 2002c; Allen and Fetterer, 2002b). However, the interaction between line and dose on plasma Vit E levels at the recovery phase in this study, such that F compared to S birds inoculated with L dose had relatively higher levels, supports an improved gut absorptive capacity for the F birds (Tallentire *et al.*, 2016). The reduction in plasma concentration of the aforementioned metabolites are attributed to malabsorption caused by the damage to the gastrointestinal mucosa (Allen and Fetterer, 2002c; Allen and Fetterer, 2002b), leading to defective fat absorption (Sharma and Fernando, 1975; Adams *et al.*, 1996a), and to oxidation by reactive oxygen species (Allen, 1997b). In the present study, these effects persisted to d13 pi for carotenoids compared to the effects on Vit A, which may have increased in concentration as a result of its release by the liver (Harrison, 2005). The significantly elevated level of NO metabolites at d6 pi was expected in *Eimeria* infections. An upregulation of NO production, predominantly from macrophages upon stimulation by pro-inflammatory cytokines, is commonly observed in the acute phase of *Eimeria* infection with their level depending on the severity of the infection (Allen, 1997b; Allen, 1997a; Sild and Hõrak, 2009). NO metabolites facilitate parasite killing (Lillehoj and Li, 2004), but their excessive production contributes to the pathology of *E. maxima* (Allen and Fetterer, 2002b) infections due to oxidative damage. Previous studies showed that their concentration is negatively correlated with ADG and carotenoid concentration at d6 pi (Zhu *et al.*, 2000).

A significant problem associated with selection for increased growth rate in modern broilers is skeletal disorders caused by reduced bone mineralisation (Angel, 2007). Previous studies established a strong correlation between growth-rate / live-weight and lameness, which is traceable to altered body conformation from the rapid growth of pectoral (breast) muscle displacing the centre of gravity cranially, and the relatively short legs in modern broilers (Kestin *et al.*, 2001; Corr *et al.*, 2003b). Also, studies using *E. acervulina* revealed a reduction in calcium (Ca) and mineral absorption and retention during the recovery phase, depending on the severity of infection (Turk, 1973; Takhar and Farrell, 1979). *E. acervulina*-infected starter chicks (i.e. infected at d2 - 8) exhibited reduced bone ash, bone Ca content, or Ca:P ratio (Giraldo *et al.*, 1987; Ward *et al.*, 1990; Watson *et al.*, 2005). Furthermore, it was reported that a combined infection of day-old chicks with *E. acervulina* and *E. tenella* via seeded litter led to an adverse effect on bone breaking strength (BS) by d21 of age (Shaw *et al.*, 2011). However, the BS data in Shaw *et al.* (2011) were not adjusted for the reduction in broilers' BW following infection, and the timing of effects, touching parasite cycle, was unknown as a natural infection model was utilised. Infections with either *E. maxima* or *E. acervulina* are known to reduce bone mineral content at d6 pi from a recent study (Fetterer *et al.*, 2013), but their effects at later time points were not examined. The present study appears to be the first to investigate effects on bone mineralisation at the acute (d6 pi) and recovery (d13 pi) time points over the course of *E. maxima* infection in broilers.

The results presented herein revealed that *E.maxima*-induced penalties on long bone mineralisation persisted across time points, were more pronounced during the recovery phase, and that the femur was affected earlier than the tibia. The differential effects on femur and tibia were in line with the different rate of mineralisation reported for these bones in growing broilers (Applegate and Lilburn, 2002). Furthermore, the significantly impaired tibia and femur mineralisation by d13 pi meant that upon recovering from a limitation of nutrient resources as a result of coccidiosis, bone development lags behind tissue accretion. This can rightly be interpreted to mean the addition of extra pressure on the under-developed skeletal framework or bone quality of modern fast-growing broilers following recovery from malabsorptive *Eimeria* infections.

Regarding long bone linear growth, the slow-growing line had longer, wider and denser tibias and femurs, which yielded more dry weight and ash weight across the two sample points, apart from femur ash weight which was initially similar between the two lines. These changes likely reflect the influence of the selection criteria of the maternal lines of the fast-growing broilers and the effect they exert on body conformation traits, including breast

muscle development (Corr *et al.*, 2003a). On the other hand, tibia and femur ash percentage were similar amongst the two genetic lines suggesting that they had similar levels of hydroxyapatite formation and bone maturation over the grower phase. It also indicates that incorporation of markers in modern schemes can potentially eliminate the impact of growth rate on bone ash percentage and they have been shown to exhibit high heritability (De Verdal *et al.*, 2013). Despite the lack of differences in bone ash percentage between lines, femur strength, which is a marker of mineralisation was higher for the slow- than the fast-growing line at d26 of age. This was expected because the more massive breast muscle with the corresponding higher BW and ADG imposed by the forward shift of the centre of gravity in fast-growing broiler lines (Corr *et al.*, 2003b), directly challenges the integrity of femur. Therefore, the quality of tibia and femur should be assessed separately in genetic schemes aiming at improving leg health

Over the post-infection period, the two lines differed by 30% in their final body weight at d26 of age (d13 pi) while they differed by 7% in their FCR, according to expectations. However, contrary to the hypothesis of the present study, the impact of infection, independent of dose magnitude, was similar between lines, apart from the feed efficiency of the S differing from the F line. A recent review (Tallentire *et al.*, 2016) summarising effects of selection for performance on the digestive physiology of broilers, stated that selection for growth rate has reduced the size of the gastrointestinal tract (GIT), but is accompanied by increased surface area due to greater intestinal villi size. Although the present study did not assess the relative size of GIT, when villi height was expressed relative to BW, F line birds had shorter villi than S line birds. This finding, coupled with the lower FCR of F line birds, suggests that they could absorb nutrients more efficiently, potentially by differential expression of nutrient transporters as there is considerable variability among different lines of chicken on the level of mRNA expression of nutrient transporters in the small intestine (Gilbert *et al.*, 2007). Furthermore, in the presence of infection, a lower impact on FCR in F line birds than S line birds could be attributed to a proportionally smaller GIT and the concomitant lower energetic and nutrient costs which would accompany the repair of damaged intestinal tissue (Sandberg *et al.*, 2007).

In the current study, although F and S broilers originated from the same paternal lines, bred under identical husbandry conditions to reduce factors of variation, the observed lack of difference between lines in relation to resistance to infection may be attributed to certain factors. It is believed that less robust phenotypes with altered immune functions and less resistance and tolerance to infection evolved from single trait productivity inclined selection in earlier genetic schemes, which led to unwanted consequences for traits that were not

selected for (Yunis *et al.*, 2000; Havenstein *et al.*, 2003; Van der Most *et al.*, 2011; Hocking, 2014). This is why modern poultry industry employs multi-trait selection schemes encompassing functional traits in the selection programmes (Hocking, 2014) which allows improvement in performance alongside health-related traits as part of a balanced breeding programme (Kapell *et al.*, 2012b). This probably is linked to previous finding that selection for immune functions is possible without compromising growth (Van der Most *et al.*, 2011), and may have been the reason as to why selection for growth rate did not affect the outcomes of the present study in relation to the resistance and ADG and ADFI when comparing these two genetic lines.

In summary, the focus of this study was not to look at immune pathways, but at the impact of a given pathogen on resistance and tolerance, and by proxy to account for their immunocompetence. Faster growth rates in multi-trait selection schemes did not lead to reduced resistance or tolerance. Contrary to the hypothesis tested, FCR was better for the F line in the face of *E. maxima* infection. Ross Ranger is a relatively new genotype destined for slow-growing broiler markets, and its nutritional specifications are not different from Ross 308 (Aviagen personal communication), albeit they are expected to be lower due to the slower growth rate. Pathogen-induced anorexia may be sensitive to dietary nutrient adequacy, and this has implications for the differential response of fast vs slow-growing genotypes (Kyriazakis, 2010). Future studies should look into the differential effects of coccidiosis in lines differing in their efficiency of feed utilisation. The subsequent chapters of this thesis examined targeted nutritional modulations to ameliorate the consequences of *E. maxima* infection in the fast-growing line, especially those affecting the long bone quality.

Chapter 3: Effects of vitamin D source and supply level on vitamin D status, bone development, performance and intestinal morphology of coccidia-infected broilers

3.1 Summary

Broiler coccidiosis impairs fat-soluble vitamin status and long bone mineralisation [Chapter 2]. The hypothesis that broilers infected with coccidiosis would benefit from increased dietary supplementation with vitamin D (VitD) or with 25-hydroxycholecalciferol (OHD) instead of the conventional cholecalciferol (D3) was tested in a $2 \times 2 \times 2$ factorial experiment. A total of 336 male Ross 308 (fast-growing) chicks were randomly assigned to diets with low (L) or high (H) VitD levels (1000 vs 4000 IU/kg) supplemented with either D3 or OHD. At d11 of age, birds were orally inoculated with water (control; C) or 7000 sporulated *Eimeria maxima* oocysts (infected; I). Each treatment group consisted of 6 replicate pens with 7 birds/pen. Pen performance was calculated over 14 days post-infection (pi) and for the early (d1 – 6 pi), acute (d7 – 10 pi) and recovery (d11 – 14 pi) periods pi. At the end of each period (d6, 10 and 14 pi), 6 birds per treatment combination were dissected to evaluate alterations in histomorphometric features, long bone quality, as well as levels of plasma OHD, Calcium (Ca) and Phosphorus (P). Parasite replication and gross lesions due to *E. maxima* infection were assessed at d6 pi. Performance, bone mineralisation and plasma OHD levels were significantly reduced during infection ($P < 0.05$). Offering L diets compromised feed efficiency pi, reduced femur breaking strength (BS) and plasma P levels at d10 pi in I birds. H diets or diets with OHD raised plasma OHD, improved performance and aspects of bone mineralisation. There was a significant 3-way interaction ($P < 0.05$) between VitD source, supply level and infection status on tibia ash weight in proportion to BW (ash/BW) at d14 pi; I compared to C broilers had significantly lower ($P < 0.05$) values only amongst those receiving the LD3 and HOHD treatment combinations. VitD level interacted with infection status on FCR ($P < 0.05$) calculated over the period of infection (d1 – 14 pi) such that I compared to C birds showed inferior FCR, which was more pronounced amongst broilers receiving the L than the H diets. VitD level and infection status interacted ($P < 0.05$) similarly for femur BS at d6 pi and plasma P at d10 pi; I compared to C birds receiving L diets had significantly lower value whilst H-fed I and C birds had similar values. Furthermore, VitD source and infection status interacted for plasma Ca levels ($P < 0.05$) at d10 pi such that values were higher in I than C birds receiving OHD diets but similar amongst D3-fed I and C broilers. As expected, VitD level and source interacted for OHD status ($P < 0.05$) across time points with birds receiving the high OHD diets having the highest values consistently.

Contrastingly, offering H diets or diets with OHD resulted in higher parasite loads ($P < 0.05$) at the acute phase of infection, which was accompanied by reduced jejunal villi height at d10 pi and significant atrophy in small intestine morphology. In conclusion, diets with 4000 IU/kg content or OHD improved broiler performance and bone mineralisation, irrespective of infection status. Diets with 4000 IU/kg VitD level further improved performance and mineralisation in the presence of coccidial infection. The positive correlation which appears to exist between increased VitD activity and higher parasite burden warrants further investigation.

3.2 Introduction

In broiler chickens, coccidia infections impair absorption and utilisation of fat-soluble vitamins (Allen and Fetterer, 2002a; Jafari *et al.*, 2012a) [Chapter 2] alongside other vital nutrients (reviewed in chapter 1). As a consequence, coccidiosis impairs bone growth and mineralisation (Watkins *et al.*, 1989; Watson *et al.*, 2005) [Chapter 2]. Malabsorptive coccidia species affecting broilers include *Eimeria acervulina, E. maxima E. mitis* and *E. praecox* (Blake and Tomley, 2014), but *E. maxima* is considered the most pathogenic and infects the largest region of the intestinal tract amongst these three species (Cervantes, 2002). Chapter 2 of this thesis suggests that *E. maxima* infection may impair the VitD status of growing broilers because other fat-soluble vitamins status (A and E), as well as bone mineralisation, were impaired in infected birds. According to previous studies, impaired digestibility and malabsorption of fat following coccidia infection may be linked to the lowered pH (< 5.0) of intestinal lumen (Russell and Ruff, 1978) which retards actions of pancreatic carboxyl ester hydroxylases responsible for hydrolysis of cholesterol and fat-soluble vitamins (Lombardo *et al.*, 1980; Mathias *et al.*, 1981). Fat malabsorption may also arise from qualitative changes to the absorptive surface due to villous atrophy as trapped globules of fat were seen in parasitised villous epithelial cells with a concomitant high fat concentration in voided excreta confirming impaired absorption (Sharma and Fernando, 1975; Allen, 1987).

Broiler diets especially under intensive management systems (usually no exposure to sunlight) needs to be fortified with VitD, and for many years cholecalciferol (D3) has been the conventional source. However, studies have shown that replacing D3 with OHD in diets can amplify VitD effects for broilers (Fritts and Waldroup, 2003; Fritts *et al.*, 2004; Fritts and Waldroup, 2005). A possible reason for this is that OHD absorption, compared to D3, is less fat dependent and approximately 1.5 times faster (Maislos *et al*., 1981). This implies that OHD may be the preferred VitD source during malabsorptive conditions such as coccidia

infections. Moreso, the fat independent absorption of OHD has been scientifically proven, as oral administration of OHD was employed to successfully treat osteomalacia in humans suffering from steatorrhea (Wagonfeld *et al*., 1976; Compston and Thompson, 1977; Reed *et al*., 1980). Other D3 metabolites such as 1,25-dihydroxycholecalciferol (1,25D3) (Rennie *et al*., 1993) and especially those with hydroxyl group in 1-C position (Honma *et al.*, 1983; Edwards, 1990) do have higher efficacy to mediate VitD effects compared to D3 (Edwards, 1989; Morris *et al.*, 2015).

Given that coccidian infections impair fat-soluble vitamin status and bone mineralisation in broilers (chapter 2), this study aimed to investigate dietary modulation of fat-soluble and bone-related VitD during coccidia infection. The modulation involved increasing dietary VitD supply, supplementing with a more efficiently absorbed metabolite (OHD) of D3, or both, during *E. maxima* infection. It was hypothesised that: 1) VitD status will be impaired in *E. maxima* infected birds. 2) Dietary supplementation with OHD will be more efficient than D3 in improving VitD status and bone mineralisation due to its higher metabolic potency; the effects will be more pronounced in infected broilers. 3) Improved VitD status may mediate improvements in ADG and FCR due to the reduction of the mucosal pro-inflammatory response which will be accompanied by alterations in gut morphology, and 4) Improved VitD status may impact positively on broiler resistance to coccidian infection as indicated by reduced parasite replication.

3.3 Materials and Methods

3.3.1 Birds, Husbandry and Diets

All procedures were conducted under the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU for animal experiments after obtaining the Home Office authorisation (P441ADF04). A total of 336 male day-old Ross 308 chicks were raised from d1 until 26 days old at the Newcastle University Cockle Park farm. Birds originating from parent flock subjected to similar husbandry were obtained from a local hatchery. Unlike chapter 2, the birds in this study and the experiments reported in chapters 4 and 5 of this thesis, were raised in a single round and housed in 48 rectangular 1.4 m x 0.6 m (0.84 m^2) pens arranged in a windowless, thermostatically controlled room. The pens were equipped with tube feeders and bell-drinkers and arranged such that the treatments were allocated uniformly to represent the different sides of the room in the various experiments. Wood shavings were used as litter in the pens to a depth of 5cm. Birds were offered *ad libitum* access to water as well as a starter (d0 – 10) and grower (d11 – 26) diets (Table 3.1). Routine husbandry procedures were as described in section 2.3.1 of chapter 2.

The diets were manufactured according to Aviagen nutrition specifications (Aviagen, 2014a) but with altered VitD content. Two sources of VitD (D3 and OHD) supplemented at two levels (low(L); 1000 and high(H); 4000 IU/kg of feed) were added to the diets. Hence, four different dietary treatments: LD3 (low level of D3; 1000 IU/kg), HD3 (high level of D3; 4000 IU/kg), LOHD (low level of OHD; 1000 IU/kg), HOHD (high level of OHD; 4000 IU/kg) were formulated. The choice of 1000 and 4000 IU/kg of feed as levels of VitD supply was jointly influenced by the commonly fed levels in the industry today ranging from 2000 to 4000 IU/kg for broilers (Fritts *et al.*, 2004), and the EFSA maximum authorised level of 5000 IU/kg (EFSA, 2012). Birds were randomly assigned to one of four diets upon arrival. The starter diet was offered in crumbled form and the grower and finisher diets in pelleted form.

3.3.2 Experimental design and inoculations

The experiment was a 2 × 2 × 2 factorial design with VitD level, source and infection status as the independent variables. Day-old birds were randomly assigned to treatment groups receiving 1000 or 4000 IU/kg level of dietary VitD supplementation, using D3 or OHD as sources of VitD activity, and two levels of infection (non-infected control group (C) vs infected group (I)). At d11 of age (d0 post-infection, pi) birds were orally inoculated with a single 0.5 ml oral dose of water (C) or 7000 (I) of sporulated *E. maxima* oocysts of the Weybridge strain using 1 ml syringes. The infection dose was selected based on previous experiments and aimed at avoiding severe clinical disease and prepared using a previously described method (Pastor-Fernández *et al*., 2018). Each treatment group consisted of 6 replicate pens with an initial stocking density of 7 birds/pen.

3.3.3 Sampling

Bird individual weight and pen feed intake were measured pi; at d0, d6, d10 and d14 pi. One bird per pen with a BW close to the pen average was selected at weighing on d6, d10 and d14 pi. The selected sampling days roughly represents the end of the early, acute and recovery periods, respectively, for broiler performance during *E. maxima* infection [chapter 2]. After weighing, the selected birds were blood-sampled via the wing vein and subsequently euthanised with a lethal injection of sodium pentobarbitone (Euthatal®, Merial Harlow, United Kingdom). Blood was collected in 5 ml sodium heparin plasma tubes (BD Vacutainer, SST II Advance Plus Blood Collection Tubes - BD, Plymouth, United-Kingdom), then immediately placed on ice and centrifuged for 10 mins at 1500 g at 4 °C within 1.5 h after collection. Aliquoted plasma samples were stored at -80 °C pending analyses.

During necropsy of each selected bird, the gastrointestinal tract was removed, and the duodenum, jejunum and ileum were scored for any lesions according to the method described by Johnson and Reid (1970). Following lesion scoring of the selected birds at d6 pi, 6 cm of intestinal tissue from the immediate region of Meckel's diverticulum, which is the mid-point of the intestinal area infected by *E. maxima* (Long *et al.*, 1976), was excised, opened longitudinally and digesta contents were gently removed. Thereafter, 5 cm of excised tissue was submerged in 7 ml bijous, and 1 cm proximal to the jejunum in 1.5 ml screw cap microtubes (Thermo Scientific) filled with RNAlater® (Life Technologies; Carlsbad, CA, USA). Samples were immediately stored at -80 °C pending analyses. Also, three 1 cm segments; one from the duodenal loop, one from mid-jejunum (midway between Meckel's diverticulum and the end of the duodenal loop), and one from mid-ileum (midway between Meckel's diverticulum and the ileocecal junction), were sampled from birds dissected on d6, 10 and 14 pi. They were fixed in 10% phosphate-buffered formalin maintained at a pH of 7.0 for histomorphometric assessment. Following intestinal tissue sampling, right femur and tibia were dissected and defleshed using scalpels, and stored at -20 °C in airtight individually labelled polythene bags pending evaluation.

3.3.4. Sample analysis

Experimental diet

Feed samples were analysed for VitD3 and OHD contents (see Table 3.2) at the DSM Laboratory (Basel, Switzerland) using a previously published method (Jakobsen *et al.*, 2007).

Morphometric analysis of gut

Excised, formalin-fixed intestinal sections from the duodenum, jejunum and ileum were dehydrated in increasing concentrations (50%, 75%, 95% and 100%) of ethanol followed by xylene in a Shandon™ Excelsior™ ES Tissue Processor (Thermo Fisher Scientific Inc., Waltham, Massachusetts). Tissues were oriented longitudinally and cross-sectionally before being embedded in paraffin wax, and 4 µm of sections were cut using a Leica microtome RM2235 (Mannheim, Germany). Slides left to dry overnight, deparaffinised in xylene, gradually rehydrated in ethanol (100%, 80%, 70% and 50%) and then rinsed in water. After that, slides were immediately stained for 5 mins in hematoxylin, rinsed with water and then counterstained in eosin for 3 mins.

After mounting slides in distyrene plasticiser xylene, they were left to settle and dry for two days before scanning with a Leica SCN400 slide scanner (Leica Microsystems, Germany), which has SlidePath Gateway Client Viewer 2.0 software version. Images were viewed to measure morphometric parameters of intestinal architecture at 20× magnification. From the stained sections,

the villus height (VH) and crypt depths (CD) were determined using ImageScope® software (Aperio Technologies, Vista, CA, USA). VH was estimated by measuring the vertical distance from the villus tip to the villus-crypt junction level and CD by measuring the vertical distance from the villus-crypt junction to the lower limit of the crypt (Figure 3.1). Ten villi with their corresponding crypts were measured per section to obtain their estimated lengths (micrometres, µm) in duodenal, jejunal and ileal samples of 6 birds per treatment combination.

Parasite replication

Quantitative real-time PCR (qPCR). Predicted *E. maxima* (Reid *et al.*, 2014) and *G. domesticus* (Furlong, 2005) genome sizes were used, and total genomic DNA (gDNA) from sporulated *E. maxima* oocysts and uninfected chicken intestinal tissue was extracted using the method of (Blake *et al.*, 2008). Ten-fold DNA dilution series was created using prescribed methods (Blake *et al.*, 2006; Nolan *et al.*, 2015), and other procedures were as described in section 2.3.4 of chapter 2.

Blood metabolites

Plasma concentrations of Ca and P (mmol/l) were analysed using ABX Horiba Pentra 400 automatic analyser (Horiba Medical, Irvine, CA, USA) in duplicate according to the manufacturer's instructions. Also, the plasma concentration of OHD (ng/ml) was determined using the 25-Hydroxy VitD Direct EIA kit (IDS Diagnostics, Fountain Hills, AZ, USA).

Bone evaluation

Bones were thawed at 4^{o}C in a fridge overnight. Following thawing, they were placed at room temperature for 1 hour before defleshing of the remaining adhering soft tissues using scalpels. Tibia and femur length, and diameter (maximum and minimum) at the centre of the diaphysis were measured using digital callipers and recorded. Fresh weight of each bone was measured using an analytical balance. Robusticity (Riesenfeld, 1972) and Seedor indices (Seedor *et al.*, 1991) were calculated using the formulas in section 2.3.4 of chapter 2. Bone breaking strength (BS) was also tested using an Instron testing machine (Instron 3340 Series, Single Column-Bluehill) and the method described in section 2.3.4 of chapter 2.

Following BS evaluation, tibia and femur bones were boiled for 5 minutes in deionised water at 100 °C to facilitate removal of cartilage caps, were split in two for manual removal of bone marrow, and then further hand-broken into smaller bits. Subsequently, bones were placed in vessels containing 10 ml of acetone (VWR) and 10 ml of Petroleum ether (VWR) and were subjected to fat extraction in a Mars 6 Microwave Assisted Reaction System 6 (CEM,

Matthews, USA) with a set temperature of 180 °C for 80 minutes. Fat-extracted bones were then placed in an oven at 105 °C for 18 hours, and the dry defatted bone weight was measured thereafter. After that, they were placed in a Phoenix CEM ashing microwave furnace (CEM, Matthews, USA) at 850 °C for 1.5 hours to obtain the ash weight (g). The above procedure was repeated for tibia and femur bones of 6 birds per treatment combination.

3.3.5 Calculations and statistics

All statistical analyses were carried out using IBM SPSS Statistics for Windows, Version 22.0. (Armonk, NY: IBM Corp). For all statistical assessments, the pen was considered the experimental unit and all variables were analysed with VitD level, source and infection status as main effects and their interactions using general linear model (GLM). Pen performance data included average BW pre-infection (d11 of age) and at the end of the experiment (d25 of age), as well as average daily feed intake (ADFI; g/d), average daily gain (ADG; g/d), and feed conversion ratio (FCR) calculated over the pre-infection (d0 – 11 of age) and post-infection (pi) periods (d1 – 14 pi). The performance was also measured for specific phases that roughly corresponds to the early (d1 – 6 pi), acute (7 – 10 pi) and recovery (d11 – 14 pi) periods pi. The Shapiro-Wilk test was used for assessing the normality of the studentized residuals and non-normalised data were log-transformed.

Data generated from birds sampled at d6, 10 and 14pi such as plasma levels of Ca, P and OHD, VH, CD and villi height: crypt depth ratio (VCR), qPCR for *E. maxima* genome copy number (parasite replication) at d6 pi, and all bone-derived measurements were also analysed using GLM and the aforementioned factors. When significant differences were detected, treatment means were separated and compared by the Tukey's multiple comparison tests. For assessing the normality of the studentized residuals, the Shapiro-Wilk test was used. Significance was determined at $P < 0.05$, and a tendency was defined as $0.5 < P < 0.1$. Intestinal lesion score was evaluated as described in section 2.3.5 of chapter 2.

Table 3. 1 Ingredients and analysed chemical composition of the starter (d0–10) and grower (d11–25) diets offered to broiler chickens.

Item	Starter	Grower
Ingredient (%)		
Wheat	47.9	51.6
Corn	10.0	10.0
Soybean meal (48 % CP)	32.0	25.3
Soybean full fat	4.0	7.0
Soy crude oil	1.84	2.32
Dicalcium phosphate	1.82	1.60
Limestone	0.64	0.67
Vitamin and mineral premix	0.40	0.40
DL methionine	0.33	0.30
L-Lysine	0.27	0.25
Sodium bicarbonate (27 %)	0.21	0.20
Sodium chloride (39 %)	0.19	0.20
L-Threonine	0.14	0.12
Choline chloride (60 %)	0.05	0.05
L-Valine	0.03	0.02
Nutrient composition (%) *		
ME (kcal/kg) (calculated)	3,000	3,100
Crude protein	23.1	21.4
Crude fat	5.03	4.87
Crude fibre	2.39	2.13
Ash	5.43	4.83
Calcium	1.03	0.80
Phosphorus	0.74	0.62
Available phosphorus (calculated)	0.48	0.44
Sodium	0.18	0.15
Manganese (mg/kg)	218.2	168.8

The nutrient composition was in accordance with Aviagen nutrient specifications (Aviagen, 2014a) apart from the VitD source and level of supply.
*Analysed nutrient composition (%) unless otherwise stated.

Table 3. 2 Analysed D3 and OHD contents (IU/kg of feed) of the dietary treatment: low level of D3 (LD3; 1000 IU/kg), low level of OHD (LOHD; 1000 IU/kg D), high level of D3 (HD3; 4000 IU/kg) and high level of OHD (HOHD; 4000 IU/kg).

VitD	Level	Starter	Grower
D3	Low	1560	1020
	High	4910	4520
OHD	Low	844	652
	High	2828	2720

Abbreviations: D3, cholecalciferol; OHD, 25-hydroxycholecalciferol

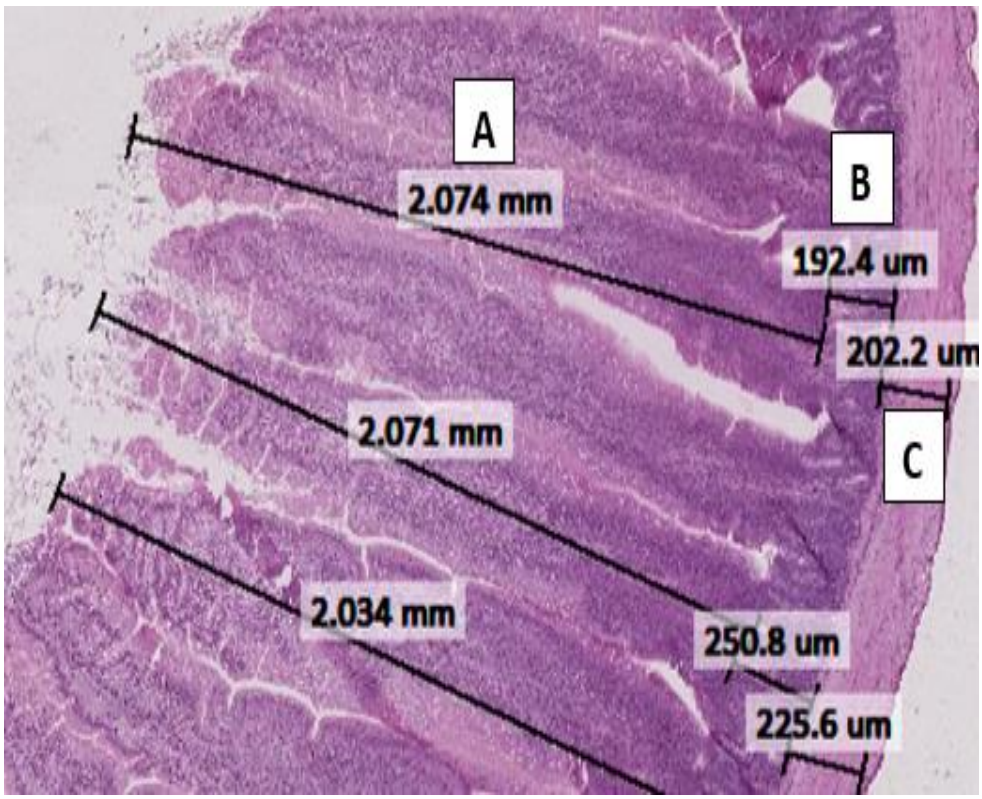

Figure 3. 1 Measurements for villi height (A), crypt depth (B) and serosa thickness (C) from duodenal section

3.4 Results

3.4.1 Bird health and performance variables

No bird was euthanised due to health-related disorders, and coccidiosis caused anorexia and reduced weight gain according to expectations. The effects of VitD source, level of supply and infection status on performance: BW, ADG, ADFI, and FCR pre- and post-infection are presented in Tables 3.3 and 3.4. There were no 3-way interactions between the experimental factors for performance variables pre- or post-infection.

Pre- and post-infection periods

No significant difference between treatment groups was detected for chick BW at placement (average 43.5 g; SEM = 0.41; $P > 0.1$). Performance data analysed over d0 to 11 of age show that there was no effect of VitD source on BW, ADG, ADFI and FCR pre-infection. However, dietary VitD level significantly affected ($P < 0.05$) ADG and ADFI pre-infection, as well as the BW at the end of the pre-infection period at d11 of age (d0 pi); birds receiving the H compared to the L diets exhibited superior performance.

On the other hand, growth performance analysed over the pi period (d1 – 14 pi) indicate that VitD level significantly interacted with infection for FCR ($P < 0.05$) pi, being poorest amongst L-fed infected birds (Figure 3.2). There were no other significant 2-way interactions between VitD level, VitD source and infection status for performance variables. As expected, infection significantly affected ($P < 0.001$) all performance variables measured over the infection period; ADG, ADFI, FCR and final BW at d14 pi.

There was a main effect of VitD level on final BW and FCR, and source on final BW, ADG and FCR. Birds on the H diets had significantly higher BW ($P < 0.05$) and lower FCR ($P < 0.001$) than birds on the L diets. Birds receiving OHD achieved higher BW ($P < 0.05$) and ADG ($P < 0.01$) and lower FCR ($P < 0.05$) than birds receiving D3.

Early, acute and recovery phases of infection

The main effects of VitD source, level of supply and infection status on ADG, ADFI, and FCR during an estimated early, acute and recovery phases of infection are presented in Table 3.4. Infection penalised ($P < 0.001$) ADG, ADFI and FCR over the early and acute phases of infection whilst there was no effect of infection on these variables during the recovery period. ADG, ADFI and FCR were not affected by VitD level or source during the recovery phase.

There was an effect of VitD source only on ADG during the acute phase and an effect of VitD level on ADG (acute) and FCR (early and acute) pi. Birds offered OHD achieved higher ADG ($P < 0.05$) than birds offered D3 diets. Birds on H diets had higher ADG ($P < 0.05$) and lowered FCR ($P < 0.05$) than birds receiving L diets.

3.4.2 Plasma Calcium, Phosphorus and OHD

The main effects of VitD level, VitD source and infection status on plasma levels of Ca, P and OHD over the timepoints pi are presented in Table 3.5, and all significant interactions are presented in Figures 3.3 and 3.4. There were no significant 3-way interactions between the experimental factors for plasma levels of Ca, P and OHD.

Calcium and Phosphorus status

VitD level and infection interacted ($P < 0.05$) on P level whilst VitD source and infection interacted on Ca status at d10 pi. To summarise, I compared to C birds had lower plasma P levels amongst L-fed broilers but similar P levels amongst H-fed broilers (Figure 3.3A). Also, plasma Ca levels were higher in I than C birds amongst OHD-fed but similar amongst D3-fed broilers (Figure 3.3B).

Infection significantly reduced ($P < 0.001$) levels of Ca and P only at d6 pi, which is the day of peak replication for *E. maxima* in infected broilers. VitD supply level affected ($P < 0.05$) plasma Ca level at d6 and 10 pi such that broilers on H compared to L diets had higher levels. Furthermore, VitD source had no significant effect ($P > 0.05$) on Ca and P levels at d6, 10 and 14pi (Table 3.5).

OHD status

VitD level and source interacted ($P < 0.001$) for plasma OHD levels at d10 pi. Broilers receiving the HD3 and LOHD diets had statistically similar plasma OHD levels, which were significantly higher ($P < 0.001$) than the levels in LD3 and lower ($P < 0.001$) than the levels in the HOHD-fed broilers (Figure 3.4A). Also, level and infection interacted for plasma OHD levels ($P < 0.05$) at d10 pi, being more significantly reduced ($P < 0.05$) for the control L than H birds compared to their infected L- and H-fed counterparts (Figure 3.4B). Furthermore, plasma levels of OHD were significantly affected ($P < 0.05$) at d6, 10 and 14 pi by VitD level, source of VitD supply and infection; being significantly higher at all time points in birds on OHD than birds on D_3 treatments, H than L VitD supply level, and in C than I birds respectively.

3.4.3 Histology and lesion scores

Histology

The main effects of VitD level, VitD source and infection on duodenum, jejunum and ileum are presented in Tables 3.6 – 3.8 respectively and all significant interactions are presented in Figures 3.5 – 3.7. There were no significant 3-way interactions between the experimental factors for histological measurements in this study. VitD level and source interacted ($P < 0.01$) for jejunal VH at d10 pi and jejunal VCR at d14 pi. Birds receiving D3 compared to OHD diets had longer villi ($P < 0.05$) at the low supply level, but VH remained statistically similar ($P > 0.05$) for birds receiving OHD and D3 diets at the high supply level (Figure 3.5A). Also, VCR was significantly lower ($P < 0.05$) for H compared to L D3-fed broilers whilst values were statistically similar for H and L OHD-fed broilers (Figure 3.5B). Level and infection interacted ($P < 0.01$) for jejunal VH at d10 pi with significantly longer VH for birds offered the L than the H diets amongst the infected birds, and statistically similar VH for L and H birds amongst the control birds (Figure 3.6). VitD source and infection interacted for ileal VH ($P < 0.05$) and VCR ($P < 0.01$) at d6 pi. To summarise, ileal VH and VCR values were significantly greater for C than I birds receiving OHD diets, whilst D3-fed C compared to I birds had statistically similar VH, and a smaller (though significant) difference in VCR compared to the difference in the VCR of their OHD-fed counterparts (Figures 3.7A and 3.7B, respectively).

Infection significantly decreased ($P < 0.05$) VH and VCR, and increased CD of the duodenum at d6 and 10 pi. At d14 pi effects persisted only on CD ($P < 0.05$) and VCR ($P < 0.01$). The same direction of effects, on the same days, was observed on histomorphological measurements of the jejunum and the ileum, albeit the ileal VCR was significantly affected ($P < 0.001$) only at d6 pi (Table 3.8).

Lesion score

No intestinal lesions were detected in C birds (score 0), so they were excluded from the regression model. The main effects of vitD level (L = 1.53 vs H = 1.69; SEM = 0.112), VitD source (D = 1.56 vs OHD = 1.67; SEM = 0.112), or their interaction, on small intestine lesions derived from an average score of the three individual sections was not significant ($P > 0.1$) amongst the infected broilers in this study.

3.4.4 Parasite replication

E. maxima genome copies were not detected in control birds. There was no significant interaction *(P > 0.05*) between VitD level and source for parasite replication at d6 pi, which was the only sampling point for this variable in this study and the day of peak parasite replication for *E. maxima* in infected broilers. However, the main effects of level and source of VitD supply significantly affected ($P < 0.05$) parasite replication. OHD compared to D3-fed broilers showed higher parasite burdens (11.5 vs 11.1; SEM = 0.08), whilst L compared to H level significantly reduced ($P < 0.05$) parasite burdens (11.6 vs 11.0; SEM = 0.08).

3.4.5 Bone variables

The main effects of VitD level, VitD source and infection on bone variables over the timepoints pi are presented in Tables 3.9 – 3.11 and all the significant interactions are presented in Figures 3.8 and 3.9.

Linear growth in proportion to BW

There were no 2- or 3-way interactions between the experimental factors on the length and width of femur or tibia expressed in proportion to BW at dissection in the current study. Infection increased femur and tibia length and width per BW at d6, 10 and 14 pi, which was an artefact of infection due to the significant weight loss. VitD level affected (*P < 0.05*) tibia and femur length per BW at d10 and 14 pi, and femur width per BW at d10 pi; values were lower for the H than the L birds. Length and width of tibia or femur were not affected by VitD source.

Breaking strength in proportion to BW

Level and infection interacted (*P < 0.05*) for the femur, but not tibia, BS expressed in proportion to BW at d6 pi. Amongst infected birds, femur BS/BW was significantly higher for birds receiving the H than the L VitD diets; this was not the case with the control birds, as values did not differ between birds receiving H and L diets (Figure 3.8). Furthermore, infected birds receiving the H diets had a statistically similar BS/BW as uninfected birds receiving either the H or the L diets.

Infection at d6 and 14 pi, and L diet at d10 pi significantly reduced ($P < 0.05$) femur BS/BW. Tibia BS/BW was significantly affected ($P < 0.05$) by VitD level at d6 pi and infection at d10 and 14 pi. Birds receiving H diets, and C birds had higher values compared to birds offered L diets and I birds respectively.

Weight/length (Seedor) and robusticity indices

Infection significantly affected Seedor index of both femur and tibia at all time points pi ($P < 0.05$). Robusticity index, on the other hand, was significantly affected ($P < 0.05$) for both femur and tibia at d6 and 14 pi but not at d10 pi. VitD level significantly affected ($P < 0.05$) femur Seedor and Robusticity indices at d10 pi but did not affect these variables for tibia. Similarly, VitD source significantly affected ($P < 0.05$) tibia seedor index at d6 pi and robusticity index at d14 pi but did not affect these variables for the femur. Other effects were not statistically significant ($P > 0.05$).

Ash in proportion to BW, and ash percentage

VitD level, VitD source and infection significantly interacted ($P < 0.01$) for tibia ash weight relative to BW (ash/BW) at d14 pi with I birds on the LD3 treatment displaying the lowest values (Figure 3.9). VitD source significantly affected ($P < 0.05$) tibia ash/BW only at d6 and 10 pi. The values were significantly higher for birds receiving OHD than D3 diets. The main effect of infection significantly reduced ($P < 0.001$) ash/BW only at d14 pi. There were no other statistically significant effects ($P > 0.05$) of VitD level on tibia ash/BW in this study.

Infection significantly reduced ($P < 0.05$) ash percentage at d6, 10 and 14 pi. VitD source affected ash percentage only at d10 and 14 pi; birds receiving D3 compared to OHD diets had the lower values consistently. Furthermore, H compared to L diets significantly increased ($P < 0.05$) percentage tibia ash at d10 and 14 pi, but not at d6 pi.

Table 3. 3 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken performance pre-infection (d0-11 days of age) and over the period (d1 - 14) post-infection (pi), body weight at the end of the pre- (d11 of age) and post-infection period (d25 of age). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi)**.**

		Body weight (g)		Average daily gain (g/d)		Average daily feed intake (g/d)		Feed conversion ratio	
	Period	Pre-infection	Post-infection	Pre-infection	Post-infection	Pre-infection	Post-infection	Pre-infection	Post-infection
	Day	d11 of age (d0pi)	d25 of age (d14pi)	d0-11 of age	d1-14pi	d0-11 of age	d1-14pi	d0-11 of age	d1-14pi
Level									
	1000 (IU/kg)	434	1570	35.5	80.7	39.4	122	1.11	1.48
	4000 (IU/kg)	444	1608	36.4	82.4	40.4	122	1.11	1.43
Source									
	D3	437	1563	35.8	79.9	39.7	121	1.11	1.47
	OHD	441	1614	36.1	83.1	40.0	123	1.11	1.44
Infection									
	Control	-	1729	-	91.8	-	130	-	1.36
	Infected	-	1448	-	71.2	-	114	-	1.57
	SEM	3.58	12.8	0.307	0.794	0.319	1.10	0.0001	0.0101
					Probabilities				
Level		**0.041**	**0.042**	**0.038**	0.128	**0.037**	0.800	0.225	**0.003**
Source		0.501	**0.008**	0.415	**0.007**	0.501	0.105	0.059	**0.018**
Infection		-	**<0.001**	-	**<0.001**	-	**<0.001**	-	**<0.001**
Level × Source		0.531	0.348	0.382	0.846	0.351	0.963	0.947	0.297
Level × Infection		-	0.954	-	0.578	-	0.322	-	**0.039**
Source × Infection		-	0.518	-	0.995	-	0.437	-	0.833
Level × Source × Infection		-	0.711	-	0.883	-	0.851	-	0.966

SEM: Pooled standard error of the mean.

See Figure 3.2 for graphical illustration of significant interaction ($P < 0.05$) between the experimental factors post-infection (pi).

The period from d1 to 14 pi equates to d12 to 25 of age

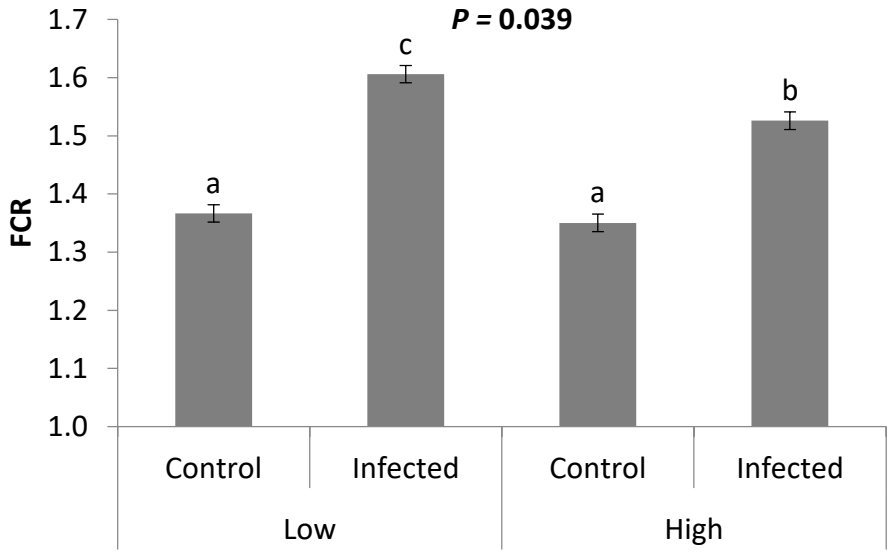

Figure 3. 2 Interaction between level of vitamin D supply that being low (1000 IU/kg) or high (4000 IU/kg) and infection status on feed conversion ratio (FCR) over the course of primary infection (d1-14 post-infection, pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d11 post-hatch.

Table 3. 4 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken performance over the early (d0 - 6), acute (d6 - 10) and recovery (d10 - 14) periods post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi)**.**

		Average daily gain (g/d)			Average daily feed intake (g/d)			Feed conversion ratio		
	Day pi	d0-6	d6-10	d10-14	d0-6	d6-10	d10-14	d0-6	d6-10	d10-14
Level										
	1000 (IU/kg)	64.8	77.9	109	91.5	116	160	1.43	1.55	1.47
	4000 (IU/kg)	67.2	82.3	108	91.1	117	158	1.37	1.47	1.47
Source										
	D3	65.5	77.1	106	90.6	113	158	1.40	1.54	1.49
	OHD	66.5	83.2	110	92	119	159	1.41	1.48	1.45
Infection										
	Control	76.2	102	107	96.9	134	158	1.27	1.32	1.49
	Infected	55.8	57.9	110	85.7	97.8	159	1.54	1.70	1.45
	SEM	0.844	1.53	1.82	1.06	2.00	1.93	0.0139	0.0268	0.017
						Probabilities				
Level		0.056	**0.047**	0.638	0.783	0.714	0.506	**0.004**	**0.035**	0.934
Source		0.409	**0.008**	0.096	0.379	0.068	0.650	0.952	0.075	0.087
Infection		**<0.001**	**<0.001**	0.242	**<0.001**	**<0.001**	0.716	**<0.001**	**<0.001**	0.135
Level × Source		0.247	0.540	0.362	0.379	0.803	0.586	0.355	0.690	0.110
Level × Infection		0.531	0.611	0.383	0.441	0.529	0.607	0.436	0.352	0.078
Source × Infection		0.474	0.606	0.216	0.545	0.650	0.220	0.129	0.272	0.568
Level × Source × Infection		0.353	0.461	0.653	0.620	0.849	0.566	0.358	0.506	0.997

SEM: Pooled standard error of the mean.
The period from d0 to 14 pi equates to d11 to 25 of age

Table 3. 5 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken plasma Ca and P concentration (mmol/l) and log-transformed plasma levels of OHD (ng/ml) at d6, 10 and 14 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi)

		Ca (mmol/l)			P (mmol/l)			Log OHD (ng/ml)		
	Day pi	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level										
	1000 (IU/kg)	2.56	2.79	2.75	2.05	1.96	1.98	1.51	1.46	1.59
	4000 (IU/kg)	2.66	2.89	2.77	2.08	2.01	2.05	2.05	1.88	2.1
Source										
	D3	2.61	2.82	2.74	2.05	1.95	1.97	1.62	1.45	1.67
	OHD	2.61	2.85	2.79	2.08	2.02	2.06	1.94	1.88	2.02
Infection										
	Control	2.81	2.81	2.73	2.2	2.01	2.04	1.84	1.94	1.88
	Infected	2.4	2.87	2.75	1.93	1.97	1.99	1.71	1.39	1.81
	SEM	0.032	0.034	0.0284	0.0466	0.0459	0.0360	0.0220	0.0230	0.0200
						Probabilities				
Level		**0.040**	**0.040**	0.593	0.730	0.442	0.161	**<0.001**	**<0.001**	**<0.001**
Source		0.861	0.564	0.185	0.675	0.296	0.090	**<0.001**	**<0.001**	**<0.001**
Infection		**<0.001**	0.194	0.109	**<0.001**	0.512	0.376	**<0.001**	**<0.001**	**0.019**
Level × Source		0.889	0.205	0.095	0.094	0.279	0.137	**<0.001**	**<0.001**	**<0.001**
Level × Infection		0.088	0.416	0.625	0.163	0.**046**	0.742	0.366	**0.033**	0.448
Source × Infection		0.34	**0.040**	0.511	0.287	0.833	0.665	0.253	0.283	0.838
Level × Source × Infection		0.259	0.877	0.326	0.307	0.521	0.534	0.31	0.571	0.844

SEM: Pooled standard error of the mean.

Abbreviations: Ca, Calcium; P, Phosphorus, OHD, 25-hydroxycholecalciferol

D6, 10 and 14 pi equates to d17, 21 and 25 of age respectively

See Figure 3.3 and 3.4 for graphical illustration of significant interaction ($P < 0.05$) between the experimental factors at d10 post-infection (pi).

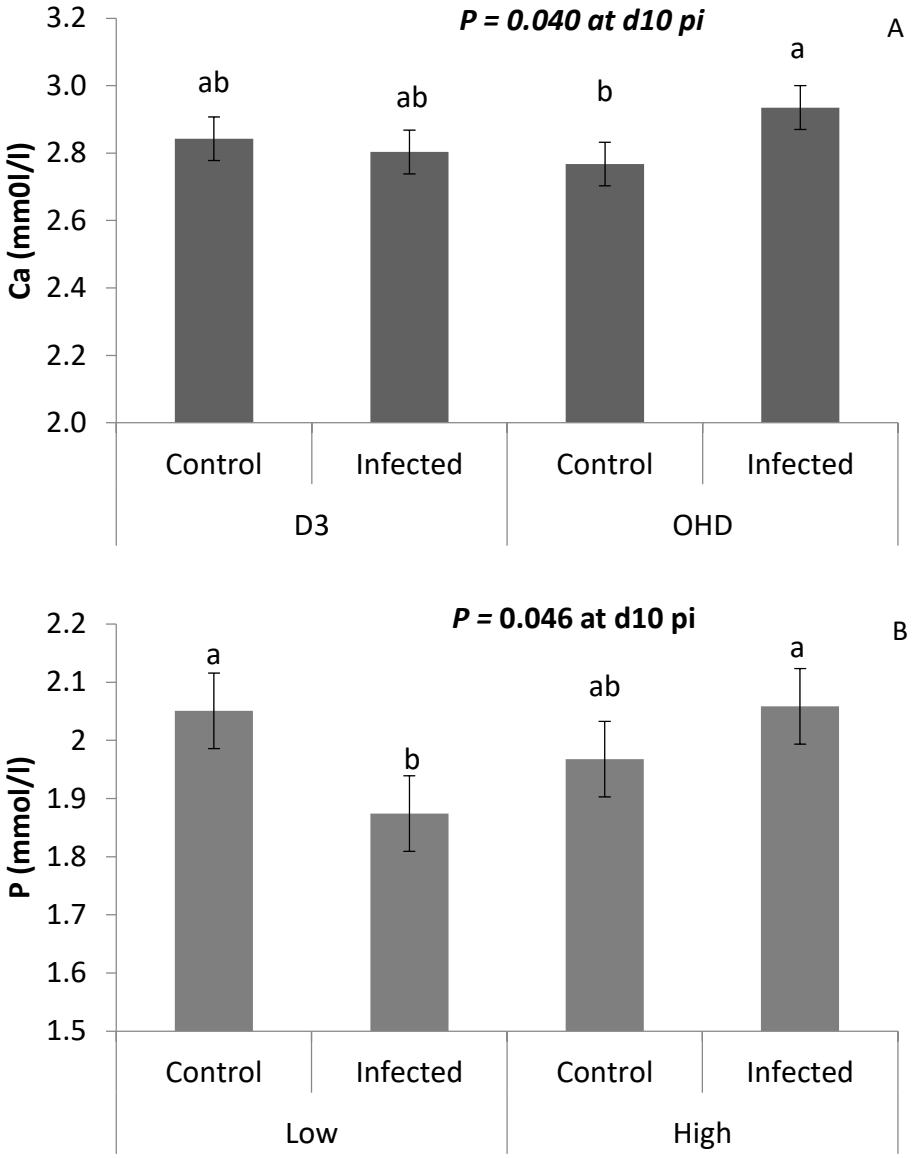

Figure 3. 3 (A) Interaction between source of vitamin D supply (OHD or D3) and infection status on plasma Ca concentration (mmol/l) and (B) between level of vitamin D supply that being low (1000 IU/kg) or high (4000 IU/kg) and infection status on plasma P concentration (mmol/l) at d10 post-infection. Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch.

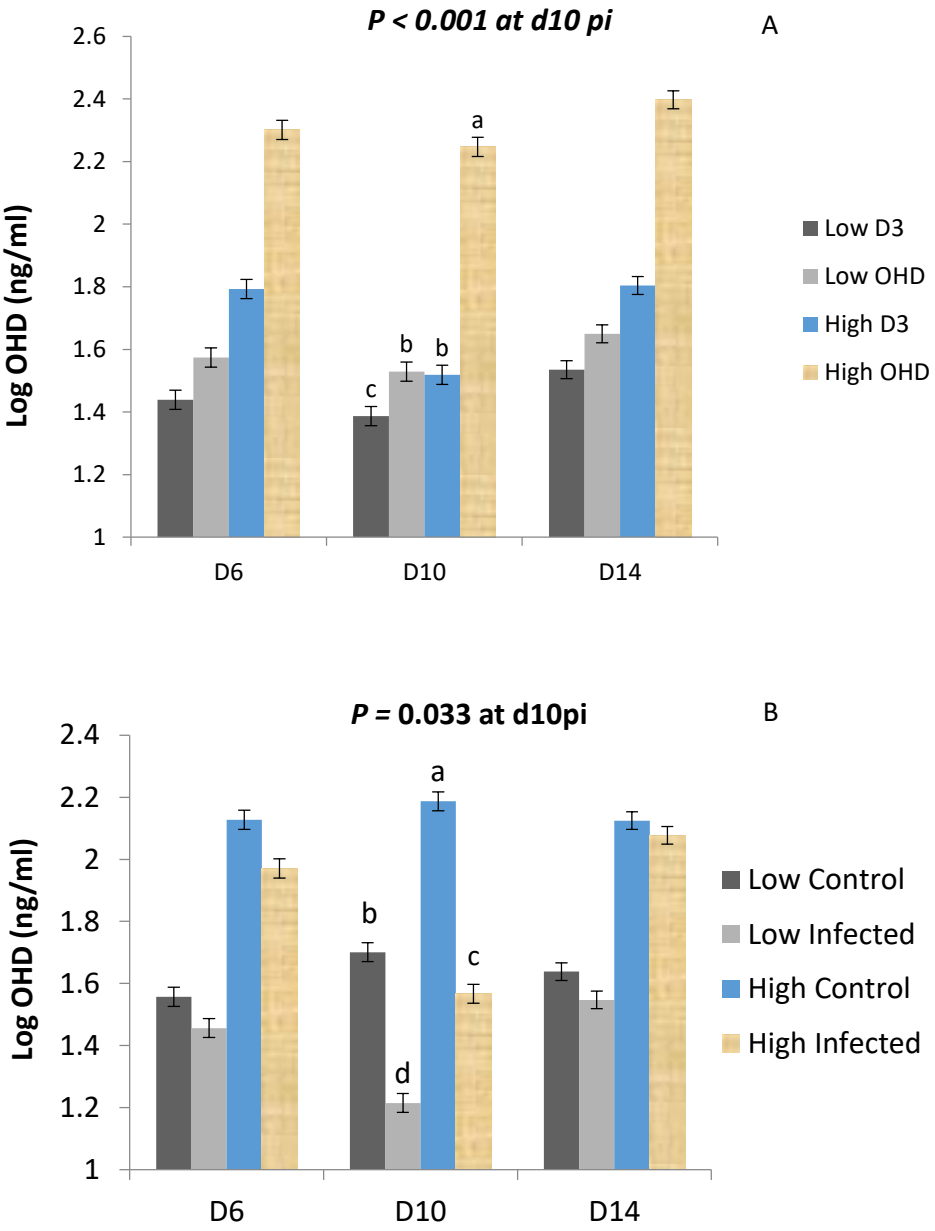

Figure 3. 4 (A) Interaction between low (1000 IU/kg) or high (4000 IU/kg) vitamin D supply and source (OHD or D3) and (B) between level and infection on log-transformed circulating levels of OHD at d10 post-infection. Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d11 post-hatch.

Table 3. 6 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken duodenum morphology on d6, 10 and 14 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi).

		Duodenum								
		Villi length (μm)			Crypt depth (μm)			Villi length: Crypt depth		
	Day pi	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level										
	1000 (IU/kg)	1617	1880	2234	263	223	197	7.81	8.62	11.6
	4000 (IU/kg)	1590	1802	2181	276	239	204	7.49	7.96	10.7
Source										
	D3	1578	1867	2215	267	238	204	7.56	8.23	11.0
	OHD	1629	1814	2200	272	229	197	7.74	8.34	11.3
Infection										
	Control	2009	1987	2216	170	199	190	11.99	10.18	11.7
	Infected	1198	1695	2199	369	269	211	3.31	6.39	10.6
	SEM	40.9	50.6	44.0	9.04	7.46	5.63	0.272	0.278	0.246
						Probabilities				
Level		0.644	0.280	0.397	0.327	0.352	0.355	0.418	0.100	**0.024**
Source		0.379	0.462	0.810	0.675	0.386	0.418	0.644	0.768	0.498
Infection		**<0.001**	**<0.001**	0.788	**<0.001**	**<0.001**	**0.012**	**<0.001**	**<0.001**	**0.002**
Level × Source		0.241	0.616	0.225	0.552	0.821	0.241	0.901	0.961	0.764
Level × Infection		0.913	0.767	0.940	0.459	0.499	0.354	0.804	0.427	0.311
Source × Infection		0.745	0.792	0.856	0.729	0.590	0.595	0.564	0.641	0.561
Level × Source × Infection		0.948	0.485	0.301	0.099	0.631	0.953	0.256	0.166	0.404

SEM: Pooled standard error of the mean.

D6, 10 and 14 pi equates to d17, 21 and 25 of age respectively

Table 3. 7 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken jejunum morphology on d6, 10 and 14 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi).

		Jejunum								
		Villi length (μm)			Crypt depth (μm)			Villi length: Crypt depth		
	Day pi	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level										
	1000 (IU/kg)	839	1045	1182	236	212	190	4.85	5.40	6.89
	4000 (IU/kg)	835	995	1186	230	203	175	4.75	5.03	6.44
Source										
	D3	808	1019	1214	229	211	184	4.94	5.10	6.65
	OHD	866	1022	1154	236	203	181	4.66	5.34	6.68
Infection										
	Control	1069	1112	1208	139	172	165	7.69	6.52	7.42
	Infected	605	928	1160	326	242	200	1.91	3.92	5.91
	SEM	31.5	27.1	32.5	10.4	8.12	6.56	0.167	0.154	0.191
						Probabilities				
Level		0.916	0.198	0.934	0.684	0.476	0.106	0.681	0.096	0.108
Source		0.202	0.927	0.197	0.631	0.448	0.711	0.234	0.275	0.918
Infection		**<0.001**	**<0.001**	0.290	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Level × Source		0.504	**0.004**	0.420	0.379	0.127	0.113	0.795	0.727	**0.008**
Level × Infection		0.499	**0.008**	0.095	0.408	0.896	0.079	0.614	0.178	0.995
Source × Infection		0.152	0.632	0.462	0.859	0.540	0.121	0.212	0.491	0.364
Level × Source × Infection		0.551	0.718	0.460	0.469	0.722	0.097	0.393	0.473	0.546

SEM: Pooled standard error of the mean.

See Figures 3.5 and 3.6 for graphical illustration of significant interaction ($P < 0.05$) between the experimental factors at d10 and 14 post-infection (pi).

D6, 10 and 14 pi equates to d17, 21 and 25 of age respectively

Table 3. 8 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken ileum morphology on d6, 10 and 14 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi).

		Ileum								
		Villi length (μm)			Crypt depth (μm)			Villi length: Crypt depth		
	Day pi	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level										
	1000 (IU/kg)	479	556	663	189	132	136	3.10	4.25	4.93
	4000 (IU/kg)	497	583	649	186	139	146	3.2	4.23	4.53
Source										
	D3	479	575	671	169	135	142	3.26	4.33	4.83
	OHD	496	563.5	642	206	137	141	3.03	4.14	4.64
Infection										
	Control	543	519	645	121	124	133	4.46	4.2	4.89
	Infected	432	619	667	253	148	149	1.84	4.27	4.57
	SEM	24.4	19.9	27.7	10.1	4.73	5.48	0.128	0.144	0.181
						Probabilities				
Level		0.605	0.345	0.709	0.834	0.308	0.216	0.579	0.930	0.124
Source		0.636	0.679	0.463	**0.015**	0.703	0.885	0.208	0.350	0.467
Infection		**0.003**	**0.001**	0.575	**<0.001**	**0.001**	**0.041**	**<0.001**	0.731	0.212
Level × Source		0.533	0.827	0.634	0.226	0.913	0.951	0.875	0.853	0.680
Level × Infection		0.465	0.795	0.908	0.605	0.867	0.672	0.618	0.838	0.960
Source × Infection		**0.022**	0.512	0.920	0.099	0.753	0.087	**0.005**	0.305	0.093
Level × Source × Infection		0.596	0.993	0.941	0.438	0.778	0.464	0.674	0.845	0.368

SEM: Pooled standard error of the mean.

See Figure 3.7 for a graphical illustration of significant interaction ($P < 0.05$) between the experimental factors at d6 post-infection (pi).

D6, 10 and 14 pi equates to d17, 21 and 25 of age respectively

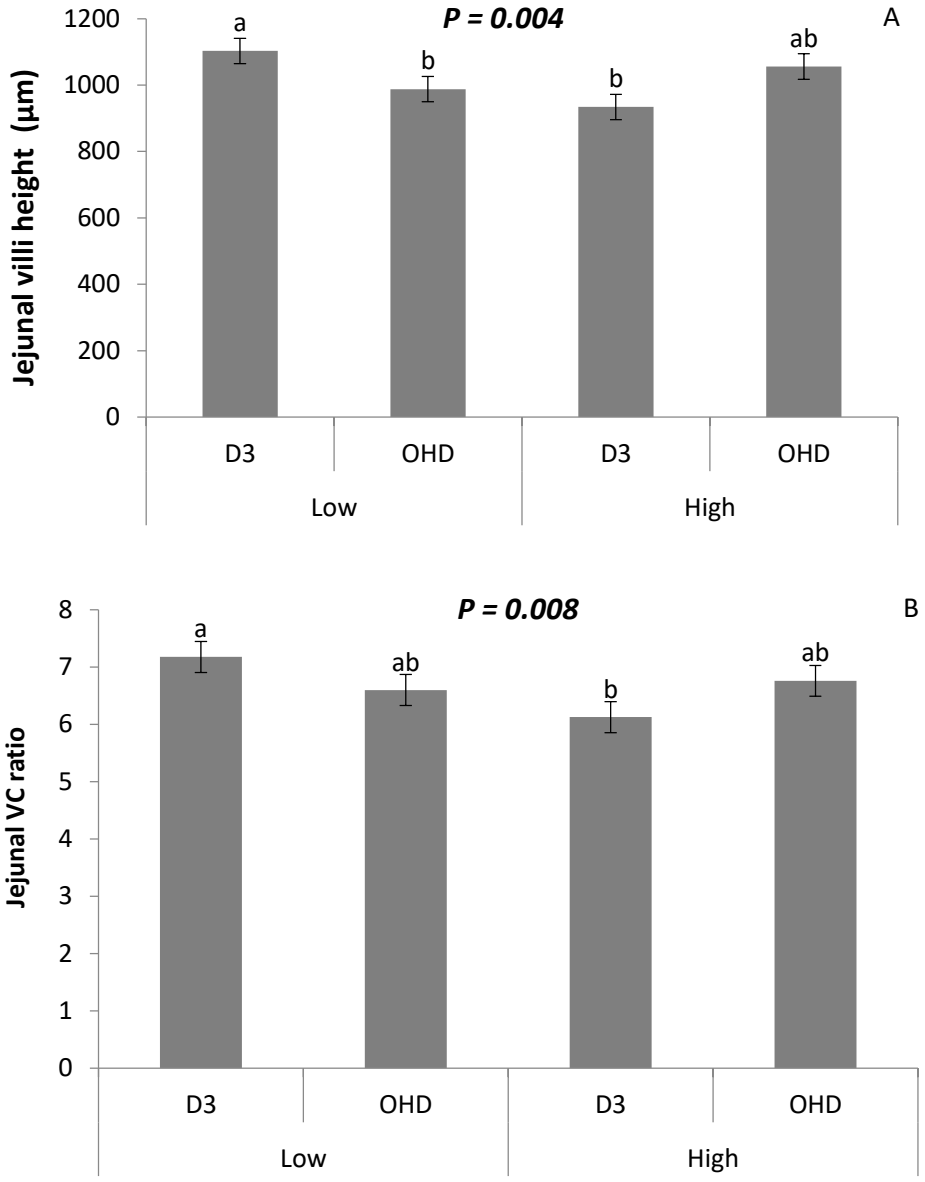

Figure 3. 5 (A) Interaction between level of vitamin D supply that being low (1000 IU/kg) or high (4000 IU/kg) and source (OHD or D_3) on jejunal villi height at d10 post-infection and (B) on jejunal villi height to crypt depth ratio (VCR) at d14 post-infection. Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d11 post-hatch.

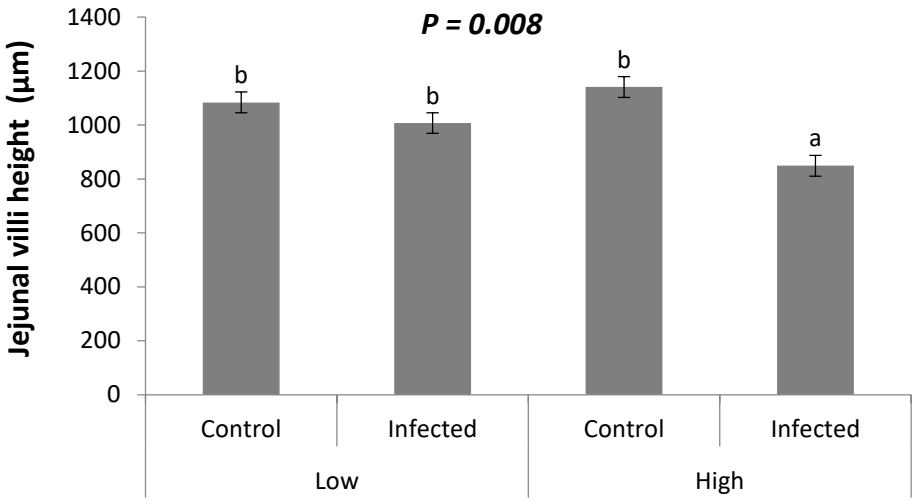

Figure 3. 6 Interaction between level of vitamin D supply that being low (1000 IU/kg) or high (4000 IU/kg) and infection on jejunal villi height at d10 post-infection. Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d11 post-hatch.

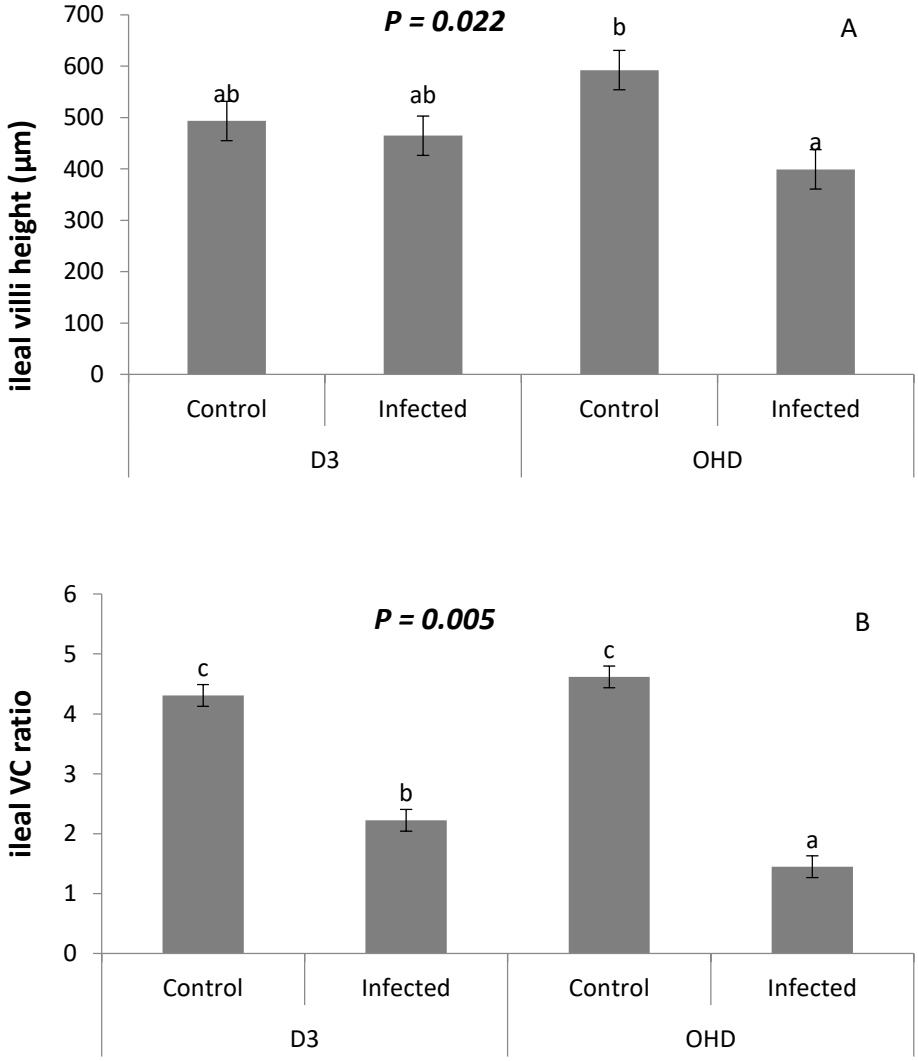

Figure 3. 7 (A) Interaction between source of vitamin D supply (OHD or D_3) and infection status on ileal villi height and (B) ileal villi height to crypt depth ratio (VCR) at d6 post-infection. Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d11 post-hatch.

Table 3. 9 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken femur characteristics on d6, 10 and 14 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi).

		Femur														
		Length/BW			Width/BW			BS (N/kg of BW)			Seedor index*			Robusticity index*		
	Day pi	d6	d10	d14	d6	d10	d14	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level																
	1000 (IU/kg)	6.62	5.20	4.12	0.772	0.63	0.510	188	167	140	78.5	90.0	109	3.35	3.34	3.36
	4000 (IU/kg)	6.46	4.90	3.93	0.764	0.598	0.503	198	182	146	79.7	96.9	113	3.34	3.26	3.31
Source																
	D3	6.49	5.07	4.09	0.755	0.612	0.505	192	171	139	78.5	91.5	110	3.34	3.31	3.36
	OHD	6.59	5.04	3.96	0.781	0.616	0.508	194	178	147	79.8	95.5	112	3.35	3.29	3.31
Infection																
	Control	6.04	4.60	3.76	0.722	0.575	0.483	205	179	152	82.3	101	119	3.29	3.28	3.30
	Infected	7.04	5.50	4.29	0.814	0.653	0.531	181	169	133	76.0	85.6	103	3.39	3.32	3.37
	SEM	0.0905	0.0620	0.0667	0.0130	0.00765	0.00978	4.31	4.52	3.47	0.983	2.02	1.70	0.0179	0.0217	0.021
												Probabilities				
Level		0.245	**0.001**	**0.043**	0.662	**0.005**	0.622	0.105	**0.028**	0.206	0.392	**0.019**	0.100	0.715	**0.017**	0.068
Source		0.473	0.712	0.148	0.176	0.701	0.813	0.681	0.289	0.110	0.362	0.169	0.598	0.711	0.439	0.072
Infection		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	0.124	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.163	**0.025**
Level × Source		0.684	0.249	0.559	0.423	0.861	0.490	0.783	0.187	0.89	0.942	0.742	0.829	0.731	0.825	0.571
Level × Infection		0.637	0.751	0.915	0.496	0.091	0.294	**0.002**	0.573	0.236	0.369	0.321	0.959	0.814	0.412	0.128
Source × Infection		0.767	0.990	0.712	0.711	0.431	0.636	0.064	0.767	0.614	0.438	0.101	0.674	0.537	0.211	0.226
Level × Source × Infection		0.826	3.302	0.354	0.598	0.723	0.317	0.213	0.542	0.731	0.202	0.396	0.130	0.080	0.360	0.227

SEM: Pooled standard error of the mean.

Abbreviations: BW, body weight; BS, breaking strength; N/kg, Newton per kilogram

D6, 10 and 14 pi equates to d17, 21 and 25 of age respectively

See Figure 3.8 for graphical illustration of significant interaction ($P < 0.05$) between the experimental factors at d6 post-infection (pi).

*Robusticity index = (bone length (mm)) / (bone weight (mg))$^{1/3}$; Seedor index = (bone weight (mg)) / (bone length (mm))

BS, Breaking strength

Table 3. 10 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken tibia characteristics on d6, 10 and 14 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi).

		Tibia														
		Length/BW			Width/BW			BS (N/kg BW)			Seedor index*			Robusticity index*		
	Day pi	d6	d10	d14	d6	d10	d14	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level																
	1000 (IU/kg)	8.87	7.20	5.78	0.685	0.549	0.455	204	205	186	83.4	99.8	120	3.99	4.00	4.08
	4000 (IU/kg)	8.73	6.85	5.51	0.686	0.528	0.440	230	215	196	84.5	104	123	4.00	3.98	4.04
Source																
	D3	8.75	7.02	5.73	0.676	0.535	0.447	212	204	189	82.3	101	110	4.01	3.98	4.09
	OHD	8.50	7.04	5.56	0.696	0.542	0.448	223	217	193	85.5	103	122	3.98	4.00	4.03
Infection																
	Control	8.22	6.39	5.29	0.641	0.507	0.435	220	221	211	87.3	108.9	131	3.96	3.97	4.01
	Infected	9.38	7.66	6.00	0.730	0.569	0.460	214	199	171	80.5	94.9	110	4.03	4.00	4.11
	SEM	0.107	0.0790	0.0910	0.0116	0.00920	0.00932	5.70	6.10	5.05	1.01	1.73	2.10	0.0174	0.0192	0.0210
												Probabilities				
Level		0.362	**0.004**	**0.044**	0.971	0.118	0.279	**0.002**	0.241	0.166	0.438	0.085	0.331	0.695	0.437	0.173
Source		0.535	0.836	0.217	0.230	0.57	0.907	0.179	0.145	0.630	**0.028**	0.375	0.364	0.227	0.491	**0.026**
Infection		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.072	0.431	**0.014**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.005**	0.385	**0.001**
Level × Source		0.406	0.150	0.445	0.880	0.672	0.508	0.748	0.382	0.051	0.264	0.975	0.978	0.452	0.750	0.489
Level × Infection		0.484	0.725	0.489	0.682	0.228	0.381	0.768	0.652	0.825	0.752	0.453	0.467	0.292	0.555	0.327
Source × Infection		0.749	0.893	0.501	0.980	0.537	0.529	0.583	0.835	0.224	0.724	0.745	0.975	0.710	0.889	0.150
Level × Source × Infection		0.992	0.257	0.583	0.813	0.610	0.758	0.785	0.619	0.518	0.909	0.539	0.188	0.480	0.354	0.576

SEM: Pooled standard error of the mean.

Abbreviations: BW, body weight; BS, breaking strength; N/kg, Newton per kilogram

D6, 10 and 14 pi equates to d17, 21 and 25 of age respectively

*Robusticity index = (bone length (mm)) / (bone weight (mg))$^{1/3}$; Seedor index = (bone weight (mg)) / (bone length (mm))

BS, Breaking strength

Table 3. 11 Main effects of level (1000 vs 4000 IU/kg) and source (D3 vs OHD) of vitamin D supply, and *Eimeria* infection status on chicken tibia mineralisation on d6, 10 and 14 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch (d0 pi).

		Tibia					
		Ash (g/kg BW)			Ash percentage (%)		
	Day pi	d6	d10	d14	d6	d10	d14
Level							
	1000 (IU/kg)	0.997	0.985	0.929	50.7	50.9	50.4
	4000 (IU/kg)	1.04	0.999	0.953	51.6	51.9	52.0
Source							
	D3	0.989	0.957	0.938	50.7	50.9	50.6
	OHD	1.05	1.03	0.944	51.6	51.8	51.8
Infection							
	Control	1.01	1.00	1.012	51.9	53.0	52.3
	Infected	1.02	0.981	0.870	50.3	49.8	50
	SEM	0.0167	0.0185	0.0166	0.346	0.280	0.367
Level		0.072	0.595	0.316	0.066	**0.021**	**0.003**
Source		**0.018**	**0.011**	0.799	0.070	**0.030**	**0.030**
Infection		0.664	0.399	**<0.001**	**0.002**	**<0.001**	**<0.001**
Level × Source		0.118	0.681	0.52	0.720	0.981	0.153
Level × Infection		0.071	0.776	0.635	0.674	0.570	0.456
Source × Infection		0.858	0.786	0.416	0.569	0.984	0.945
Level × Source × Infection		0.724	0.883	**0.005**	0.869	0.745	0.237

SEM: Pooled standard error of the mean.

D6, 10 and 14 pi equates to d17, 21 and 25 of age respectively

See Figure 3.9 for a graphical illustration of significant interaction ($P < 0.05$) between the experimental factors at d14 post-infection (pi).

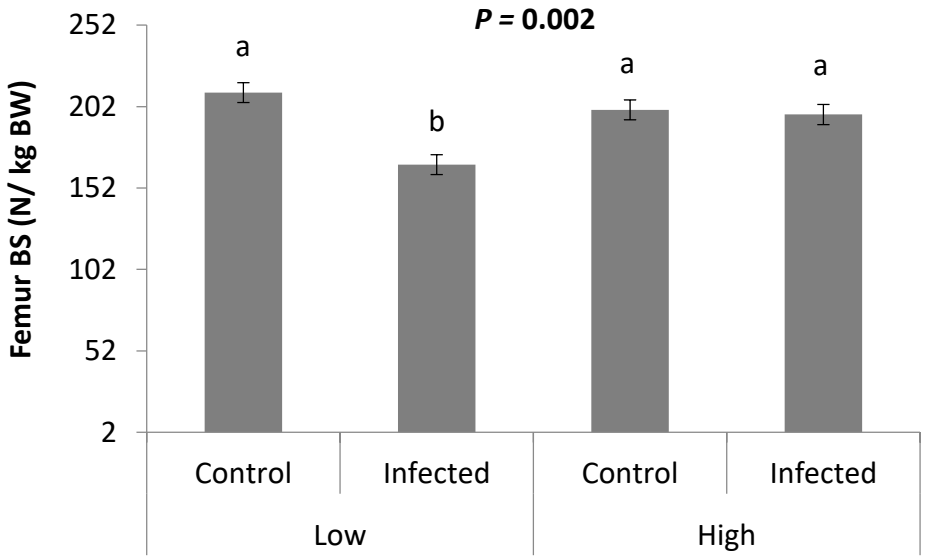

Figure 3. 8 Interaction between the level of vitamin D supply that being low (1000 IU/kg) or high (4000 IU/kg) and infection status on femur bone breaking strength (BS) at d10 post-infection (P = 0.002). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d11 post-hatch.

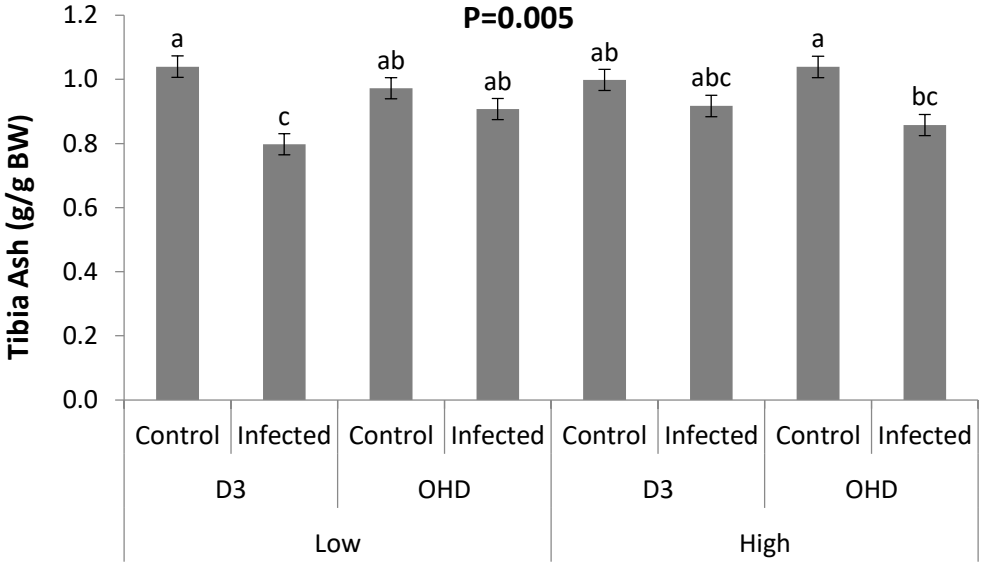

Figure 3. 9 Interaction between level of vitamin D supply that being low (1000 IU/kg) or high (4000 IU/kg), source (OHD or D3) and infection status on ash weight (g) expressed as a proportion of body weight at dissection (g/kg BW) at d14 post-infection (P = 0.005). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated *E. maxima* oocysts (Infected) at d11 post-hatch.

3.5 Discussion

In chapter 2, *E. maxima* infection reduced bone mineralisation and impaired fat-soluble vitamins status in fast and slow-growing broiler lines. The present study tested whether offering differing dietary VitD levels (4000 vs 1000 IU/kg), different forms (OHD vs D3), or both, would alleviate the effects of *E. maxima* infection on bone mineralisation and growth performance of fast-growing broilers. Also, the effects of the above dietary modulations on the parasite burden were assessed using parasite GC at peak replication. The basis of the hypothesis was that fat-soluble vitamin status is impaired during coccidiosis, which in turn may further aggravate a marginal VitD deficiency, and that OHD may be absorbed in a more fat-independent manner, being more potent than D3 in mediating VitD activity (Wagonfeld *et al.*, 1976; Reed *et al.*, 1980; Fritts and Waldroup, 2003).

Consistent with chapter 2, infection penalised the performance of infected chickens during the early and acute periods of infection but infected and control birds a similar performance during the recovery period. Gastrointestinal damage occurred across all segments of the small intestine around peak parasite replication (Blake *et al.*, 2006). The effects were more pronounced and persisted longer in the proximal and mid-intestine, which is the predilection site for *E. maxima* (Williams, 2005b; Cornelissen *et al.*, 2009). Compensatory ileal villi development took place as described previously in similar studies with the same parasite (Idris *et al.*, 1997b), but not at the acute stage of infection (d6 pi). The higher long bone linear growth (length and width) relative to BW amongst infected birds were artefacts of infection. The values were, to a negligible extent, due to the shorter and thinner bones, but more importantly due to the significant weight loss of birds following infection. Hence, longer and wider tibia and femur bones per BW did not correspond with improved strength and mineralisation for infected birds in this study. Incomplete infilling of the periosteal surface by osteoblasts (Williams *et al.*, 2004) is believed to have caused the weakness and poor mineralisation of long bones for the infected birds. In other words, a higher porosity of long bones could be rightly inferred for the infected than the control birds in this study

In terms of bone mineralisation, the effects of infection were present throughout the pi period for both femur and tibia with both showing inferior robusticity and seedor indices. Femur BS responded to infection earlier than tibia BS, which could be attributed to the faster mineralisation rate of the former in comparison to the latter at initial stages of broiler growth (Applegate and Lilburn, 2002). Even though the proportion of tibia ash to BW at dissection was constant for control birds throughout d17 - 25 post-hatch (Bar *et al.*, 2003), this was not

the case for infected birds where a progressive decrease was noted. By d14 pi infected birds matched the growth rates of their non-infected counterparts, but their tibias carried 14 % less ash (g). Moreover, tibia ash % was severely depressed at all timepoints, being more pronounced at d10 pi but persisting at d14 pi. These results bear significance considering that although ADG was comparable between infected and control birds over the recovery period, the BW of infected birds was significantly lower, indicating that proportionally more stress was applied to their long bones.

Consistent with the hypothesis of this study, VitD status was impaired in response to infection with *E. maxima*. Infection reduced plasma levels of OHD across the pi period, reaching the lowest level at d10 pi. Studies in mammalian species suggest that some storage of OHD occur in the liver, adipose and muscle tissues, which can be released slowly in periods of VitD deficiency to raise plasma OHD levels: the rate of release is higher when subjected to a negative energy balance (Brouwer *et al.*, 1998; Heaney *et al.*, 2009; Burild *et al.*, 2016). Although there is no information on VitD storage and kinetics in avian species, these reserves are depleted within a week in the absence of dietary supply in minipigs (Heaney *et al.*, 2009). The results of the present study suggest that within a few days of coccidian challenge, systemic circulating OHD levels become severely depressed. At d6 pi, levels of plasma Ca and P, and bone mineralisation was penalised, likely due to their reduced absorption following gastrointestinal tissue damage. However, homeostasis of both Ca and P was attained later during infection, while penalties on plasma OHD concentration and bone mineralisation persisted throughout the infection period.

The results of feed analysis suggested that the amount of dietary OHD was consistently lower than D3, in both the starter and grower diets. The reason for this discrepancy is believed to be analytical, i.e. related to the methodology for estimating OHD contents. Ultimately, plasma OHD concentration was significantly higher for birds offered the OHD than the D3 diets. Therefore, the results present in the current study can be interpreted with confidence. Overall, OHD status was significantly increased by higher VitD supplementation and by offering OHD as the source of VitD activity in both control and infected birds. The interaction between level and source indicates that offering OHD is more efficient than D3 in raising OHD status and is consistent with previous reports in chickens where serum or plasma OHD concentrations were assessed (Yarger *et al.*, 1995; Vignale *et al.*, 2015; Sakkas *et al.*, 2018). Although there was no formal interaction between level, source and infection on circulating levels of OHD, at d10 pi when effects of infection were maximised, OHD levels were similar between HD3 and LOHD birds suggesting a better absorption efficiency for dietary OHD (Figure 3.4a). On the

other hand, VitD supply interacted with infection status for levels of OHD at d10 pi, being significantly depressed in L infected birds but maintained in H infected birds to similar levels as L controls. Infected birds on low VitD diets also showed inferior FCR across the pi period, and had the lowest femur BS and circulating P levels on the same day pi. The effect of VitD on phosphate absorption is thought to be mediated via the saturable transcellular mechanism as increased levels of NaPiIIb in the brush border membrane have been measured in response to biologically-active VitD (1,25D3) treatment of patients with renal failure and in VitD deficient rats (Davis *et al.*, 1983; Kurnik and Hruska, 1984). The only formal interaction between VitD level, VitD source and infection was detected at d14 pi for tibia ash weight per BW. Infected birds receiving LD3 diets showed the lowest ash/BW overall. Collectively, these results indicate that a low VitD supply penalised bone development in infected chickens, with the greatest impact at later stages of infection when offered in D3 form. On the other hand, although dietary OHD was more efficient for maintaining VitD status, it did not offer additional benefits in the presence of infection. Previous studies involving increased dietary supply of Ca (Watkins *et al.*, 1989) and P (Willis and Baker, 1981) have been unsuccessful in improving bone mineralisation in coccidiosis-infected birds while phytase supplementation has limited efficacy (Watson *et al.*, 2005; Shaw *et al.*, 2011). There are limitations in the capacity of infected birds to compensate for penalties imposed on their bone development, at least within the period studied.

Although final BW was improved by both VitD level and source, VitD level affected ADG only over the period pre-infection and VitD source only during the period pi, whilst FCR was affected only during the period pi by both. These results are consistent with those of previously published studies (Yarger *et al.*, 1995; Whitehead *et al.*, 2004) and suggest that VitD requirements of broilers for growth functions may remain high throughout the grower period. Although increasing VitD supplementation, or offering OHD, improved all markers of bone mineralisation, effects were not consistent across sampling points. Nonetheless, tibia ash% which is the most important marker of bone mineralisation was significantly increased by d10 and d14 pi when offering high levels of vitD or in the form of OHD. These results show that benefits from increased VitD supply extend beyond the starter period and are in agreement with a recently published study evaluating effects of VitD supply in fast-growing broiler lines (Sakkas *et al.*, 2018). A higher level of VitD supply also increased plasma concentration of Ca but not of P. Although this could have occurred due to increased bone resorption or enhanced Ca and P absorption, ultimately bones were more mineralised promoting mineralisation of the bone matrix (St-Arnaud, 2008; Bikle, 2012; Haussler *et al.*,

2013). The efficiency of Ca absorption is low in VitD deficient animals (Pansu *et al.*, 1983) and has been related to transcellular and the paracellular absorption mechanisms (Christakos, 2012).

In the present study, offering a higher level of VitD, or replacing D3 with OHD, associated with a higher degree of parasite replication. Likewise, a higher degree of gastrointestinal damage was observed with higher levels of VitD activity. In the presence of infection offering HD3 diets evoked greater jejunal VH than LD3 diets at d10 pi, and OHD diets resulted in smaller ileal VH and VCR at d6 pi than D3 diets. Unsurprisingly, there was no correlation between the histomorphometric analysis and lesion scores of the small intestine in the current study. The main effects and interaction of VitD level and source on intestinal lesions were not significant for the infected birds. A previous study had reported the lack of correlation between microscopic and gross morphological assessments, recommending microscopic to be more reliable (Idris *et al.*, 1997a).

Eimeria maxima evokes a complex cytokine response characterized by increased production of Th1 pro-inflammatory cytokines such as IL-1b, IL-6, IL-8, IL-17, and IFN-γ in the small intestine, as well as Th2 anti-inflammatory cytokines such as IL-4 and IL-10 (Williams, 2005b; Hong *et al.*, 2006b; Min *et al.*, 2013). In particular, increased IFN-γ mRNA levels are thought to associate with antigen-specific resistance to coccidiosis, promoting Th1 cell production, whilst preventing Th2 cell production (Laurent *et al.*, 2001; Cornelissen *et al.*, 2009), balanced by IL-10 (Rothwell *et al.*, 2004). Elevated IL-10 mRNA levels have been described in susceptible compared to resistant broiler chicken lines (Rothwell *et al.*, 2004), whilst dietary fed oral antibody to chicken IL-10 prevents growth depression due to a mixed *Eimeria* spp. infection (Sand *et al.*, 2016). On the other hand, $1,25D_3$ may support conversion of naïve T cells into T regulatory cells, which produce IL-10 and TGF-β that inhibit the expression of pro-inflammatory cytokines such as IFN-γ and IL-17 (Jeffery *et al.*, 2009) and to upregulate IL-10 production in macrophages (Sharma and Fernando, 1975; Korf *et al.*, 2012). Previous research has shown that increased supplementation of OHD, above 2000 IU/kg of feed, in white Leghorn chicks infected with a mixed *Eimeria spp.* resulted in smaller penalties on their ADG, similar to the present study (Morris *et al.*, 2015). However, decreased IL-1β and increased IL-10 transcripts were detected in the cecal tonsils. It is possible that in the present study a delayed upregulation of IFN-γ, or an earlier upregulation of IL-10, rather than variation in their absolute levels at the peak of the infection may have affected parasitological outcomes and degree of GIT damage.

An earlier study (Trout and Lillehoj, 1995) had demonstrated the involvement of $CD8^+$ T cell in functioning as a transporter for sporozoite and increasing oocyst shedding, and recently Morris *et al.* (2015) reported that feeding OHD increased the percentage of $CD8^+$ cells in the cecal tonsils of layers inoculated with multiple coccidia species. However, contrary to results presented herein, Morris *et al.* (2015) found no effect of dietary OHD on faecal oocyst shedding at their level of infection even though OHD was supplemented at similar levels as the current study. Further investigation of the immune response at earlier stages of infection is required to elucidate the observed effects. In addition, outcomes may differ according to the parasite species in question; *E. maxima*, in particular, induces a strong pro-inflammatory response as opposed to the more balanced Th1/Th2 phenotype which characterises infections with *E. acervulina* and *E. tenella* (Williams, 2005b). Interestingly, parasitological and histological findings did not corroborate performance outcomes. It has been previously shown that a higher VitD status results in an increased fractional rate of synthesis and increased breast muscle yield in broilers (Yarger *et al.*, 1995). Therefore, the reduced FCR observed in high VitD-fed infected broilers could be attributed to their increased VitD status and their improved ability to accrete body protein in the presence of infection (Yarger *et al.*, 1995). The lack of an interactive effect of source of vitD supply and infection status on performance variables indicates that the source is likely less critical than the level of vitD supply within these experimental conditions.

In conclusion, the present study shows that an *E. maxima* infection penalises broiler chicken performance, bone mineralisation and VitD status, whilst a low VitD supply seems to aggravate the adverse effects of infection. In contrast, a higher VitD supply resulted in higher parasite loads and compromised gut architecture in the absence of adverse effects on performance variables. Chapter 4 further examined the effects of the high VitD supply level (4000 IU/kg) from D3 and OHD source in coccidia-infected broilers receiving marginally deficient Ca/P diets. No doubt, additional studies are required to unravel the effects of vitD supply on immune responses over time in different host/pathogen systems.

Chapter 4: Interactions between dietary Calcium and Phosphorus level, and vitamin D source on bone mineralisation, performance and intestinal morphology of coccidia-infected broilers

4.1 Summary

Coccidiosis penalises Calcium (Ca), Phosphorus (P) and vitamin D (VitD) status, as well as bone mineralisation in broiler chickens [Chapter 3]. It was hypothesised that dietary VitD supplementation in the form of 25-hydroxycholecalciferol (OHD), compared to cholecalciferol (D3), would improve bone mineralisation in broilers receiving marginally deficient Ca/P diets, with more pronounced effects during malabsorptive coccidiosis. In a 2 VitD source × 2 Ca/P levels × 2 levels of infection factorial experiment (n = 6 pens per treatment, 6 birds/pen), 288 day-old Ross 308 broilers were assigned to an Aviagen-specified diet supplemented with 4000 IU/kg of either OHD or D3. From d11 to 24 of age, the diet contained adequate (A; 8.7:4.4g/kg) or marginally deficient (M; 6.1:3.1g/kg) total Ca and available (av)P levels. At d12 of age, birds were inoculated with either 7,000 *Eimeria maxima* oocysts (I) or water (C). Pen performance was measured over 12 days post-infection (pi), and during the early (d0 to 6 pi) and late (d6 to 12 pi) stages of infection. Six birds per treatment combination were assessed for parameters of bone mineralisation and intestinal histomorphometric features (d6 and 12 pi), as well as *E. maxima* replication and gross lesions of the small intestine (d6 pi). There was no 3-way interaction between VitD source, Ca/P level, and infection status for any variable examined. VitD source and infection interacted for plasma Ca levels at d6 pi; OHD- compared to D3-fed broilers had higher levels ($P < 0.05$) of plasma Ca amongst C birds whilst levels were statistically similar amongst I birds receiving D3 and OHD diets. VitD level interacted with infection status for tibia breaking strength (BS) at d12 pi; M compared to A birds had significantly lower BS ($P < 0.05$) amongst C birds but similar values amongst I birds. VitD source and Ca/P level also interacted for tibia ash weight relative to BW (ash/BW), and ash percentage (AP) at d12 pi. Values for ash/BW and AP were similar for A and M broilers receiving D3 diets but M compared to A broilers had significantly lower ($P < 0.05$) values amongst birds on OHD diets. Overall, BS/BW, Ash/BW and AP were highest in broilers fed the OHD-supplemented A diets irrespective of infection status. *E. maxima* infection significantly impaired ($P < 0.05$) average daily gain (ADG) and feed conversion ratio (FCR) pi; Ca and P levels in blood plasma at d6 pi; OHD status, BS, AW and AP at d12 pi; and intestinal morphology at d6 and 12 pi. A- compared to M-fed broilers had higher BS, AW and AP at d6 pi, and AW at d12 pi. VitD source affected only OHD status, being higher ($P < 0.001$) for OHD- than D3-fed broilers at d6 and 12 pi. In

conclusion, offering OHD and adequate levels of Ca and P improved bone mineralisation, with no effect on performance. Dietary D3 and OHD supplemented at 4000IU/kg had similar effects on coccidiosis-infected and uninfected broilers, which led to the rejection of the hypothesis.

4.2 Introduction

Vitamin D nutrition plays a critical role in Ca and P metabolism, bone mineralisation, and other physiological processes related to performance and immunity against infections in broiler chickens (Whitehead *et al.*, 2004; Khan *et al.*, 2010; Shao *et al.*, 2019). Studies have shown that higher-level supplementation and replacing cholecalciferol (D3), which is the conventional source of dietary VitD, with 25-hydroxycholecalciferol (OHD), can amplify VitD effects for broilers (Fritts and Waldroup, 2003; Fritts *et al.*, 2004; Fritts and Waldroup, 2005). This is because D3 requires liver-located hydroxylases for its conversion to OHD, which is the major VitD circulated metabolite (Barchetta *et al*., 2012; Bergada *et al*., 2014), and OHD may be the preferred source in young animals where the liver function has not reached its full adult capacity. Furthermore, OHD, compared to D3 absorption, is less fat dependent, occurs at a faster rate, and more efficiently (Maislos *et al.*, 1981). The above implies that OHD may be the preferred dietary source of VitD in young broilers, as well as during malabsorptive conditions, such as coccidia infections.

Malabsorptive coccidiosis adversely affects aspects of bone mineralisation in broilers [Chapter 2], and is linked to reduced absorption of Ca and P (Turk, 1973; Turk, 1978), depressed levels of circulating OHD [Chapter 3], and increased bone resorption (Akbari Moghaddam Kakhki *et al.*, 2018). Furthermore, this thesis revealed that higher levels of VitD supply, and offering VitD in the form of OHD instead of D3, significantly improved bone mineralisation, while reduced VitD supply penalised long bone mineralisation to a similar degree in coccidian-infected and uninfected broilers [Chapter 3].

In chapter 3, lower VitD supplementation (1000 vs 4000 IU/kg) while offering Ca/P adequate diets reduced bone mineralisation and growth performance with more significant penalties occurring amongst coccidiosis-infected than uninfected broiler. Moreover, previous studies reveal that OHD is more efficient at improving Ca and P absorption and OHD status at comparable levels of D3 supplementation (Fritts and Waldroup, 2005; Sakkas *et al.*, 2018). Indeed, adequate levels of Ca and P in diets, as well as a Ca/P ratios from 1.8 to 2:1, are crucial for their retention by broilers (Rao *et al.*, 2006; Delezie *et al.*, 2012).

There is increasing evidence of the need for P reduction in broiler diets for economic and environmental reasons (Knowlton *et al.*, 2004; Wironen *et al.*, 2018). This relates to the high prices of feed-grade P supplements but more importantly, the environmental pollution caused by unutilized phytate phosphorus (PP) excretion. PP also chelates essential minerals including Zinc, Copper, Iron and Magnesium in feedstuffs thereby limiting their availability to broilers, and increasing the amount of these minerals excreted into the environment (Reddy *et al.*, 1982; Shafey *et al.*, 1991; Maenz *et al.*, 1999). Therefore, studies aiming to reduce the exogenous supply of P from phosphate sources focus on strategies for improving P, and invariably Ca, utilisation via increased VitD supply, phytase enzyme supplementation, or both.

The present study investigated whether dietary VitD supply at commercially supplemented levels (4000 IU/kg) in the form of OHD, would result in improved bone mineralisation for coccidia-infected broilers receiving marginally deficient Ca/P diets. The hypothesis was that VitD supplementation in the form of OHD compared to D3 will promote bone mineralisation due to improved Ca and P utilisation, as well as enhance performance, for broilers on marginally deficient Ca and P diets; effects will be more pronounced in the presence of coccidiosis.

4.3 Materials and Methods

4.3.1 Birds, Husbandry and Diets

All experimental procedures complied with the UK Animals (Scientific Procedures) Act 1986, as well as the EU Directive 2010/63/EU for animal experiments, and were carried out under Home Office authorisation (P441ADF04). Two hundred and eighty-eight male Ross 308 day old chicks were raised from hatch to 24 d of age at the Newcastle University Cockle Park farm. Birds were housed in 48 rectangular pens (0.84 m^2) situated in a windowless thermostatically controlled building such that the treatments were allocated uniformly to represent the different sides of the room. Each pen was equipped with a tube feeder and a bell-drinker and had wood shavings to a depth of 5 cm as litter. The temperature at pen level was monitored daily and maintained to meet Aviagen recommendations for spot brooding (Aviagen, 2014b), starting at 34 °C at chick placement and gradually reduced to 20 °C by 24 d of age. Light intensity at pen level ranged from 60 to 80 lux, while a lighting schedule of 23L:1D was applied for the first 7 days of age and switched to 18L:6D for the remainder of the trial. Feed and water were offered to birds *ad libitum* for the duration of the experiment.

Upon arrival, birds were allocated to a standard starter (d1 to 10) diet and then to a grower (d11 to 24) diet (see Table 4.1), which offered either adequate (A) levels of total Ca and avP or marginally

deficient (M) levels of approximately 30% below Aviagen recommendations. The M level was classed as marginally deficient because it has a similar effect on the performance of broilers as the Aviagen-recommended A levels but results in reduced bone mineralisation (Rao *et al*., 2006; Delezie *et al*., 2015; Valable *et al*., 2018). Hence, there were four dietary treatments; AD3, MD3, AOHD, and MOHD. VitD was supplemented at 4000 IU/kg as per commercial practice in the EU (Fritts *et al.*, 2004) and is within the EFSA authorised limits (EFSA, 2012). The dietary specifications of the starter and the grower diets were according to Aviagen nutrition recommendations (Aviagen, 2014a) apart from the total Ca and avP levels of M diets. Starter diets were offered in crumbled form and grower diets in pelleted form.

4.3.2 Experimental design and inoculations

The experiment was arranged in a 2×2×2 factorial pattern with dietary VitD source (D3 vs OHD), Ca/P levels (adequate vs deficient) and infection status (control vs infected) of birds as the independent variables. The diets containing adequate total Ca and avP (A; 8.7: 4.4 g/kg), or deficient levels (M; 6.1: 3.1 g/kg) were introduced at 10 d of age, while all birds received 4000 IU/kg VitD either as D3 or OHD throughout the experiment. At 12 d of age, birds were orally inoculated with a single 0.5 ml dose of water (Control; C) or 7000 (Infected; I) sporulated *E. maxima* oocysts of the Weybridge laboratory reference strain, using 1ml syringes. The treatment groups were replicated in six pens stocked with six birds per pen.

4.3.3 Sampling

To assess performance, pen feed intake was measured post-infection (**pi**), and birds were individually weighed at infection (d0pi), and at time points (d6 and 12pi), representing the early and late stages of *E. maxima* infection in broilers. One bird per pen weighing close to pen average was selected at d6 and 12pi for blood sampling via the wing vein and subsequently euthanised with a lethal injection of sodium pentobarbitone (Euthatal®, Merial Harlow, United Kingdom). Blood samples were collected in 5 ml sodium heparin plasma tubes (BD Vacutainer, SST II Advance Plus Blood Collection Tubes-BD, Plymouth, United-Kingdom), then immediately placed on ice and centrifuged for 10 mins at 1500 g at 4 °C within 1.5 h after collection. Plasma sample aliquots were stored at -80 °C pending analyses. The gastrointestinal tract (**GIT**) of the selected birds was removed during necropsy and duodenum, jejunum and ileum were scored separately for any lesions, according to the method described by Johnson and Reid (1970). Following lesion scoring at d6pi, 6 cm of intestinal tissue from the immediate region of Meckel's diverticulum, the mid-point of the intestinal area infected by *E. maxima* (Long *et al.*, 1976), was excised and opened longitudinally to remove digesta contents. Excised tissue (5 cm) was submerged in 7 ml bizous,

and 1 cm in 1.5 ml screw cap microtubes (Thermo Scientific) filled with RNAlater® (Life Technologies; Carlsbad, CA, USA) and stored at -80 ^{o}C pending analyses. Thereafter, three segments of 1 cm each from the duodenal loop, mid-jejunum (midway between Merkel's diverticulum and the end of the duodenal loop) and mid-ileum (midway between Merkel's diverticulum and the ileocecal junction) were sampled from birds dissected on d6 and d12pi and fixed in 10% buffered formalin pending microscopic morphological assessment. Following GIT removal, right femur and tibia of the selected birds were dissected to evaluate physical development and mineralisation. Much of the adhering tissues were removed from dissected bones before storing in airtight individually labelled polythene bags at -20 °C pending evaluation.

4.3.4. Sample analysis

Experimental diet

Feed samples were analysed for VitD3 and OHD contents at the DSM Laboratory (Basel, Switzerland) using the method described in section 3.3.4 of chapter 3. The analysed D3 and OHD contents of the experimental diets were 4910 and 2828 IU/kg (starter) and 4520 and 2720 IU/kg (grower), respectively.

Morphometric analysis of gut

The same method described in section 3.3.4 of chapter 3, was used.

Parasite replication

Quantitative real-time PCR (qPCR). The number of *Eimeria maxima* genome copies (GC) in intestinal tissue was measured using the same method described in section 2.3.4 of chapter 2.

Blood metabolites

As in chapter 3, concentrations of Ca and P (mmol/l) in blood plasma were analysed in duplicate using an ABX Horiba Pentra 400 automatic analyser (Horiba Medical, Irvine, CA, USA) according to the manufacturer's instructions. Plasma concentration of OHD (ng/ml) was determined using the 25-Hydroxy Vitamin D Direct EIA kit (IDS Diagnostics, Fountain Hills, AZ, USA) according to the manufacturers' instructions.

Bone evaluation

Bones were thawed at 4 ^{o}C overnight and place at room temperature for 1 h before the adhering soft tissues were removed using scalpels. All procedures, measurements and derived variables were as described in section 3.3.4 of chapter 3.

4.3.5 Calculations and Statistics

All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 22.0. (Armonk, NY: IBM Corp). The pen was considered the experimental unit for all statistical assessments. Average daily feed intake (ADFI; g/d), gain (ADG; g/d) and feed conversion ratio (FCR; ADFI/ADG) were calculated pre-infection (d0 – 10 and d11 – 12 post-hatch), over the entire period post-infection (d1 – 12 pi), as well as for the early (d1 – 6 pi) and late (d7 – 12 pi) stages of infection . Furthermore, BW, ADG and ADFI post-infection were expressed in proportion to body weight at infection (BWd0 pi) to account for diet-change-induced tendencies of *a priori* differences in BW at infection, and because BW and ADFI increased proportionally with age. Data were analysed with VitD source, level of Ca: P supply and infection status as fixed factors, and their interactions using the General Linear Model procedure (GLM).

The concentration of Ca, P and OHD in blood plasma, villi height (VH), crypt depth (CD) and villi height: crypt depth ratio (VCR), GC number from qPCR analysis (parasite replication), and all bone-derived data generated from birds sampled at d6 and 12 pi were also analysed with the above factors using GLM. When significant differences were detected, treatment means were separated and compared by the Tukey's multiple comparison test. The Shapiro-Wilk test was used for assessing the normality of the studentised residuals and non-normalised data, such as plasma OHD concentration and parasite genome copy numbers were log-transformed before statistical analysis. The log-transformed values are presented herein. Significance was determined at $P < 0.05$, and a tendency was defined as $P < 0.1$.

Intestinal lesion score was evaluated on a scale of 0 (no lesion) to 4 (very severe lesions); 1, 2 and 3 represented mild, moderate and severe lesions respectively. As only infected birds had lesions, data were analysed with level and source of VitD supply as fixed variables and their interaction, using ordinal logistic regression procedure of SPSS and significance was determined at $P < 0.05$.

Table 4. 1 Ingredients and chemical composition of the starter (d0–10) and the two grower (d11–24) diets offered to broiler chickens.

	Starter	Grower	
Item		Adequate	Deficient
Ingredient (%)			
Wheat	47.9	51.6	52.5
Corn	10.0	10.0	10.0
Soybean meal (48 % CP)	32.0	25.3	25.3
Soybean full fat	4.00	7.00	7.00
Soy crude oil	1.84	2.32	2.32
Dicalcium phosphate	1.82	1.60	0.87
Limestone	0.77	0.67	0.45
Vitamin and mineral premix	0.40	0.40	0.40
DL methionine	0.33	0.30	0.30
L-Lysine	0.27	0.25	0.25
Sodium bicarbonate (27 %)	0.21	0.20	0.20
Sodium chloride (39 %)	0.19	0.20	0.20
L-Threonine	0.14	0.12	0.12
Choline chloride (60 %)	0.05	0.05	0.05
L-Valine	0.03	0.02	0.02
Nutrient composition (%) *			
ME (kcal/kg) (calculated)	3,000	3,067	3,095
Crude protein	23.1	21.37	21.00
Crude fat	5.03	4.87	5.12
Crude fibre	2.39	2.13	2.22
Ash	5.43	4.83	4.80
Calcium	1.03	0.80	0.63
Phophorus	0.74	0.62	0.55
Available phosphorus (calculated)	0.48	0.44	0.30
Sodium	0.18	0.15	0.15
Manganese (mg/kg)	218	169	160

The nutrient composition was in accordance with Aviagen nutrient specifications (Aviagen, 2014a), but the two grower diets contained different levels of Ca and P: an adequate and a deficient level.
*Analysed nutrient composition (%) unless otherwise stated.

4.4 Results

4.4.1 Bird health and performance variables

Two birds with gait scores of 4 out of 5 were culled and euthanised while coccidiosis caused anorexia and reduced weight gain according to expectations. The main effects of dietary VitD source, Ca/P level and infection status on performance (BW, ADG, ADFI, and FCR) pre- and post-infection, are presented in Tables 4.2 – 4.4. There were no significant 2- or 3-way interactions ($P > 0.05$) between the experimental factors for performance variables in this study. No performance variable was affected by VitD source ($P > 0.1$).

Pre-infection

VitD source did not significantly affect ($P > 0.1$) performance over the starter period (d0 – 10 of age). However, Ca/P levels affected performance. Between d11 and 12 of age when dietary Ca/P levels were altered, birds receiving the M in comparison to the A diets had significantly higher ($P < 0.05$) ADFI, and tended to have higher ADG ($P = 0.069$) and BW ($P = 0.067$) prior to inoculation at d12 of age (Table 4.2).

Post-infection period

Infection significantly reduced ($P < 0.001$) ADG and ADFI, and increased FCR ($P = 0.005$) calculated over the infection period (d1 – 12 pi). BW at d12 pi was significantly reduced for I compared with C birds. Ca/P level significantly affected ($P < 0.05$) ADG post-infection, as well as BW at d12 pi; values were significantly higher for birds receiving the M in comparison to the A diets (Table 4.3).

Early and late stages of infection

E. maxima infection impaired ($P < 0.05$) ADG, ADFI and FCR during the early and late stages of infection, whereas the effect of Ca/P level on these variables was not significant ($P > 0.05$) during both phases. Moreover, ADG and ADFI tended ($0.5 < P < 0.1$) to be higher for the M than the A- fed broilers during the acute phase (Table 4.4). VitD source and infection status tended to interact ($P = 0.052$) for ADG per unit BWd0 pi during the early stage of infection: values for OHD- compared to D3-fed broilers tended to be higher amongst control birds, but lower amongst infected birds.

4.4.2 Plasma Calcium, Phosphorus and OHD concentrations

The main effects of dietary VitD source and Ca/P level, and infection status on plasma Ca, P, and OHD concentrations are presented in Tables 4.5. The 2-way interaction between VitD

source and infection status for Ca (*P = 0.014*) at d6 pi is illustrated in Figure 4.1. Amongst C birds, plasma Ca was significantly higher for birds receiving OHD than D3 diets, whilst D3 and OHD diets had a similar effect on plasma Ca concentration for I birds. There were no other 2- way or any 3-way interactions between the experimental factors.

Infection significantly reduced (*P < 0.05*) Ca and P concentration (mmol/L) in the blood plasma of broilers only at d6 pi, whereas VitD source and Ca/P level had no significant effect on Ca or P status at both d6 and12 pi. Infection significantly reduced (*P < 0.05*) plasma OHD concentration (ng/ml) at d6 and d12 pi. Broilers receiving OHD compared to D3 diets had higher (*P < 0.001*) plasma OHD concentration at d6 and d12 pi. Ca/P level tended to increase (*P = 0.098*) plasma OHD concentration for M- compared to A-fed broilers at d6 pi, but did not affect OHD status at 12 pi (Table 4.5).

4.4.3 Histology and lesion scores

Histology

Results of the duodenum, jejunum and ileum morphological analysis are presented in Table 4.6. There were no significant 2- or 3- way interaction between dietary VitD source, Ca:P level and infection status for VH, CD or VCR in any section of the small intestine at d6 or 12 pi. Dietary VitD source and Ca/P level did not affect (*P > 0.05*) small intestine morphological architecture assessed at d6 and 12 pi.

Infection significantly reduced (*P < 0.05*) duodenal VH at d6 and 12 pi, and jejunal VH at d6 pi. Infection increased (*P < 0.05*) jejunal and ileal CD at d6 and 12 pi, and duodenal CD only at d6 pi. Furthermore, infection reduced (*P < 0.05*) VCR of duodenum, jejunum and ileum at d6 and d12 pi (Table 4.6).

Lesion score

Uninfected broilers had no intestinal lesions (score 0). The main effects of dietary VitD source (D3 = 1.58 vs OHD = 1.69; SEM = 0.0887) and Ca/P level (M = 1.64 vs A = 1.64; SEM = 0.0887), and their interaction, was not significant (*P* > 0.1) for *E. maxima* lesions in the small intestine. Individually, lesions in the duodenal, jejunal or ileal sections were also not affected (*P* > 0.1) by the experimental factors (the same scenario occurred in chapters 2 and 3). Nevertheless, severe *E. maxima* lesions (score 3) occurred only in the jejunum, whereas the duodenum had only mild lesions (score 1), and the ileum had mild to moderate lesions (score 2).

4.4.4 Parasite replication

There were no traces of *E. maxima* parasites in the gut of C birds at the point of sampling: d6 pi. There was no interaction ($P > 0.05$) between Ca/P level and VitD source for *E. maxima* GC number. M and A diets (M = 5.16 vs. A = 5.32; SEM = 0.0692), as well as D3 and OHD VitD sources (D3 = 5.34 vs. OHD = 5.16; SEM = 0.0692), had no effect ($P > 0.05$) on parasite replication of broilers.

4.4.5 Bone evaluation

The main effects of dietary VitD source and Ca/P level, and infection status on bone variables are presented in Tables 4.7 and 4.8. The significant interactions between the experimental factors for markers of bone mineralisation are illustrated in Figures 4.2 and 4.3. There were no significant 3- way interaction between VitD source and Ca/P level, and infection status. The main effect of VitD source was not significant ($P > 0.05$) for all femur and tibia parameters assessed in this study.

Linear growth in proportion to BW

Dietary Ca/P level interacted with infection status ($P < 0.05$) for relative tibia length, i.e. in proportion to body weight at dissection (length/BW), at d6 pi. Infected birds on the M compared to the A diets had significantly lower values ($P < 0.05$) for tibia length/BW, which were statistically similar to values for control birds irrespective of Ca/P level in the diet.

Infection significantly increased ($P < 0.05$) femur length/BW, as well as femur width/BW at d6 and 12 pi (Table 4.7). Infection also significantly increased ($P < 0.05$) tibia length/BW at d6 and 12 pi and tibia width/BW at d6 pi (Table 4.8).

Dietary Ca/P level affected tibia and femur linear growth only at d6 pi. M compared to A diets significantly reduced ($P < 0.05$) width/BW of tibia and femur (Tables 4.7 and 4.8).

Breaking strength in proportion to BW

Ca/P level in diet interacted with infection status ($P = 0.024$) for tibia BS in proportion to body weight d12 pi. C birds offered A compared to M diets had significantly higher values ($P < 0.05$), whereas BS/BW of infected A and M birds were statistically similar (Figure 4.2).

Infection significantly reduced BS/BW ($P < 0.05$) of tibia and femur only at d12 pi. M diet reduced ($P < 0.05$) tibia and femur BS/BW compared to A diet at d6 pi. BS/BW values

remained lower ($P < 0.05$) for tibia and femur of M- than A-fed broilers at d12 pi (Tables 4.7 and 4.8).

Weight/length (Seedor) and robusticity indices

Dietary Ca/P level interacted with infection status ($P < 0.05$) for tibia and femur seedor and robusticity indices only at d12 pi. In general, results of both indices suggest that tibia and femur density amongst birds receiving the A diet were significantly higher ($P < 0.05$) for the C than the I birds. On the other hand, tibia and femur density were statistically similar ($P > 0.05$) for C and I birds receiving the M diet. Infection reduced ($P < 0.05$) seedor index of tibia and femur at d12 pi, whereas the main effect of dietary Ca/P level on tibia and femur seedor or robusticity indices was not statistically significant ($P > 0.05$).

Ash percentage and ash in proportion to BW

VitD source and Ca/P level interacted ($P < 0.05$) for tibia AP and ash/BW only at d12 pi. Amongst birds receiving OHD diets, AP and ash/BW values were significantly reduced ($P < 0.05$) for birds offered the M compared to the A diets. These reduced values for MOHD birds were at statistically similar levels as the AP and ash/BW values for birds receiving MD3 and AD3 diets (Figure 4.4A and B).

Infection reduced ($P < 0.05$) tibia ash/BW only at d12 pi, and AP at d6 and d12 pi for I in comparison to C birds (Table 4.8). Dietary Ca/P level affected ($P < 0.05$) tibia ash/BW at d6 and 12 pi, and tibia AP at d6 pi. M- compared to A-fed broilers had reduced ($P < 0.05$) tibia Ash/BW at d6 and 12 pi, as well as AP at d6 pi (Table 4.8).

Table 4. 2 Main effects of source of VitD supply (D3 vs OHD) and Ca/P level (M = 6.1:3.1 vs A = 8.7:4.4 g/kg) on body weight (BW, g) at d10 and 12 of age, and on average daily feed intake (ADFI, g/d), average daily gain (ADG, g/d) and feed conversion ratio (FCR, ADFI/ADG) of broiler chickens pre-infection with *E. maxima*

		Pre-infection							
		d0 to10 of age				d11 to 12 of age			
		ADG	ADFI	FCR	BWd10	ADG	ADFI	FCR	BWd12
Level	M	*NA*	*NA*	*NA*	378	58.4	74.3	1.28	495
	A	*NA*	*NA*	*NA*	373	55.5	70.5	1.27	484
Source	D3	32.7	36.4	1.11	372	57.8	72.6	1.26	488
	OHD	33.4	36.6	1.10	379	56.1	72.2	1.29	491
	SEM	0.288	0.287	0.00591	2.96	1.08	0.943	0.0171	4.01
		Probabilities							
Level		*NA*	*NA*	*NA*	0.242	0.069	0.006	0.890	0.067
Source		0.124	0.734	0.122	0.135	0.246	0.781	0.193	0.628
Level × Source		*NA*	*NA*	*NA*	0.201	0.187	0.061	0.893	0.105

SEM: Pooled standard error of the mean. *NA* means not applicable

Table 4. 3 Main effects of dietary VitD source (D3 vs OHD), Ca/P level (M = 6.1:3.1 vs A = 8.7:4.4) and infection status on body weight at infection (BWd0pi), and d12 post-infection (BWd12pi), and on average daily gain (ADG, g/d), feed intake (ADFI, g/d) and feed conversion ratio (FCR, ADFI/ADG) over the post-infection period (d1 to d12 pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch.

					Post-infection period (d1 to 12pi)				
		BWd0pi (g)	BWd12pi (g)	Bwd12pi/BWd0pi	ADFI (g/d)	ADG (g/d)	ADFI/ BWd0pi	ADG/ BWd0pi	FCR
Level	M	495	1510	3.05	119	84.6	0.241	0.171	1.85
	A	483	1461	3.03	115	81.5	0.238	0.169	1.86
Source	D3	488	1476	3.03	116	82.4	0.237	0.169	1.79
	OHD	490	1494	3.05	118	83.6	0.242	0.171	1.92
Infection	Control	484	1582	3.27	122	91.5	0.253	0.189	1.69
	Infected	493	1389	2.81	112	74.5	0.226	0.151	2.02
	SEM	3.98	14.2	0.0208	1.84	0.993	0.00288	0.00175	0.078
				Probabilities					
Level		0.042	0.020	0.353	0.111	0.037	0.493	0.387	0.975
Source		0.758	0.378	0.400	0.331	0.391	0.302	0.465	0.262
Infection		0.100	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005
Level × Source		0.065	0.503	0.165	0.145	0.799	0.513	0.195	0.433
Level × Infection		0.825	0.245	0.133	0.142	0.125	0.067	0.078	0.332
Source × Infection		0.740	0.342	0.359	0.287	0.223	0.218	0.249	0.336
Level × Source × Infection		0.167	0.131	0.760	0.407	0.210	0.941	0.863	0.548

SEM: Pooled standard error of the mean
The period from d1 to 12 pi equates to d12 to 24 of age

Table 4. 4 Main effects of source of VitD supply (D3 vs OHD), Ca/P level (M = 6.1:3.1 vs A = 8.7:4.4 g/kg) and infection status on average daily feed intake (ADFI, g/d), average daily gain (ADG, g/d) and feed conversion ratio (FCR, ADFI/ADG) during the early (d0 to 6pi) and late (d7 to 12pi) stages post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch.

		Early stage (d0 to 6pi)					Late stage (d7 to 12pi)				
		ADFI (g/d)	ADG (g/d)	ADFI/ BWd0pi	ADG/ BWd0pi	FCR	ADFI (g/d)	ADG (g/d)	ADFI/ BWd0pi	ADG/ BWd0pi	FCR
Level	M	101	74.2	0.204	0.150	1.37	137	94.0	0.278	0.190	1.50
	A	96.6	71.6	0.200	0.148	1.37	133	91.8	0.276	0.190	1.47
Source	D3	97.9	72.6	0.201	0.149	1.35	134	91.8	0.274	0.188	1.50
	OHD	99.7	73.1	0.203	0.149	1.39	137	94.0	0.280	0.192	1.47
Infection	Control	102	78.9	0.211	0.163	1.30	143	104	0.295	0.216	1.39
	Infected	95.5	66.8	0.193	0.135	1.44	128	81.5	0.259	0.165	1.58
	SEM	1.74	1.05	0.00284	0.00173	0.0189	2.29	1.40	0.00381	0.00268	0.015
		Probabilities									
Level		0.076	0.086	0.290	0.478	0.741	0.224	0.267	0.805	0.992	0.100
Source		0.455	0.712	0.473	0.793	0.172	0.320	0.263	0.302	0.321	0.224
Infection		0.011	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Level × Source		0.094	0.294	0.291	0.993	0.070	0.284	0.841	0.840	0.155	0.293
Level × Infection		0.341	0.113	0.260	0.052	0.337	0.102	0.336	0.052	0.307	0.531
Source × Infection		0.206	0.084	0.151	0.059	0.436	0.455	0.693	0.426	0.825	0.905
Level × Source × Infection		0.724	0.084	0.972	0.305	0.395	0.288	0.442	0.669	0.893	0.680

SEM: Pooled standard error of the mean.
The period from d0 to 6pi and d7 to 12pi equates to d12 to 18, and d19 to 24 of age, respectively.

Table 4. 5 Main effects of source of VitD supply (D3 vs OHD), Ca/P level (M = 6.1:3.1 vs A = 8.7:4.4 g/kg) and infection status on concentration of Calcium (mmol/l), Phosphorus (mmol/l) and log-transformed 25-hydroxycholecalciferol (OHD; ng/ml) in blood plasma on days 6 and 12 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post hatch,

		Calcium (mmol/l)		Phosphorus (mmol/l)		25-hydroxy cholecalciferol(ng/ml)[1]	
		d6pi	d12pi	d6pi	d12pi	d6pi	d12pi
Level	M	2.73	2.86	1.82	1.86	2.08	1.91
	A	2.75	2.87	1.78	1.92	1.99	1.92
Source	D3	2.71	2.86	1.76	1.91	1.78	1.67
	OHD	2.78	2.87	1.84	1.87	2.29	2.15
Infection	Control	2.90	2.85	1.88	1.89	2.09	2.10
	Infected	2.58	2.88	1.71	1.89	1.98	1.73
	SEM	0.0301	0.0255	0.0371	0.0400	0.0373	0.0479
				Probabilities			
Level		0.691	0.723	0.436	0.312	0.098	0.883
Source		0.099	0.740	0.125	0.534	<0.001	<0.001
Infection		<0.001	0.311	0.003	0.889	0.036	<0.001
Level × Source		0.765	0.936	0.122	0.742	0.268	0.082
Level × Infection		0.903	0.418	0.067	0.624	0.516	0.477
Source × Infection		**0.014**	0.623	0.536	0.698	0.141	0.411
Level × Source × Infection		0.598	0.900	0.701	0.634	0.711	0.546

[1]Log-transformed values.
SEM: Pooled standard error of the mean.
D6 and 12 pi equate to d18 and 24 of age, respectively.
See Figure 4.1 for a graphical illustration of the interaction (P = 0.014) between VitD source and infection status on plasma Ca concentration at d6pi.

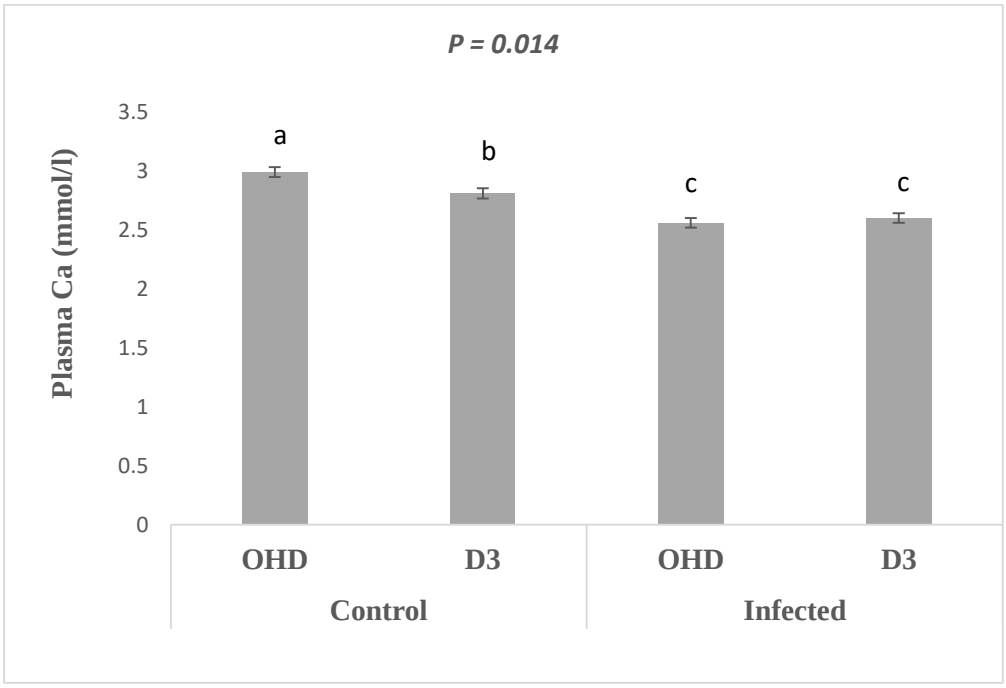

Figure 4. 1 Interaction between dietary VitD source (D3 and OHD) supplemented at 4000 IU/kg and infection status on plasma calcium concentration at d6 post-infection. Broilers chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch,

Table 4. 6 Main effects of source of VitD supply (D3 vs OHD), Ca/P level (M = 6.1:3.1 vs A = 8.7:4.4 g/kg) and infection status on small intestine morphology on days 6 and 12 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch.

Experimental factors		**Duodenum**						**Jejunum**						**Ileum**					
		Villi length (μm)		Crypt depth (μm)		Villi/Crypt ratio		Villi length (μm)		Crypt depth (μm)		Villi/Crypt ratio		Villi length (μm)		Crypt depth (μm)		Villi/Crypt ratio	
		d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi
Level	M	1767	2016	255	204	9.15	11.0	880	1135	226	175	5.44	6.70	634	711	159	136	4.49	5.39
	A	1766	2131	268	185	8.57	12.1	957	1174	248	177	5.48	6.81	640	700	162	140	4.28	5.37
Source	D3	1761	2070	264	212	8.89	10.9	952	1140	239	183	5.69	6.42	626	711	162	135	4.25	5.48
	OHD	1772	2077	260	176	8.83	12.2	886	1168	234	169	5.23	7.09	647	700	158	141	4.53	5.27
Infection	Control	2113	2156	153	183	13.8	13.2	1099	1142	126	156	8.73	7.43	617	697	116	114	5.45	6.21
	Infected	1420	1991	370	205	3.88	9.90	738	1167	347	197	2.19	6.07	657	714	205	163	3.32	4.54
	SEM	39.6	51.0	6.96	15.4	0.244	0.455	29.6	42.9	8.25	7.14	0.176	0.254	19.7	19.7	7.69	5.99	0.179	0.224
											Probabilities								
Level		0.998	0.119	0.181	0.384	0.098	0.108	0.073	0.525	0.070	0.864	0.860	0.758	0.836	0.708	0.829	0.688	0.417	0.957
Source		0.855	0.917	0.673	0.107	0.854	0.054	0.121	0.641	0.693	0.161	0.069	0.068	0.461	0.698	0.739	0.471	0.295	0.512
Infection		<0.001	0.028	<0.001	0.308	<0.001	<0.001	<0.001	0.683	<0.001	<0.001	<0.001	0.001	0.177	0.859	<0.001	<0.001	<0.001	<0.001
Level × Source		0.070	0.457	0.318	0.871	0.223	0.645	0.821	0.122	0.962	0.356	0.347	0.535	0.966	0.103	0.475	0.155	0.294	0.392
Level × Infection		0.853	0.388	0.806	0.247	0.213	0.690	0.320	0.306	0.362	0.235	0.128	0.657	0.668	0.968	0.571	0.598	0.506	0.842
Source × Infection		0.559	0.906	0.338	0.280	0.693	0.266	0.754	0.137	0.473	0.589	0.488	0.794	0.864	0.379	0.945	0.588	0.815	0.383
Level×Source×Infection		0.100	0.187	0.579	0.164	0.112	0.072	0.695	0.853	0.482	0.757	0.628	0.966	0.629	0.590	0.780	0.554	0.661	0.325

SEM: Pooled standard error of the mean.
D6 and 12 pi equate to d18 and 24 of age, respectively.

Table 4. 7 Main effects of source of VitD supply (D3 vs OHD), Ca/P level (M = 6.1:3.1 vs A = 8.7:4.4 g/kg) and infection status on femur length (mm), width (mm) and breaking strength (N) in proportion to body weight (BW; cg) as well as seedor and robusticity indices on d6 and 12 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch,

Experimental factors		Femur									
		Length/BW (mm/cg)		Width/BW (mm/cg)		Breaking Strength/BW (N/cg)		Seedor index		Robusticity index	
		d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi
Level	M	5.95	4.28	0.687	0.512	146	122	80.0	113	3.41	3.35
	A	6.16	4.40	0.719	0.521	171	140	81.7	112	3.39	3.33
Source	D3	6.01	4.36	0.691	0.513	164	130	80.2	113	3.41	3.36
	OHD	6.10	4.32	0.715	0.520	153	132	81.6	112	3.39	3.33
Infection	Control	5.80	4.15	0.684	0.498	162	140	82.1	115	3.40	3.36
	Infected	6.32	4.53	0.722	0.535	155	122	79.7	110	3.40	3.32
	SEM	0.0767	0.0646	0.0101	0.0067	5.04	3.77	1.23	1.63	0.0182	0.0173
		Probabilities									
Level		0.070	0.203	0.030	0.344	0.002	0.002	0.339	0.932	0.490	0.597
Source		0.441	0.656	0.104	0.458	0.124	0.685	0.454	0.725	0.534	0.246
Infection		<0.001	<0.001	0.011	<0.001	0.338	0.002	0.168	0.034	0.705	0.186
Level × Source		0.583	0.944	0.674	0.650	0.589	0.052	0.454	0.383	0.653	0.805
Level × Infection		0.078	0.251	0.636	0.064	0.477	0.596	0.294	0.037	0.595	0.048
Source × Infection		0.634	0.370	0.639	0.350	0.509	0.316	0.472	0.066	0.736	0.217
Level × Source × Infection		0.235	0.859	0.941	0.363	0.136	0.470	0.157	0.326	0.187	0.341

SEM: Pooled standard error of the mean.
D6 and 12 pi equate to d18 and 24 of age, respectively.

Table 4. 8 Effects of source of VitD supply (D3 vs OHD), Ca/P level (M = 6.1:3.1 vs A = 8.7:4.4 g/kg) and infection status on tibia length (mm), width (mm), breaking strength (N) and ash weight (mg) in proportion to body weight (BW; cg) as well as ash percentage, seedor and robusticity indices on d6 and 12 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch.

Experimental factors		**Tibia**													
		Length/BW (mm/cg)		Width/BW (mm/cg)		Breaking Strength/BW (N/cg)		Seedor index		Robusticity index		Ash/BW (mg/g)		Ash percentage	
		d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi	d6pi	d12pi
Level	M	8.01	5.80	0.619	0.450	214	167	86.7	118	4.05	4.04	0.904	0.909	50.1	50.5
	A	8.23	5.96	0.66	0.455	236	191	86.8	115	4.04	4.04	1.00	0.995	51.7	51.2
Source	D3	8.07	5.90	0.639	0.455	227	176	86.4	117	4.05	4.04	0.951	0.946	51.0	50.5
	OHD	8.17	5.86	0.640	0.451	224	182	87.1	115	4.04	4.04	0.955	0.958	50.8	51.2
Infection	Control	7.83	5.62	0.623	0.454	230	207	87.8	121	4.06	4.03	0.969	0.992	51.4	52.5
	Infected	8.42	6.15	0.656	0.452	220	151	85.7	111	4.03	4.06	0.937	0.911	50.4	49.2
	SEM	0.0858	0.0794	0.00965	0.00722	6.01	4.05	1.15	1.78	0.0164	0.017	0.0154	0.0152	0.321	0.308
		Probabilities													
Level		0.081	0.180	0.006	0.605	0.013	<0.001	0.952	0.338	0.674	0.928	<0.001	<0.001	0.001	0.080
Source		0.394	0.761	0.992	0.672	0.734	0.306	0.701	0.398	0.690	0.909	0.868	0.587	0.593	0.172
Infection		<0.001	<0.001	0.023	0.851	0.254	<0.001	0.189	<0.001	0.216	0.294	0.145	0.001	0.042	<0.001
Level × Source		0.426	0.865	0.870	0.734	0.776	0.602	0.143	0.214	0.834	0.274	0.831	**0.023**	0.740	**0.022**
Level × Infection		0.035	0.335	0.084	0.095	0.965	**0.024**	0.715	0.017	0.363	0.008	0.391	0.528	0.763	0.933
Source × Infection		0.393	0.348	0.863	0.109	0.502	0.83	0.471	0.143	0.432	0.144	0.750	0.396	0.710	0.904
Level × Source × Infection		0.171	0.789	0.965	0.326	0.805	0.143	0.123	0.284	0.126	0.965	0.501	0.812	0.948	0.477

SEM: Pooled standard error of the mean.
D6 and 12 pi equate to d18 and 24 of age, respectively.
See Figures 4.2 and 4.3 for a graphical illustration of significant interactions ($P < 0.05$) between the experimental factors on bone mineralisation parameters at d12pi.

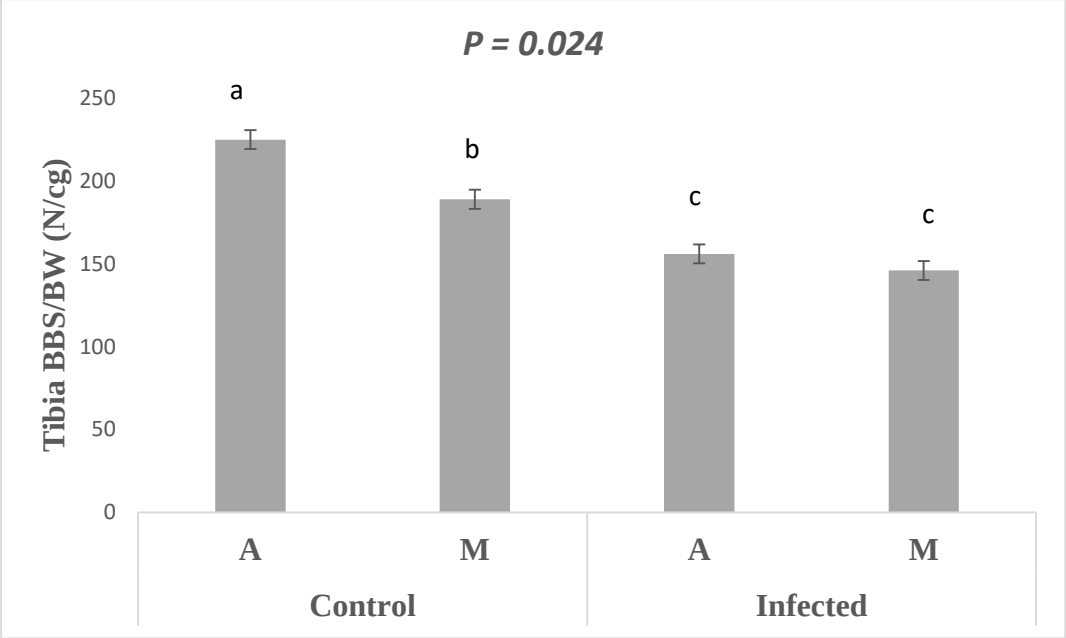

Figure 4. 2 Interaction between dietary Ca:P level (A = 8.7:4.4 vs M = 6.1:3.1) and infection status on tibia bone breaking strength in proportion to body weight (BS/BW) at d12 post-infection. Broilers chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch.

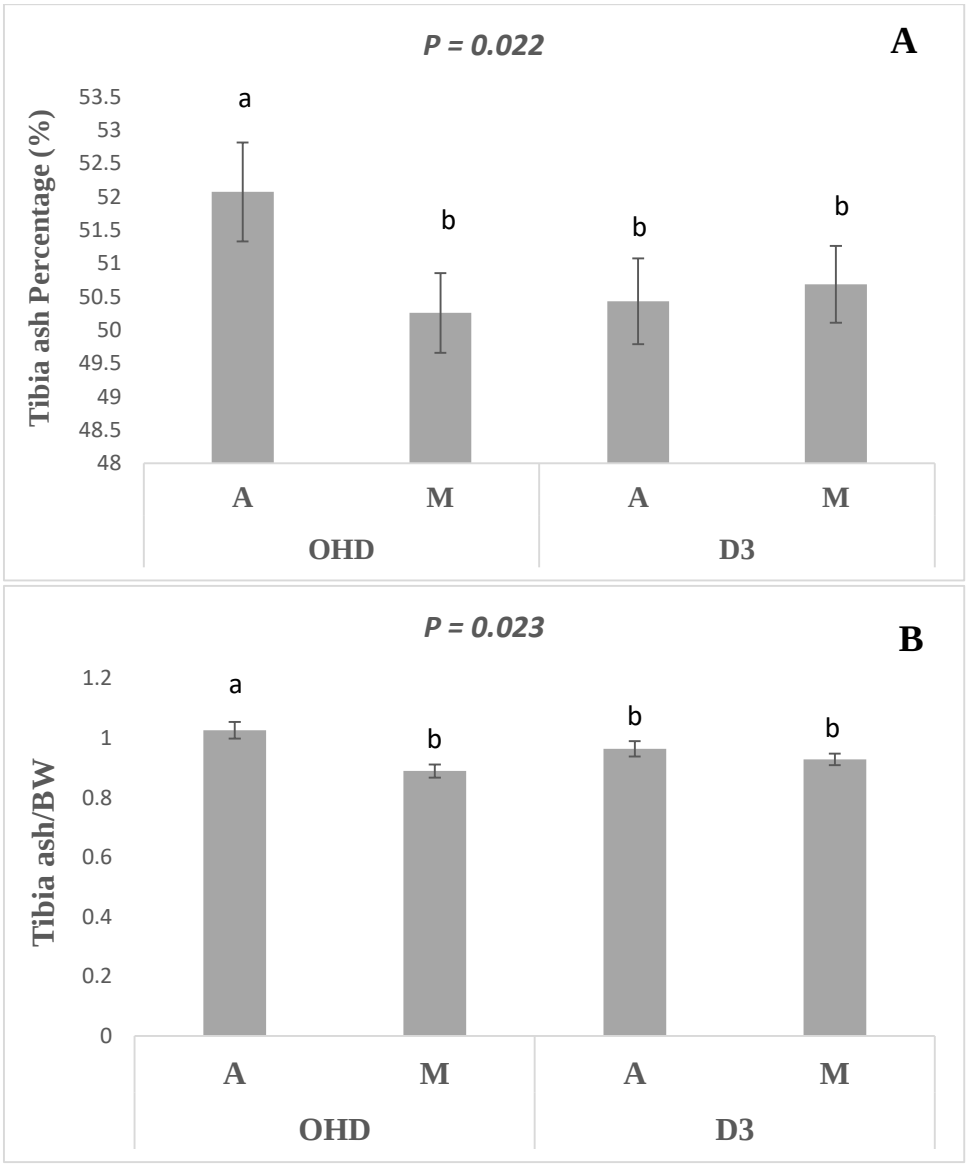

Figure 4. 3 Interaction between dietary VitD source (D3 and OHD) supplemented at 4000 IU/kg and dietary Ca:P level (A = 8.7:4.4 vs M = 6.1:3.1) on tibia ash percentage (A) and ash in proportion to body weight (B) at d12 post-infection. Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d12 post-hatch.

4.5 Discussion

This study aimed at inducing a marginal Ca and P deficiency in growing broilers to test the hypothesis that dietary VitD supplementation at 4000 IU/kg using OHD instead of D3 will enhance long bone mineralisation and productive performance of broilers on such diets during coccidiosis. By this hypothesis, more pronounced effects of VitD source was also expected in the coccidiosis-infected than in the uninfected broilers. Furthermore, a 3-way interaction between VitD source, Ca/P level and infection status, as well as a 2-way interaction between VitD source and Ca/P level, for parameters of long bone mineralisation and performance of broilers was anticipated in this study. The higher biological potency of OHD in mediating VitD activity compared to D3 (Wagonfeld *et al.*, 1976; Reed *et al.*, 1980; Fritts and Waldroup, 2003), and the observed penalties on bone mineralisation following malabsorptive coccidian infections [Chapters 2 and 3] formed the basis of the hypothesis. In addition, the present study investigated the effect of 4000 IU/kg dietary OHD compared to D3 supplementation on parasite replication and gut integrity during broiler coccidiosis.

Although the analysed OHD levels (section 4.3.4) in the experimental diets were consistently lower than the intended values, the cause of this may likely be analytical as observed in chapter 3: the same laboratory and equipment were used to analyse feed samples in both studies. Notwithstanding, blood OHD concentration, which is considered to be the most reliable indicator of dietary VitD intake (Hollis, 2005; Autier *et al*., 2014), were consistently and significantly higher for broilers receiving the OHD compared to the D3 diets in accordance with the aims and hypothesis of the study. Therefore, the results presented herein can be discussed confidently without any reservations in this regard. Also, the effects of the Ca/P levels on parameters of long bone mineralization, which was significantly reduced for the M compared to the A-fed broilers, were as expected for femur and tibia BS (d6 and 12 pi), as well as tibia ash weight (d6 and 12pi) and ash percentage (d6pi). Furthermore, *E. maxima* infection significantly impaired performance and long bone mineralisation, and altered gut morphological features, as observed in chapters 2 and 3.

In the present study, circulating OHD was assessed as a marker for VitD status because it is the main storage form of VitD in animals and research has shown that it is a more accurate indicator of VitD nutritional status than the biologically active metabolite, 1,25D3 (Yarger *et al*., 1995; Young *et al*., 1997; Norman, 2008). A reason for the above is that the formation process of OHD from D3 in the liver is not regulated, so blood level rightly reflects dietary intake. Conversely, renal or extra-renal production of 1,25D3 from OHD is tightly regulated;

being influenced by several factors including circulating Ca, P and parathyroid and other related hormones (Holick, 2005; Hollis and Horst, 2007; Autier *et al*., 2014).

The poorer performance, regarding BW, ADG, ADFI, and FCR, of the I than the C broilers reported herein, is traceable to several factors including pathogen-induced anorexia (Kyriazakis, 2014), impaired nutrient absorption and utilization (Hernández-Velasco, *et al*., 2014; Preston-Mafham and Sykes, 1970; Rochell, *et al*., 2016) associated with the parasite-induced morphological alterations in the small intestine [Chapter 3]. The penalised performance may also be attributed to the activation of an immune response (Allen, 1997) amongst other disease-related expenditures such as altered expression of nutrient transporters and digestive enzymes in the intestine (Su *et al*., 2015).

The absence of an effect of dietary Ca/P level on relative ADG and ADFI in the current study was anticipated. This is because 25 to 33% and 15 to 23% reductions of dietary total Ca and of avP respectively, below the recommended levels (Aviagen, 2014a), whilst maintaining a Ca/P ratio close to 2:1, have not previously penalised growth performance in growing broilers (10 to 30 days of age) (Delezie *et al.*, 2012; Delezie *et al.*, 2015; Valable *et al.*, 2018). Valable *et al.* (2018) suggested that this lack of effect on growth performance is because a decrease in Ca and avP at the levels mentioned above reduces lean body content and increases body fat content. It is expected that effects on bone mineralisation would be seen before the impact on performance because Ca and P requirements for mineralisation exceed those for lean body mass development (Larbier and Leclercq, 1992).

The absence of a significant 3-way interaction between Ca/P level, VitD source and infection status for any of the measured variables in this study led to the rejection of the hypothesis of this study. Therefore, the results suggested a similar effect of dietary OHD and D3 supplied at 4000 IU/kg, on long bone quality, growth performance, and gut morphology of broilers infected with *E. maxima* or not. Previous studies (Fritts and Waldroup, 2003; Sakkas *et al.*, 2018) had reported a similar capacity for OHD and D3 to mediate VitD activities in healthy birds when supplied at the level in the current study. It is unclear why the effects of OHD and D3 on bone mineralisation did not differ due to coccidian infection that led to impaired Ca and P status, amongst birds offered deficient or adequate Ca/P diets. However, this suggests that coccidiosis also impairs the utilisation of these nutrients for bone mineralisation.

The infection-induced reductions in plasma concentrations of Ca, P and OHD in the current study are consistent with the previous findings [Chapter 3]. In the present study, VitD source did not affect plasma Ca levels in infected broilers, whereas OHD- in comparison to D3-fed

broilers had higher plasma Ca concentration amongst uninfected broilers. This interaction between VitD source and infection status that occurred for plasma Ca at d6pi (Figure 4.1) was implicated in the similar Ca/P level and infection status interaction that occurred for tibia BS at d12pi (Figure 4.2). It was also connected with the 2-way interaction between VitD source and Ca/P level for tibia AP and AW/BW at d12pi in this study: values for AD3, MD3 and MOHD were statistically similar but significantly lower than AOHD (Figure 4.3). The specific mechanism by which OHD and D3 diets can have similar effects on plasma Ca status for coccidia-infected, but not for healthy broilers, as shown in the current study remains unclear. However, similar to the effects of phytase reported in Watson *et al.* (2005), these results suggest that OHD compared to D3 is more efficient in improving Ca and P absorption in the absence of coccidiosis, and that Ca and P utilisation may be impaired by coccidiosis. The above finding for the control birds agreed with the hypothesis of the current study, but that of the infected birds warrants further investigations.

As expected, the OHD- compared to the D3-fed broilers had significantly higher OHD concentration in blood plasma at d6 and 12pi, but this was the case irrespective of bird infection status and dietary Ca/P level. Although plasma OHD status of birds may be influenced by VitD content of the diet ingested, there is no evidence to suggest any correlation between concentrations of blood OHD and its biologically active metabolite, 1,25D3, in broilers (Yarger *et al.*, 1995). In line with Yarger *et al.* (1995), despite the higher plasma OHD concentration for the OHD- than the D3-fed broilers, the effects of VitD source on measured variables did not always reflect this in the current study. This offers a likely explanation as to why the main effect of VitD source was not significant for parameters of bone mineralisation (BS/BW, Ash/BW, and AP) or development (linear growth and density) in this study. However, the OHD and A diet combination in comparison to the other diet combinations significantly increased long bone mineralisation for broilers as discussed in the preceding paragraph.

Consistent with the delayed effect of infection on bone strength in relation to the performance reported in chapters 2 and 3, *E. maxima* infection significantly weakened long bone BS of broilers at d12pi in the current study. There was a corresponding significant reduction in tibia AP for the infected than uninfected broilers at d6 and 12pi and Ash/BW at d12pi. However, contrary to expectations, dietary OHD and D3 supplemented at 4000 IU/kg affected bone mineralisation to a similar degree in both infected and control broilers. This conflicting evidence likely stem from the fact that the higher potency of OHD compared to D3 is better displayed when offering diets with suboptimal (< 500 IU/kg) VitD levels (Fritts and

Waldroup, 2003; Fritts *et al.*, 2004; Fritts and Waldroup, 2005). Similar to the findings herein, previous studies reported a reduction in bone mineral content, AP in dry defatted bone, and BS in the absence of effects on performance when offering diets with suboptimal levels of Ca and avP at a steady 2:1 ratio (Yan *et al.*, 2005; Valable *et al.*, 2018).

The significantly higher parasite burden for OHD than D3 broilers observed in chapter 3 was absent in the current study, although the total parasite replication was almost double in this study. However, the results herein are in line with the previous report that dietary D3 and OHD mediate VitD functions similarly when supplemented at 4000 IU/kg (Fritts and Waldroup, 2003; Fritts *et al.*, 2004; Sakkas *et al.*, 2018). A possible reason why 4000 IU/kg OHD and D3 diets had similar effects on parasite replication in this study may be connected to the significantly higher parasite load herein. It may be the case that VitD immunomodulatory effects are more pronounced at lower levels of *E. maxima* replication, or that the outcome of a higher level of infection was influenced by the *Eimeria* crowding effect (Williams, 2001), possibly negating any experimental differences. Further studies will unravel this, as the scientific literature has no specific information to offer regarding this issue.

In conclusion, *E. maxima* infection and offering marginally deficient diets in Ca/P reduced bone mineralisation. Broilers offered OHD as the source of dietary VitD and adequate Ca/P diets showed the highest degree of bone mineralisation. However, the hypothesis that high-level (4000 IU/kg) dietary OHD instead of D3 supplementation will enhance mineralisation to a higher degree for broilers during *E. maxima* infection was rejected as both sources had similar effects on parameters of long bone mineralisation of broilers in the presence or absence of coccidiosis. This suggests that the high-level OHD and D3 diets used in commercial broiler production might not affect *E. maxima* replication differently.

Chapter 5: Effects of reducing early growth rate via diet dilution on bone development, performance and relative carcass yield of coccidia-infected broilers

5.1 Summary

Coccidia infection and genetic selection for faster growth rate (GR) compromise long bone quality in modern broiler chickens [Chapter 2]. The hypothesis that artificial reduction in GR via diet dilution with lignocellulose during peak bone development will improve bone mineralisation in both coccidiosis-infected and uninfected broilers was tested in this study. Male Ross 308 day-old chicks totalling 384 were allocated to a basal grower diet (3107 kcal/kg ME and 19.4% CP) diluted with 0, 5, 10 or 15% lignocellulose at d10 of age. Before this, birds in each group received half the intended diet-dilution levels from d8 to 10 of age, and a standard starter diet from d1 to 7 of age. At d13 of age (d0 post-infection, pi), birds were orally inoculated with either 7000 sporulated *Eimeria maxima* oocysts (I) or water (C), forming a 4 diet-dilution level × 2 infection status factorial experiment. Each treatment group consisted of 6 replicate pens with 8 birds per pen. Broiler performance was evaluated using average daily feed intake (ADFI) and average daily gain (ADG) measured over 12 days pi and scaled to BW at infection (d0 pi). The scaling was done to account for *a priori* BW differences due to diet dilution. ADFI and ADG relative to BW were also assessed over the early (d1 – 6 pi) and late (d7 – 12 pi) stages of infection. At d12 pi (d25 of age), 6 birds per treatment combination were sampled to assess tibia and femur mineralisation relative to BW, as well as carcass yield. There was no interaction ($P > 0.1$) between infection status and diet-dilution level on ADFI/BW measured over d1 – 12 pi, or on any bone variable. ADG/BW pi decreased ($P < 0.01$) with diet dilution amongst C birds, but was statistically similar ($P > 0.05$) amongst I birds. I compared to C birds had reduced breast meat ($P < 0.05$) and eviscerated carcass yield ($P < 0.01$), femur ($P < 0.05$) and tibia ($P < 0.01$) relative breaking strength (BS/BW), and femur relative ash weight (ash/BW) ($P < 0.05$). Diet dilution with lignocellulose did not affect carcass yield, but improved femur BS/BW ($P < 0.001$), and tended to improve ($0.05 < P < 0.1$) femur and tibia Ash/BW. Overall, diet dilution significantly affected more femur than tibia variables: relative BS, robusticity index, and ash percentage. Artificial diet-dilution induced reduction in GR affected marker of long bone mineralisation to a similar degree in the presence or absence of coccidiosis in broilers.

5.2 Introduction

Intensive genetic selection for performance in modern broilers has increased GR, such that the time required to reach 2 kg BW reduced by 3 weeks between the 1950s and 2014 (Tallentire *et al.*, 2016), but at the same time appears to have compromised skeletal development and integrity (Julian, 1998; Pratt and Cooper, 2018). Rapid GR causes rapid periosteal bone deposition, impaired mineralisation, altered biomechanical properties and radial vascular canal orientation, which increases the porosity of the cortical bone (Williams *et al*., 2004; Pratt and Cooper, 2018). Skeletal problems, particularly those affecting leg bones are associated with chronic pain in broilers that negatively affect their welfare (Julian, 1998; Danbury *et al*., 2000; Dibner *et al*., 2007). Moreover, their increased prevalence causes substantial financial losses due to lameness, which limits bird access to feed (Knowles *et al*., 2008) and increased mortality, and culling rates due to leg fractures and vertebral column abnormalities, as well as increased carcass condemnations caused by muscle haemorrhage and the presence of bone fragments in meat portions (Driver *et al*., 2006; Knowles *et al*., 2008; Pines and Reshef, 2015).

Broilers with slow compared to fast GR have lower incidence of leg disorders (Corr *et al.*, 2003b; Caplen *et al.*, 2012; Kapell *et al.*, 2012a), better adaptation to increased mechanical load (Pitsillides *et al*., 1999), higher bone ash and reduced occurrence of cortical bone porosity (Leterrier and Nys, 1992; Williams *et al*., 2004; Pratt and Cooper, 2018). Chapter 2 revealed a similar bone ash percentage (AP), but a higher amount of ash in proportion to body weight (ash/BW) and improved femur strength for the slower-growing of two broiler lines divergently selected for GR. Generally, studies suggest that the axial bone of fast-growing modern-day broilers remains under development throughout its lifetime, never reaching the stage of homeostasis and remodeling as seen in other vertebrates (Rath *et al.*, 2000; Roberson *et al.*, 2005), and factors including infection, flock management system and nutrition affect healthy bone development (reviewed in Kierończyk *et al*. (2017)).

Bone development in broilers is at the peak during the first three weeks of life (Lilburn, 1994; Williams *et al*., 2000). Reducing GR during this period may facilitate bone development in fast-growing broilers. GR reduction via feed restriction has been shown to improve bone mineralisation, skeletal development and impact positively on the bone quality of healthy broilers (Pratt and Cooper, 2018). Quantitative feed restriction as a means of delaying GR achieved lower bone porosity and higher mineralisation (Williams *et al*., 2004). However, this methodology raises welfare concerns due to the association of chronic hunger with prolonged

restrictions (Tolkamp and D'Eath, 2016). Another effective method of GR reduction is the use of qualitative feed restriction which involves diluting the diet with an inert ingredient or with feed ingredients of low nutritional value (Rezaei and Hajati, 2010; Atapattu and Silva, 2016; Xu *et al*., 2017). This method reduces the adverse effects of starvation or chronic hunger on broiler welfare that are associated with the quantitative restriction method.

Coccidiosis penalises bone mineralisation by limiting intestinal absorption and utilisation of vital bone minerals (Turk, 1973; Turk, 1978) [Chapter 2], as well as increasing bone resorption in broilers (Akbari Moghaddam Kakhki *et al*., 2018). Chapters 2 to 4 showed that significant effects of malabsorptive coccidiosis on bone mineralisation are delayed until the later stages (beyond 12 days) of infection. This suggests that previous studies may have underestimated the adverse effect of coccidiosis on broiler bone mineralisation as they typically assess bone mineralisation within the first 6 days post-infection (pi**)** when productive performance is grossly impaired (Blake and Tomley, 2014).

In the present study, the aim was to investigate whether a diet-dilution induced reduction in early GR, i.e. during weeks 2 and 3 of age when bone development peaks, would improve bone mineralisation of broilers in the presence, as in the absence, of coccidiosis. Grower diets were diluted with lignocellulose, an inert substance with high water holding capacity (WHC) and no added nutritive value to the feed, to limit the nutrient intake and consequently ADG. The hypothesis was that GR reduction would improve parameters of bone mineralisation in broilers infected with coccidiosis as in their uninfected counterparts.

5.3 Materials and Methods

5.3.1 Birds, Husbandry and Diets

All procedures were conducted under the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU for animal experiments and carried out under Home Office authorisation (P441ADF04). Three hundred and eighty-four male Ross 308 chicks were raised from hatch until 25d old. They were housed in 48 rectangular 0.84 m^2 pens situated in a thermostatically controlled building at the Newcastle University Cockle Park farm. Each pen was equipped with a tube feeder and a bell-drinker offering birds *ad libitum* access to feed and water throughout the experimental trial. Wood shavings to a depth of 5 cm were used as litter. Routine husbandry procedures were carried out as described in section 2.3.1 of chapter 2.

Upon arrival, broilers were allocated to a conventional starter diet until 7 d of age. A basal grower diet with 3107 kcal/kg ME and 19.4% CP was diluted at graded levels, i.e. 0, 5, 10 or 15%, of

Arbocel ® RC Fine lignocellulose (JRS PHARMA, Rosenberg, Germany) to formulate four experimental diets (Table 5.1). The energy to crude protein ratios of the diets was maintained due to the inert nature of the diluent. Arbocel is a natural lignocellulose produced from fresh spruce trees (*Picea* species) by JRS PHARMA and is characterised by its high WHC. It was used as the feed diluent because of its inert nature and high WHC, which was expected to cause a reduction in feed intake and hence GR at the above levels of inclusion in the diet. An adaptation period was incorporated from d8 to 10 of age, during which chicks within each diet group were assigned to half of their intended diet dilution level, before allocating them to the dietary treatments at 10 d of age. All diets were offered as a coarse mash passed through a 5 mm screen.

5.3.2 Experimental design and inoculations

The experiment had a 4 × 2 factorial arrangement with four feeding treatments and two infection statuses. Birds were assigned to diets diluted with 0, 5, 10 or 15% lignocellulose (R0 – R3 respectively) at d10 of age. At 13 d of age (d0pi), birds were orally inoculated with a single 0.5 ml dose of water containing either 0 (Control; C) or 7000 (Infected; I) sporulated *E. maxima* oocysts of the Weybridge laboratory reference strain, using 1 ml syringes. The inocula were prepared using a previously described method (Pastor-Fernández *et al*., 2018) as in the preceding chapters. Each treatment group was replicated in six pens.

5.3.3 Sampling

Bird and feed weight were measured at d0, 6 and 12 pi to evaluate the growth performance of birds during the early (d1 to 6 pi) and the late (d7 to 12 pi) stages of infection, and the entire period of infection (d1 to 12 pi). To confirm the occurrence of infection, polyethene sheets were placed over the wood shavings of each pen for 90 minutes daily from d4 to 10 pi to obtain excreta samples for enumerating oocysts per gram (OPG). Approximately 10 g of pooled excreta from each pen were collected in screw cap pots and stored at 4°C pending OPG determination.

One bird/pen weighing close to pen average was selected for carcass yield evaluation at d12 pi. Birds were euthanised with a lethal injection of sodium pentobarbitone (Euthatal®, Merial Harlow, United Kingdom), and then eviscerated. The weight of eviscerated carcass and portions including breast meat, wings, thigh and drumstick were measured using a digital scale. Following carcass evaluation, right femur and tibia bones were dissected, defleshed using scalpels, and stored in airtight individually labelled polythene bags at -20°C pending evaluation. Long bones were sampled at d12 pi because *E. maxima*-infected broilers experience significantly impaired bone mineralisation at d12 pi [Chapters 2 – 4].

5.3.4. Sample analysis

Excreta oocysts count

Excreta OPG were estimated using the modified McMaster technique (Kaufmann, 2013). After thorough mixing of excreta, a 3 g sample was removed for OPG determination and the remaining excreta material was freeze-dried to estimate dry matter (DM) content. Sampled material for OPG determination was mixed with 42 ml of water and then passed through a 1mm sieve. The suspension formed was transferred to a glass tube, centrifuged at 1500 rpm for 2 mins at room temperature, the supernatant was carefully siphoned off, and then the underlying pellet was vortexed until re-suspended. After that, 10 ml of saturated sodium chloride solution was added, the suspension was mixed thoroughly, and a sample taken from the centre of the tube was carefully transferred to the chambers of a McMaster counting slide. Slides were left to stand for 10 mins to allow the oocysts to rise to the top of the slide, before being read at 100× magnification. Values obtained were further expressed per unit gram of excreta DM content to obtain 'OPG excreta DM.'

Bone evaluation

Bones were thawed at 4 ºC overnight and placed at room temperature for 1 h before further de-fleshing of adhering soft tissues. Length and width at the centre of the diaphysis were measured for tibia and femur using a digital calliper, and then the weight of each bone was measured using an analytical balance. The bones were subjected to a 3-point break test using an Instron testing machine (Instron 3340 Series, Single Column-Bluehill) to determine breaking strength (BS) in Newtons (N). The testing support consisted of an adjustable 2-point block jig, spaced at 30 mm for both tibia and femur bones. The crosshead descended at 30 mm/min until a break was determined by measuring a reduction in force of at least 5%. After BS assessment, tibia and femur ash weight (AW, g) and AP were determined using a previously described method in section 3.3.4 of chapter 3.

Table 5. 1 Ingredients and chemical composition (%) of the starter (d0 to 7) and grower (d8 to 25) diets offered to broiler chickens.

	Starter	Grower			
Ingredients (%)	**Basal**	**Basal (R0)**	**Low Lignocellulose (R1)**	**Medium Lignocellulose (R2)**	**High Lignocellulose (R3)**
Ground Maize	10.0	10.0	9.5	9.0	8.5
Ground Wheat	51.5	53.9	51.2	48.5	45.8
Soybean meal (48% CP)	26.0	23.0	21.8	20.7	19.5
Arbocel (lignocellulose)	-	-	5	10	15
Full fat Soya	5.0	5.0	4.75	4.5	4.25
Limestone	1.25	1.25	1.19	1.13	1.06
L-Lysine HCL	0.4	0.3	0.29	0.27	0.26
DL-Methionine	0.4	0.35	0.33	0.32	0.3
L-Threonine	0.15	0.15	0.14	0.14	0.13
Soya oil	3.00	3.50	3.33	3.15	2.98
Monocalcium phosphate	1.50	1.25	1.19	1.13	1.06
Salt	0.25	0.25	0.24	0.23	0.21
Sodium bicarbonate	0.15	0.15	0.14	0.14	0.13
Premix	0.4	0.4	0.4	0.4	0.4
Titanium dioxide	-	0.5	0.5	0.5	0.5
Nutrient composition (%)*					
ME (kcal/kg) (calculated)	3059	3107	2940	2796	2629
Crude protein	21.4	19.4	18.5	17.6	16.9
Crude fibre	2.9	2.3	4.5	7.2	10.7
Ether extract (oil A)	5.55	6.59	6.13	5.79	5.53
Total oil (oil B)	-	7.32	6.86	6.51	6.30
Acid detergent fibre	-	3.25	5.61	7.96	11.60
Neutral detergent fibre	-	8.3	11.5	15.7	21.9
Acid detergent Lignin	-	0.48	1.39	2.45	3.23

The nutrient composition was in accordance with Aviagen nutrient specifications (Aviagen, 2014a), but the four grower diets contained 0, 5, 10 and 15% lignocellulose supplemented at the expense of wheat and soybean meal.
*Analysed nutrient composition (%) unless otherwise stated.

5.3.5 Calculations and Statistics

All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 22.0. (Armonk, NY: IBM Corp). The pen was used as the experimental unit for all statistical assessments. Values obtained for ADFI (g/d) and ADG (g/d) were expressed relative to BW (g) at infection to account for *a priori* differences in BW between groups. Carcass yield (%) was obtained by expressing the weight of eviscerated carcass and portions including breast meat and thigh plus drumstick as a percentage of live BW at dissection (d12 pi). Breast meat and thigh plus drumstick were further expressed as a percentage of eviscerated carcass weight to obtain part yield. Tibia and femur BS, length and width (mm), and AW measured at d12 pi were expressed in proportion to BW at dissection. Robusticity index and AP were calculated for both long bones using the prescribed formula (Riesenfeld, 1972). Pen relative performance and bone data generated from sampled birds were analysed with dietary treatment and infection status as fixed factors using a general linear model. Excreta OPG data was further analysed using repeated measures mixed procedure. The model contained diet and day as the factors and the 2-way interaction between diet and day. Linear and quadratic responses to diet dilution were determined using orthogonal polynomial contrasts for all variables. Treatment means were compared by the Tukey's multiple comparison test, which maintains the desired alpha levels provided the model assumptions such as normality and homogeneity of residuals are met. For assessing the normality of the studentized residuals, the Shapiro-Wilk test was used, and non-normalized data, such as OPG were log-transformed. Significance was determined at $P < 0.05$ and tendency at $0.05 < P < 0.1$. Furthermore, allometric scaling relationships between tibia (Y) length, width, weight and ash weight, and their corresponding measurements for femur (X) bone was determined for broilers in all the treatment groups, using reduced major axis (RMA) linear regression. This method accounts for variations in both X and Y axes (Rayner, 1985; Sokal and Rolf, 1995). All regression analyses were performed on natural log-transformed data to establish the allometric equation:

$$\text{LogY (tibia variable1)} = \text{Loga} + \text{b LogX (femur variable1)}$$

Then the difference between slope (b) derived from R0 vs R1, 2 or 3 treatments was assessed separately based on infection status (i.e., control and infected) for each variable using prescribed formula (Andrade and Estévez-Pérez, 2014). The different slopes derived from R0 in control vs. R0 to 3 in infected broilers for length, width, weight and ash weight were calculated using the prescribed formula:

$$\frac{\mathrm{b1} - \mathrm{b2}}{\sqrt{\mathrm{SEb1}^2 + \mathrm{SEb2}^2}}$$

Where b1 and b2 represent the individual slopes and SEb1 and SEb2 were the respective standard errors of the slopes.

5.4 Results

5.4.1 Bird health and performance variables

No bird was euthanised due to health-related disorders, and coccidiosis caused anorexia and reduced weight gain according to expectations. Results on growth performance are presented for BW pre-infection, as well as the early, late, and entire periods pi (Tables 5.2 and 5.3).

BW pre-infection

Broilers had similar BW ($P > 0.05$) at the beginning of the adaptation period, d8 of age. Diet dilution led to a reduction ($P < 0.05$) in the BW of R1, 2 and 3 compared to R0 broilers by approximately 7, 9 and 15% respectively (estimated from the main effect of diet on BW d0pi, Table 5.2) at the point of infection, d13 of age.

Relative BW, ADG and ADFI post-infection

There was a significant interaction ($P < 0.05$) between diet and infection status for ADFI relative to BW (ADFI/BW) during the late stage of infection, relative BW at d12 pi and ADG relative to BW (ADG/BW) during the early, late and entire periods pi. During the late period of infection, the diluted diets (R1 to 3) had statistically similar effects ($P > 0.05$) on ADFI/BW of C and I birds, while the I compared to the C birds receiving undiluted, R0, diet had a lower ADFI/BW ($P < 0.05$) (Table 5.3). On the other hand, relative BW at d12 pi and ADG/BW reduced ($P < 0.05$) with increasing diet dilution amongst C birds, while values were statistically similar ($P > 0.05$) amongst the I birds.

Performance at any time point was significantly impaired ($P < 0.001$) by infection. Diet dilution affected ADFI/BW only during the early stage of infection ($P < 0.05$); R3 compared to R0 broilers had significantly higher ADFI/BW, while intake levels relative to BW were statistically similar for R0 to 2, as well as R2 and 3 broilers (Table 5.3). Furthermore, diet dilution reduced ($P < 0.05$) BW at d6 and 12 pi and relative BW at d12 pi followed a statistically linear reduction ($P < 0.001$). The effect of diet dilution on ADG/BW was statistically significant ($P < 0.05$) during the early, late and entire periods pi. Birds receiving the R3 compared to the R0 diet had significantly reduced ADG/BW or while R1, R2, and R3 birds had statistically similar ADG/BW.

5.4.2 Oocyst excretion

The effect of diet dilution on OPG is illustrated in Figure 5.1. There were no oocysts detected in the control pens. The repeated measurements analysis on daily oocyst excretion revealed no significant interaction ($P > 0.05$) between diet and day pi for OPG excreta DM output. Diet dilution did not affect ($P > 0.05$) excreted oocysts calculated per gram of excreta DM content from d4 to 10 pi (Figure 5.1). However, there was a linear decrease ($P < 0.01$) in oocysts output with increasing levels of diet dilution for OPG excreta DM at d7, 8 and 9pi. Day affected ($P < 0.05$) OPG excreta DM (4.62 vs 5.24 vs 4.37 vs 3.55 respectively; SEM = 0.0973) for d6 to 9pi; values were highest at d7pi compared to the other days pi.

5.4.3 Carcass evaluation

The main effects of diet dilution and infection status on carcass variables are presented in Table 5.4. There was no interaction between diet and infection status for carcass variables measured in this study. Infection significantly reduced ($P < 0.001$) relative eviscerated carcass and breast meat yield but did not affect ($P > 0.05$) relative thigh plus drumstick yield, breast meat and thigh plus drumstick part yield at d12 pi. Diet dilution did not affect ($P > 0.05$) relative eviscerated carcass, breast, and thigh plus drumstick yields at d12pi. There was no significant effect ($P > 0.05$) of diet dilution on breast meat and thigh plus drumstick part yield, i.e. when expressed as a percentage of eviscerated carcass weight. Furthermore, increasing diet dilution caused a linear decrease in all carcass variables measured at d12 pi, while the reduction in relative eviscerated carcass yield followed both a quadratic and linear pattern (Table 5.4).

5.4.4 Bone evaluation

The main effect of diet dilution and infection status on long bone variables at d12pi are presented in Tables 5.5 and 5.6. There were no interactions ($P > 0.05$) between diet and infection for tibia or femur bone variables measured.

Relative linear growth of long bone and robusticity index

Infection increased ($P < 0.05$) tibia length and femur width expressed as a proportion of BW at dissection: an artefact of reduced BW pi. Diet dilution caused a significant linear increase ($P < 0.05$) in tibia and femur length and width. Femur robusticity index of R3 and R2 compared to R0 broilers was higher ($P < 0.05$). Femur, but not tibia, robusticity increased

linearly ($P < 0.05$) with an increase in dilution level. Other effects were not significant ($P > 0.05$).

Breaking strength in proportion to BW

Infection significantly reduced ($P < 0.05$) femur and tibia BS. On the other hand, diet dilution increased ($P < 0.001$) femur, but not tibia BS for R3 in comparison to R0 and R1 birds (Table 5.5). Also, increasing diet dilution level caused a quadratic increase ($P < 0.001$) in femur and tibia BS relative to BW; R0 had the lowest value of 17.0 N/g.

Ash percentage and ash in proportion to BW

Infection did not affect ($P > 0.05$) femur and tibia AP, and tibia Ash/BW at d12 pi. However, it significantly reduced ($P < 0.05$) femur ash/BW. Diet dilution tended to increase ($0.05 < P < 0.1$) femur and tibia ash/BW, and femur AP for R3 in comparison to R0-2 birds (Table 5.5). Tibia AP was not affected by diet dilution ($P > 0.1$), while tibia and femur AP increased in a quadratic manner with diet dilution level in this study.

Allometric scaling of tibia vs femur

Results of differences between the slopes derived from linear regressions of the tibia (Y) vs femur (X), (i.e. comparing the dietary treatments) are shown for length, width, weight and ash weight in the appendix. There were no significant differences ($P > 0.05$) between the slope for R0 vs R1, 2 or 3 amongst the control or the infected birds. Also, the difference between the slope for R0 in control vs. R0, 1, 2, or 3 in the infected broilers was not statistically significant ($P > 0.05$) for the variables above (see Table 5.6).

Table 5. 2 Effect of diet diluted with 0, 5, 10 or 15% lignocellulose and infection status on body weight and relative body weight at infection (d0 pi) and during the early (d6pi) and late (d12pi) phases post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d13 post-hatch.

		Body Weight (g)			**Relative Body Weight (g/g)**	
Infection × Diet		d0pi	d6pi	d12pi	(d6/d0)pi	(d12/d0)pi
Control	R0	351	707	1178	2.04	3.47^{a}
	R1	340	625	982	1.97	3.27^{ab}
	R2	326	654	1063	1.95	3.17^{abc}
	R3	302	555	927	1.89	2.96^{bcd}
Infected	R0	356	617	979	1.80	2.90^{cd}
	R1	317	568	877	1.78	3.03^{bcd}
	R2	330	565	868	1.78	2.81^{d}
	R3	297	525	804	1.81	2.81^{d}
SEM		13.7	24.5	36.5	0.029	0.068
Main Effect						
Infection						
Control		330	636	1022	1.96	3.22
Infected		325	569	898	1.80	2.89
SEM		6.8	12.2	18.2	0.015	0.034
Diet						
R0		354^{a}	666^{a}	1080^{a}	1.92	3.18
R1		329^{ab}	605^{ab}	995^{ab}	1.87	3.15
R2		328^{ab}	592^{b}	928^{bc}	1.87	2.99
R3		300^{b}	545^{b}	836^{c}	1.85	2.89
SEM		9.7	17.3	25.8	0.021	0.048
				Probabilities		
Diet × Infection		0.682	0.579	0.329	0.096	**0.024**
Infection		0.626	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Diet		**0.004**	**<0.001**	**<0.001**	0.123	**<0.001**
Diet (linear)		**<0.001**	**<0.001**	**<0.001**	0.489	**<0.001**
Diet (Quadratic)		**0.025**	**<0.001**	**<0.001**	0.982	0.885

[a-c] Means in a column with different superscript differ significantly ($P < 0.05$)
R0, 1, 2 and 3 represents 0, 5, 10 and 15% lignocellulose-diluted diets respectively
Relative BW is BW divided by BW at infection
D6 and 12 pi equate to d13 and 25 of age respectively.

Table 5. 3 Effect of diet diluted with 0, 5, 10 or 15% lignocellulose and infection status on average daily gain (ADG) and average daily feed intake (ADFI) post-infection (pi): values expressed in proportion to body weight at infection (BWd0pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d13 post-hatch.

		Early stage (d1-6 pi)		**Late stage (d7-12 pi)**		**Post-infection (d1 -12 pi)**	
Infection × Diet	Diet	ADG (g/d) / BWd0pi (g)	ADFI (g/d) / BWd0pi (g)	ADG (g/d) / BWd0pi (g)	ADFI (g/d) / BWd0pi (g)	ADG (g/d) / BWd0pi (g)	ADFI (g/d) / BWd0pi (g)
Control	R0	0.170^{a}	0.223	0.225^{a}	0.329^{a}	0.197^{a}	0.276
	R1	0.149^{b}	0.226	0.198^{ab}	0.318^{ab}	0.176^{b}	0.272
	R2	0.149^{b}	0.236	0.187^{bc}	0.321^{ab}	0.168^{bc}	0.278
	R3	0.145^{bc}	0.246	0.172^{bcd}	0.317^{ab}	0.159^{bcd}	0.281
Infected	R0	0.126^{d}	0.182	0.170^{bcd}	0.266^{c}	0.148^{cd}	0.224
	R1	0.124^{d}	0.189	0.181^{bcd}	0.298^{abc}	0.152^{cd}	0.244
	R2	0.120^{d}	0.194	0.159^{cd}	0.282^{bc}	0.139^{d}	0.238
	R3	0.128^{cd}	0.211	0.153^{d}	0.308^{abc}	0.140^{d}	0.259
SEM		0.0038	0.0060	0.0063	0.0096	0.0044	0.0073
Main Effect							
Infection							
Control		0.153	0.233	0.195	0.321	0.175	0.277
Infected		0.124	0.194	0.166	0.288	0.145	0.241
SEM		0.0019	0.0030	0.0031	0.0096	0.0022	0.0037
Diet							
R0		0.148	0.202^{b}	0.197	0.298	0.173	0.250
R1		0.137	0.208^{b}	0.189	0.307	0.164	0.258
R2		0.134	0.215^{ab}	0.173	0.302	0.153	0.258
R3		0.136	0.229^{a}	0.163	0.312	0.149	0.270
SEM		0.0027	0.0043	0.0044	0.0068	0.0031	0.0052
				Probabilities			
Diet × Infection		**0.004**	0.941	**0.019**	**0.043**	**0.007**	0.198
Infection		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Diet		**0.011**	**0.001**	**<0.001**	0.468	**<0.001**	0.066
Diet (linear)		0.929	0.646	0.289	0.966	0.576	0.882
Diet (Quadratic)		0.961	0.995	0.999	0.999	0.999	0.999

$^{a-c}$ Means in a column with different superscript differ significantly ($P < 0.05$). The period from d1 to 12 pi equates to d13 to 25 of age. R0, 1, 2 and 3 represents 0, 5, 10 and 15% lignocellulose-diluted diets respectively

Table 5. 4 Main effects of diet diluted with 0, 5, 10 or 15% lignocellulose and infection status on carcass yield (%) at d12 post-infection. Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d13 post-hatch.

	Eviscerated / BW (%)	Breast muscle / BW (%)	Thigh + Drumstick / BW (%)	Breast muscle / Eviscerated (%)	Thigh + Drumstick / Eviscerated (%)
Main effect					
Infection					
Control	63.1	20.6	17.8	32.6	28.1
Infected	61.3	19.4	17.4	31.7	28.4
SEM	0.38	0.33	0.18	0.44	0.32
Diet					
R0	62.6	20.7	17.8	33.0	28.4
R1	62.5	20.3	17.9	32.5	28.5
R2	62.4	19.8	17.4	31.7	27.9
R3	61.3	19.3	17.2	31.5	28.1
SEM	0.54	0.46	0.26	0.63	0.45
			Probabilities		
Infection	**0.001**	**0.015**	0.178	0.149	0.547
Diet	0.268	0.201	0.279	0.330	0.723
Diet (Linear)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.017**
Diet (Quadratic)	**0.003**	0.989	0.376	0.658	0.999

Eviscerated/BW =Percentage of the eviscerated carcass to live body weight; Breast muscle/BW = Percentage of breast muscle weight to live body weight; Thigh + Drumstick/BW = Percentage of thigh plus drumstick weight to live body weight; Breast muscle/Eviscerated = Percentage of breast muscle weight to eviscerated carcass weight; Thigh + Drumstick/Eviscerated = Percentage of thigh plus drumstick weight to eviscerated carcass weight.
R0, 1, 2 and 3 represents 0, 5, 10 and 15% lignocellulose-diluted diets respectively
D12 pi equates to d25 of age

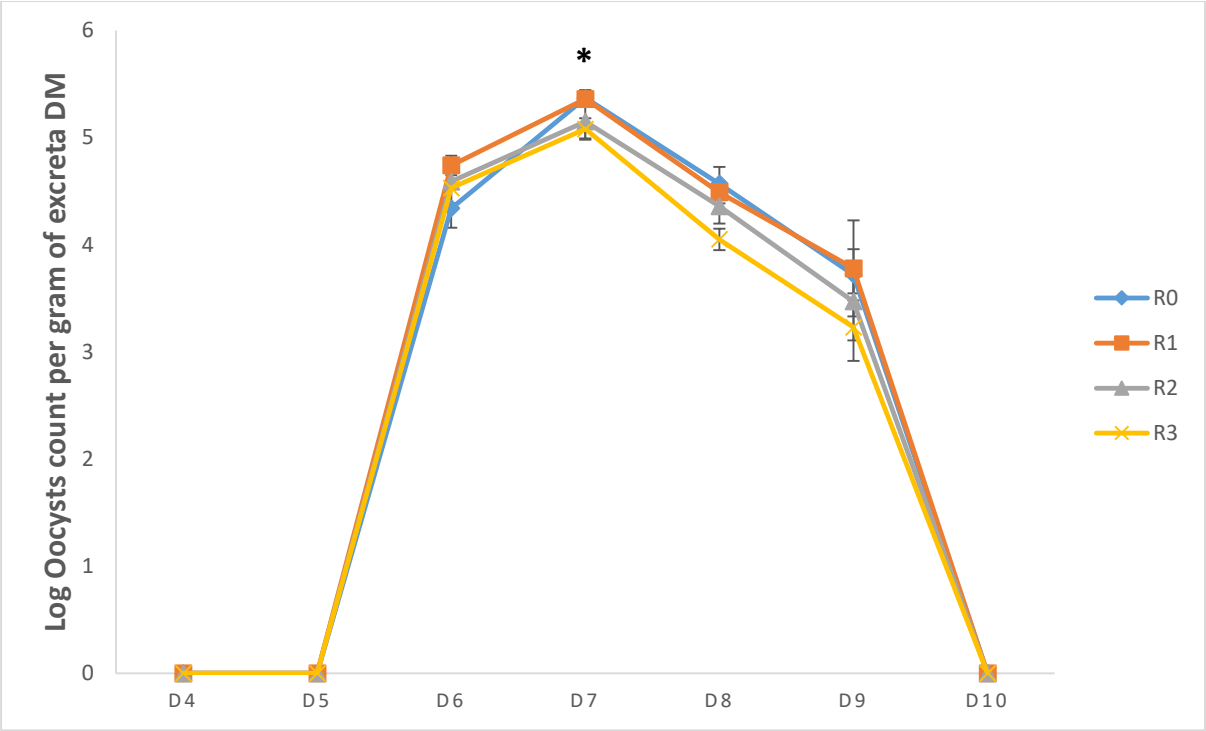

Figure 5. 1 Effects of diet diluted with 0, 5, 10 or 15% lignocellulose on excreted oocysts per gram (OPG) of dry excreta matter from d4 to 10 post-infection (pi). Broiler chickens orally inoculated with 7000 sporulated oocysts of *E. maxima* at d13 post-hatch. R0, 1, 2 and 3 represents 0, 5, 10 and 15% lignocellulose-diluted diets respectively. * represents significantly higher OPG ($P < 0.05$) on d7 compared to d8 and 9 pi.

Table 5. 5 Main effects of diet diluted with 0, 5, 10 and 15% lignocellulose and infection status on markers of femur and tibia bone development at d12 post-infection (pi). Broiler chickens orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d13 post-hatch.

	Femur						Tibia					
Main effect	Breaking Strength / BW (N/g)	Length / BW (mm/cg)	Width / BW (mm/cg)	Robusticity index	Ash / BW (mg/g)	Ash percentage	Breaking Strength / BW (N/g)	Length / BW (mm/cg)	Width / BW (mm/cg)	Robusticity index	Ash / BW (mg/g)	Ash percentage
Infection												
Control	19.6	5.98	0.652	3.51	0.776	50.4	25.0	8.01	0.586	4.17	1.02	50.4
Infected	18.0	6.36	0.692	3.49	0.741	50.2	20.8	8.45	0.607	4.18	0.97	50.2
SEM	0.52	0.134	0.0131	0.021	0.0016	0.38	0.74	0.172	0.0115	0.030	0.003	0.472
Diet												
R0	17.0^{b}	5.50^{c}	0.623^{b}	3.41^{b}	0.758	50.3	22.8	7.37^{c}	0.559^{b}	4.17	1.00	50.4
R1	17.3^{b}	5.97^{bc}	0.649^{b}	3.50^{ab}	0.746	49.2	23.0	8.02^{bc}	0.564^{b}	4.19	0.94	49.7
R2	19.2^{ab}	6.48^{ab}	0.673^{b}	3.56^{a}	0.746	50.2	22.2	8.61^{ab}	0.606^{ab}	4.21	0.97	49.5
R3	20.6^{a}	6.73^{a}	0.743^{a}	3.53^{a}	0.814	51.3	23.8	8.91^{a}	0.656^{a}	4.15	1.08	51.4
SEM	0.58	0.175	0.0169	0.027	0.0028	0.50	1.07	0.225	0.0153	0.040	0.003	0.63
						Probabilities						
Infection	**0.033**	0.055	**0.037**	0.571	**0.045**	0.689	**0.001**	0.082	0.202	0.928	0.187	0.769
Diet	**<0.001**	**0.001**	**<0.001**	**0.010**	0.064	0.076	0.737	**0.001**	**<0.001**	0.752	0.058	0.149
Diet (Linear)	**<0.001**	**<0.001**	**0.002**	**0.008**	0.948	**<0.001**	0.055	**<0.001**	**0.005**	0.987	0.943	**<0.001**
Diet (Quadratic)	**<0.001**	0.453	0.781	0.141	0.947	**<0.001**	**<0.001**	0.182	0.726	0.445	0.857	**<0.001**

[a-c] Means in a column with different superscript differ significantly ($P < 0.05$)

R0, 1, 2 and 3 represents 0, 5, 10 and 15% lignocellulose-diluted diets respectively

D12 pi equates to d25 of age

Table 5. 6 Difference between regression slopes of tibia (Y-axis) vs femur (X-axis) at d12 post-infection (pi) for length (mm), width (mm), weight (g) and ash weight (g) in broiler chicken orally inoculated with 0 (Control) or 7000 sporulated oocysts of *E. maxima* (Infected) at d13 post-hatch, and receiving diet diluted with 0, 5, 10 and 15% arbocel (R0 – 3 respectively). Regression was done using log-transformed values.

Tibia (Y) vs Femur (X)	Diets	Comparing R1-3 with R0				Comparing infected (R0-3i) with control R0 (R0c)				
		Slope (diff)	SE (diff)	t-stat	*P*	Diets	Slope (diff)	SE (diff)	t-stat	*P*
Length										
Control	R1-R0	-0.523	0.879	-0.595	0.574	R0i - R0c	-0.0311	0.955	0.0325	0.975
	R2-R0	-0.0912	0.751	-0.121	0.907	R1i - R0c	-0.0351	0.876	0.0401	0.969
	R3-R0	-0.130	1.03	-0.126	0.904	R2i - R0c	0.320	1.09	0.293	0.779
Infected	R1-R0	-0.00404	0.714	-0.00565	0.996	R3i - R0c	-0.290	1.38	0.210	0.840
	R2-R0	0.352	0.932	0.377	0.716					
	R3-R0	-0.259	1.22	-0.213	0.837					
Width										
Control	R1-R0	1.11	0.975	1.14	0.287	R0i - R0c	0.623	0.546	1.141	0.287
	R2-R0	-0.200	0.436	-0.458	0.659	R1i - R0c	-0.157	0.387	0.406	0.696
	R3-R0	-0.271	0.604	-0.448	0.666	R2i - R0c	0.00669	0.576	0.0116	0.991
Infected	R1-R0	-0.780	0.497	-1.57	0.155	R3i - R0c	-0.0261	0.399	0.0655	0.949
	R2-R0	-0.616	0.686	-0.898	0.395					
	R3-R0	-0.649	0.509	-1.27	0.238					
Weight										
Control	R1-R0	0.566	0.837	0.676	0.518	R0i - R0c	-0.0648	1.59	0.0408	0.968
	R2-R0	0.633	0.907	0.698	0.505	R1i - R0c	0.811	0.826	0.982	0.355
	R3-R0	0.543	0.939	0.578	0.579	R2i - R0c	1.18	0.973	1.21	0.262
Infected	R1-R0	0.876	0.985	0.889	0.400	R3i - R0c	0.710	1.01	0.701	0.503
	R2-R0	1.24	1.13	1.09	0.305					
	R3-R0	0.775	1.17	0.661	0.527					
Ash Weight										
Control	R1-R0	-0.0179	0.827	-0.0217	0.983	R0i - R0c	0.340	0.722	0.471	0.650
	R2-R0	0.190	0.499	0.382	0.713	R1i - R0c	0.155	0.519	0.299	0.773
	R3-R0	0.423	0.722	0.585	0.574	R2i - R0c	0.702	0.578	1.21	0.259
Infected	R1-R0	-0.185	0.496	-0.374	0.718	R3i - R0c	0.361	0.445	0.811	0.441
	R2-R0	0.362	0.556	0.652	0.533					
	R3-R0	0.0213	0.422	0.0505	0.961					

t-stat = slope (diff) divided by SE (diff), i.e. $\frac{b1-b2}{\sqrt{SEb1^2 + SEb2^2}}$; where b = slope and SEb = standard error of slope;

P = Probabilities.

5.5 Discussion

In chapter 2, genetic selection for reduced GR improved bone mineralisation with effects persisting in the presence of coccidia infection. Therefore, a similar result was expected amongst broilers in the case of an artificial diet-induced reduction in GR. In the present study, lignocellulose-diluted grower diet was used to reduce the GR of a widely used rapid-growing broiler genotype during the second and third week of life (Williams *et al*., 2000). It was expected that slowing down early GR during the above period, which coincides with peak bone development, would impact positively on bone development of uninfected broilers (Knowles *et al*., 2008; Shim *et al*., 2012; Pratt and Cooper, 2018), and coccidia-infected birds. By this hypothesis, significant improvement in long bone strength and mineral content per unit BW (BS/BW and Ash/BW), and AP, i.e., markers of bone mineralisation, was anticipated amongst broilers receiving the diluted (R1 to 3) compared to the undiluted (R0) feed in this study.

In this study, oocyst excretion was measured solely to establish the occurrence of *E. maxima* infection. However, the OPG results suggested a linear reduction in *E. maxima* parasite burden at increasing levels of diet dilution from d7 to 9pi. This inverse relationship between parasite burden and diet dilution persisted after accounting for the water content, which also increased with diet dilution, in the droppings of infected birds. The reduction in OPG at increasing levels of dilution may be ascribed to the increasing bulkiness of the feed and the indigestible nature of the diluent, which resulted in a proportionally greater quantity of excreta amongst the broilers receiving the diluted diets.

The observed effects of coccidiosis on productive performance were in line with chapters 2 – 4. Also, the reduction in GR induced by the diluted diets was as expected pre-infection, i.e., from d8 to 13 of age. The diluent, lignocellulose, only contributed to bulkiness, but not to the nutritive value of the diet (Zeitz *et al*., 2018). Nonetheless, graded diet-induced GR could not be achieved amongst the infected birds over the infection period (d1 to 12pi), as seen in the control birds. This was influenced by the significantly lower feed intake, or higher magnitude of anorexia, amongst the infected compared to the control broilers receiving the undiluted (R0) diet during the late stages of infection (d7 to 12pi), whereas there was a similar intake level amongst infected and control broilers receiving the diluted (R1, R2 or R3) diets during that period (see Table 5.3).

Anorexia was previously hypothesised to be a host defense or disease-coping strategy (Kyriazakis *et al.*, 1998; Kyriazakis, 2014). The present study provides further evidence for this hypothesis showing that broilers receiving a poor (diluted) diet exhibit a lesser reduction in voluntary feed intake (anorexia) compared to broilers receiving a balanced (undiluted) diet following coccidiosis infections. A physiological explanation for this lies in the inextricable link suggested between the immune system and anorexia (Kyriazakis *et al*., 1996; Kyriazakis, 2014). Activation of the immune system following pathogen infection, with the associated upregulation of cytokines, has been implicated as the primary cause of anorexia (Van Niekerk *et al*., 2016).

Attaining a similar degree of GR reduction in both coccidiosis-infected and uninfected broilers in the current study could have been a logical reason for expecting corresponding commensurate effects on their bone mineralisation. Unfortunately, this was not the case due to the statistically similar level of anorexia amongst the infected broilers. Moreover, there are no comparable studies in the bone literature that has successfully induced a graded reduction in GR of coccidiosis-infected broilers in order to test such a hypothesis. On the use of GR reduction as a management tool for reducing the occurrence of skeletal disorders, the suggested optimum timing economically is during the second week of life (Robinson *et al*., 1992). Although this method of mitigating skeletal disorders delays the attainment of market weight by 2 or 3 days (Robinson *et al*., 1992), it should be considered in light of the substantial economic loss associated with poor skeletal development and the welfare issues that they raise (Driver *et al*., 2006; Knowles *et al*., 2008; Pines and Reshef, 2015).

There was no evidence from the present study to suggest that a reduction in early GR improved bone mineralisation to a higher degree in uninfected than in coccidiosis-infected broilers. This was the case even though the experimental diets induced graded levels of GR reduction amongst the control broilers during the period of examination (d1 to 12pi), whereas graded GR probably lasted until the onset of anorexia and thereafter remained similar for the infected birds despite the different diet dilution levels.

A differential rate of mineralisation between tibia and femur in growing broilers had been reported previously (Applegate and Lilburn, 2002); chapter 2 also support this suggestion. Femur strength is crucial for gait stability and bearing the heavy weight of fast-growing broilers because of its position in the skeleton (Marks and Popoff, 1988; Chinsamy and Elzanowski, 2001). In modern broilers, the forward shift in the centre of gravity due to

increased breast muscle places specific demands on femur integrity (Paxton *et al*., 2014). Therefore, the finding that diet dilution increased femur strength significantly at a point (d12 pi) when penalties of an *E. maxima* infection are maximised is a remarkable novel discovery from the current study.

Other studies have investigated the effects of reducing broiler GR on markers of leg bone quality and mineralisation using various methods. A 50% restriction in feed intake (compared to consumption rate in *ad libitum* fed birds), but the same level of Ca and P as *ad lib* fed birds, caused a significant increase in tibia ash content and lower porosity at d42 of age (Williams *et al*., 2004). Sequential feeding with low and high-lysine diets during the first and second half of the day, starting from d2 to 12 of age, increased activity levels and improved leg condition at d42 of age (Bizeray *et al.*, 2002b). Offering a low energy diet did not affect the bone quality (Leterrier *et al*., 1998). A light schedule of 12L: 12D improved bone ash compared to 20L: 4D (Brickett *et al*., 2007). Although the above studies induced an artificial reduction in GR, it is unknown whether the methods employed in the above studies may ameliorate the effects of coccidiosis on bone mineralisation.

Furthermore, the conclusion of Leterrier *et al*. (1998) that limiting GR using a low energy diet did not affect bone quality was based only on tibia variables. In this study and that reported in chapter 2, there is evidence to support the previously reported different mineralisation rates for tibia and femur bones (Applegate and Lilburn, 2002), which Leterrier *et al*. (1998) did not consider in their study. Moreover, they also observed that the slower-growing broilers due to low-energy diets were more than 6-fold (3.1% vs 19.9%) less predisposed to varus-valgus deformities than their faster-growing counterparts (Leterrier *et al.*, 1998). This, therefore, suggests that slowing down GR via low energy diet did have a potentially positive effect on leg bone in that study, and perhaps on one or more of the other long bones not examined. A subsequent investigation (Bruno *et al*., 2000) revealed that about 15% reduction in dietary energy intake, during the second week of life only, improved humerus weight and density without affecting the same variables in tibia or femur bones. Restriction in protein intake also affected femur, but not tibia or humerus width (Bruno *et al*., 2000). The results above underscore the possibility that a single factor may have differential effects between long bones (Applegate and Lilburn, 2002), as was observed for GR on tibia and femur strength in the present study.

In conclusion, coccidial infection penalised long bone strength and mineralisation in modern fast-growing broilers, while a reduction in GR via diet dilution with a commercially available lignocellulose product improved bone quality to a similar degree for both coccidiosis-infected and uninfected broilers. Although delaying early GR imposes economic constraints in the intensive broiler sector due to the additional days broilers require to reach market weight, it should be considered as a means to improve skeletal integrity and broiler welfare. Although lignocellulose was utilised in the present study, alternative feed ingredients with a high WHC may also be utilised to reduce overall feed costs in markets when they are available and where bird welfare is valued more (Sakkas *et al.*, 2019). Collectively, the markers of long bone mineralisation evaluated in this study suggest a more pronounced effect of artificial GR reduction on improving femur compared to tibia mineralisation.

Chapter 6: General discussion

6.1. Introduction

Coccidiosis impairs the productivity of poultry systems in diverse ways globally (Blake and Tomley, 2014). Information on the consequences of malabsorptive coccidia infections on the long bone quality of modern fast-growing broiler chickens is scarce, and the few related studies focus mainly on the early stages of infection, i.e. from the point of infection up to day 6 post-infection (pi). It is now known that although coccidiosis has adverse effects on performance and some effects on the bone quality of broilers during the early stage of infection, more significant bone effects occur during the late stages post-infection, i.e. d12 – 14 pi [Chapters 2, 3, 4 and 5]. Indeed, the bone quality and skeletal development of meat-type (broiler) chickens have not received sufficient attention over the years (Julian, 1998; Julian, 2005), which is to the detriment of modern fast-growing broilers. Genetic selection for increased growth potential without due attention to the integrity of the skeletal framework that bears the extra weight (Applegate and Lilburn, 2002), and malabsorption of vital bone minerals during coccidiosis (Turk and Stephens, 1966; Turk, 1973) but no proper assessment of the consequences on bone quality is clear evidence of undermining skeletal development in modern broilers. Fortunately, the similar ash percentage of long bones for the fast- and slow-growing broilers reported in chapter 2 suggest that some recent genetic schemes may have included markers of skeletal health. However, other examined makers of long bone mineralisation, i.e. strength and ash weight relative to body weight, still portrayed the inferiority of the fast- compared to the slow-growing broilers [Chapter 2].

This thesis had a practical objective; to assess the effects of malabsorptive coccidiosis on the long bone quality of modern fast-growing broilers, and to investigate relevant ameliorative nutritional strategies capable of jointly mitigating the bone-related effects, as well as other known effects of coccidiosis such as impaired performance. To achieve this objective, it was of interest to first dissect the effect of selection for improved growth potentials in modern broilers on their resistance and tolerance to coccidiosis [Chapter 2]. This study sets the framework for further investigations in this thesis and is the first of its kind. The nutritional strategies explored herein involved modulations of dietary VitD [Chapter 3] and Ca/P [Chapter 4], and diluting diet with an inert substance to induce an artificial reduction in early growth rate (GR) of broilers [Chapter 5]. Overall, the thesis revealed that infected broilers are as sensitive as control broilers to genetic selection for growth rate [Chapter 2], the source of VitD supply [Chapter 3], Ca and P adequacy [Chapter 4] and to a dietary-induced reduction in

early growth rate [Chapter 5]. Also, chapter 3 provided evidence that *Eimeria*-infected broilers could be more sensitive than uninfected broilers to a high level of dietary VitD supplementation regarding femur strength (d10 pi), OHD and P status (d10 pi) and feed conversion ratio over the period of infection (d1 – 14 pi). The use of *E. maxima* infection was a case point, as the methodology employed in the study can be applied to other malabsorptive coccidia species, as well as pathogens that may elicit malabsorption of vital bone minerals in broiler chickens.

6.2 Impact of coccidiosis on long bone quality in modern broilers

In chapters 2, 3, 4 and 5, the thesis unveiled novel aspects regarding the impact of coccidiosis on broiler long bone quality. For example, no previous study categorically showed that the effects of coccidia infection on long bone mineralisation lagged behind effects on productive performance such that total recovery from coccidiosis-impaired performance occurred whilst bone mineralisation was still significantly penalised. The thesis clarified for the first time that coccidiosis penalises more markers of the femur than tibia mineralisation in chapters 2 and 5. The experiments reported in chapters 3 and 4 could not cover femur mineralisation in details due to the time-consuming analysis and short interval between trials. However, in chapters 3 and 4, the thesis examined femur breaking strength, also a potent marker of mineralisation, as well as Seedor and Robusticity indices, and then examined tibia mineralisation in further details; being the commonly used marker for long bone mineralisation in most studies. Future research may investigate whether or not VitD supplementation alters the differential effects of coccidiosis on tibia and femur mineralisation reported in chapters 2 and 5.

A recent study (Akbari Moghaddam Kakhki *et al.*, 2018) that was published at the time of writing this final chapter provided further information on the interaction between broiler coccidiosis and bone quality. Akbari Moghaddam Kakhki *et al.* (2018) investigated the effects of *Eimeria* challenge on long bone attributes using Ross 708 broilers and an infection model comprising *E. acervulina* and *E. maxima*. Such *Acervulina* / *maxima* co-infection is a perfect model for testing effects on the bone because these *Eimeria* species parasitise duodenal and jejunal sections of the small intestine (Chapman, 2014), where absorption of vital bone minerals including Ca and P occur (Van der Klis *et al.*, 1990). Unfortunately, they focused their study on the acute phase of infection, d6 pi, which has been a limitation associated with many previous studies. For this reason, although their results showing significant effects on tibia but not femur mineralisation were comparable to those for d6 pi in chapter 2, their conclusion that femur mineralisation was not affected by malabsorptive coccidiosis was quite

misleading and of course due to their sampling point (d6 pi). This thesis offers a clear, consistent and reliable picture of the effect of malabsorptive coccidiosis on long bone quality obtained by sampling at both the early (d6 pi) and the later stages (d10, 12 and 14 pi) following infection. Furthermore, their assertion that the effects of coccidiosis on femur mineralisation may differ between Ross 708 broilers used in their experiment (Akbari Moghaddam Kakhki *et al.*, 2018), and Ross 308 broilers used in this thesis, was not supported by the recent study of Sakkas *et al.* (2018): similar levels of femur mineralisation were observed for Ross 308 and 708 broilers.

Set aside the above clarifications, Akbari Moghaddam Kakhki *et al.* (2018) is one of the most relevant resources on the effects of *Eimeria* infection on the long bone quality of modern broilers available in the literature. Whilst the thesis focused mainly on nutritional measures to tackle coccidiosis-induced malabsorption of vital bone minerals [Chapters 3 and 4], and then artificial reduction in the early growth rate of modern broilers to allow for commensurate bone development and weight gain [Chapter 5], Akbari Moghaddam Kakhki *et al.* (2018) has a novel discovery to their credit. They showed a vital link between damage to the intestinal tract, mineral malabsorption and increased bone resorption during broiler coccidiosis for the first time. Based on an increased serum receptor activator of nuclear factor kappa-B ligand (RANKL) concentration and a corresponding reduction in tibia ash content, they concluded that bone resorption might contribute significantly to the penalties on long bone quality during broiler coccidiosis (Akbari Moghaddam Kakhki *et al.*, 2018). Indeed, this opens up a vast array of potential future investigations relating to coccidiosis-induced bone resorption and bone-targeting therapies for infected broilers. It points towards osteoimmunology as a logical field for further investigations relating to the bone effects elicited by broiler coccidiosis.

Osteoimmunology is an interdisciplinary field that focuses primarily on the interactions between the immune and skeletal systems. The primary connection between the bone and immune system was established based on the discovery of a regulatory mechanism via the RANKL – receptor activator of nuclear factor kappa-B (RANK) – osteoprotegerin signalling axis that is common to both systems (Lee and Choi, 2015; D'Amelio and Sassi, 2016). Another connection between the bone and immune system is that bone-resorbing osteoclast cells, as well as macrophages and myeloid dendritic cells, are derivable from the same myeloid precursor cells, and the above RANKL – RANK – osteoprotegerin regulatory mechanism applies to these cells coexisting in the bone marrow (D'Amelio and Sassi, 2016). RANKL and tumour necrosis factor (TNF)-α are renowned pro-osteoclastogenic cytokines

(Sakthiswary and Das, 2013). RANKL causes an upregulation of osteoclast formation and activity, whilst TNF-α favours RANKL production, increases RANKL-responsiveness of osteoclasts precursors, and with adequate RANKL levels induces osteoclast formation (D'Amelio *et al.*, 2008).

Recent reviews of mostly murine osteoimmunological studies provided details on the association of activated immune cells, especially T cells and dendritic cells, with RANKL upregulation causing an imbalance between bone-forming osteoblast and bone-resorbing osteoclast cells, which favours bone loss (Lee and Choi, 2015; D'Amelio and Sassi, 2016). Indeed, a negative correlation between RANKL upregulation and tibia mineralisation for coccidiosis-infected broilers at d6 pi (Akbari Moghaddam Kakhki *et al.*, 2018) warrants applying high-throughput functional genomics tools to delineate a comprehensive immune mechanism associated with the more significant coccidiosis-impaired bone quality at later stages beyond the acute phase of infection. This should enhance understanding on how coccidiosis affects bone quality in modern broilers; coupling the aspects of nutrient malabsorption [Chapters 2, 3, 4 and 5] with bone resorption (Akbari Moghaddam Kakhki *et al.*, 2018) to provide much stronger evidence. The above is commended to further experimental investigations.

6.2.1 Different effects of coccidiosis on tibia and femur mineralisation

The differing effects of coccidiosis on femur and tibia mineralisation reported in the thesis [Chapters 2 and 5] reflected a previously suggested difference in their rates of mineralisation (Applegate and Lilburn, 2002) and responsiveness to stress (Wideman and Pevzner, 2012). However, in the context of broiler coccidiosis, this information is both novel and vital for mitigating effects on leg bone quality for modern heavyweight fast-growing broilers. This thesis, as well as other studies (Bradshaw *et al.*, 2002), supported the hypothesis that long bone strength with the level of mineralisation is strongly correlated [Chapters 2, 3, 4 and 5]. Also, the thesis agreed with previous studies (Lilburn, 1994; Applegate and Lilburn, 2002) that identified femur as the weak link regarding the development of the two major leg bones because of its much slower mineralisation rate compared to the tibia.

Despite the adverse effects of coccidiosis on femur mineralisation and strength [Chapters 2 and 5], the position of the bone within the skeletal framework remains crucial for bearing the increased weight of modern broilers. Moreover, recent studies (Dozier *et al.*, 2010; Mendes *et al.*, 2014) revealed how the 100% increase in pectoralis major (breast) muscle yield between modern broilers and heritage lines (Schmidt *et al.*, 2009) could be further increased for

economic gains. All of these significantly challenge the integrity of leg bones, which are further weakened with exposure to coccidia infection, particularly the femur. This thesis showed effects of rapid growth rate and coccidiosis on both tibia and femur and the ameliorative effects of high VitD supplementation [Chapter 3] in diets with adequate Ca/P levels [Chapter 4]. It also showed a significant improvement in markers of the femur, but not tibia, mineralisation achieved by reducing early growth rate [Chapter 5]. Therefore, it is recommended that future studies on coccidiosis-impaired long bone quality of broilers do not assume femur quality from tibia variables and vice versa.

6.3 Potentials of VitD nutrition to attenuate broiler coccidiosis

There is a consensus that modulating specific nutrients in broiler diets to ameliorate the consequences of infection is sustainable and indeed safer than the use of in-feed drugs. The emphasis in such nutritional modulations would typically be on nutrients with immunomodulatory potentials (Zhang *et al.*, 2012; Zhang *et al.*, 2016) and, in the case of malabsorptive infections like coccidiosis, altering dietary intake of nutrients to mitigate the effects of malabsorption and intestinal tract atrophy is an option. This thesis investigated the benefits of dietary modulations involving VitD3 and its 25-hydroxycholecalciferol (OHD) metabolite for coccidiosis-infected broilers [Chapters 3 and 4]. Chapter 1 reviewed the key functions of the biologically active VitD metabolite (1,25-dihydroxycholecalciferol; 1,25D3). They include regulation of calcium and phosphorus homeostasis (Bienaimé *et al.*, 2011), stimulating osteoclast differentiation and calcium reabsorption from the bone, and promoting mineralisation of the bone matrix (Holick, 2004; St-Arnaud, 2008; Bikle, 2012; Haussler *et al.*, 2013). Also, the ability to improve performance (Fritts and Waldroup, 2003; Whitehead *et al.*, 2004), integrity of intestinal mucosa barrier (Kong *et al.*, 2008), small intestine morphology in chicks (Ding *et al.*, 2011), as well as helping recovery during mucosal injury (Zhao *et al.*, 2012) were identified as derivable benefits of dietary VitD supplementation in broilers [Chapter 1]. All of these VitD functions are relevant to coccidiosis-induced effects on broiler long bone quality, which was the focal point of this thesis.

6.3.1 VitD and growth performance

This thesis provided experimental evidence that higher dietary VitD supplementation (4000 vs 1000 IU/kg), and replacing D3 with its metabolite, OHD [Chapter 3] can improve both the feed utilisation efficiency and bone mineralisation of coccidiosis-infected broilers. It further showed in chapter 4 that there is no added effect of VitD source on the growth performance of infected broilers offered D3- and OHD-supplemented diets at 4000 IU/kg, i.e. commercial

levels [Chapter 4]. Previous studies did identify these potentials of VitD in healthy broilers (Fritts and Waldroup, 2003; Whitehead *et al.*, 2004; Colet *et al.*, 2015), which the thesis now shows their applicability in the context of broiler coccidiosis. At least three known functions of VitD; the ability to 1) improve muscle development, 2) improve Ca and P absorption and utilisation, and 3) perform immunoregulatory functions, may have interactively contributed to improving feed utilisation and long bone mineralisation in coccidiosis-infected broilers. As higher parasite loads and an increased mucosal injury was observed in response to increased levels of VitD supplementation and VitD activity (OHD), it is more likely that its function on muscle development mediated the improvements in performance. The subsequent sections of the discussion attempt to account for the observed effects on parasite replication (see section 6.3.2).

Regarding VitD-improved muscle development, Vignale *et al.* (2015) reported that an increased level of OHD in circulation is associated with an increased fractional rate of protein synthesis and higher expression of VitD receptors (VDRs) in breast muscles. These effects offer valid explanations as to how VitD supplementation improved performance in terms of weight gain and FCR in chapter 3. Overall, what the thesis shows is that the benefits of dietary VitD on broiler performance in the absence of coccidiosis are also derivable to a similar degree by infected birds. It further agrees with Colet *et al.* (2015) on no additional benefit of OHD over D3 on broiler growth performance beyond 3500 IU/kg dietary supplementation level, confirming this amongst coccidiosis-infected broilers.

6.3.2 VitD, gut morphology, parasite replication and immune response

VitD is a potent immunoregulator of both innate and adaptive immune responses (Baeke *et al.*, 2010). Regarding its immunoregulatory roles, the biologically active 1,25D3 metabolite acts as an immune system modulator preventing the excessive expression of cytokines and increasing the oxidative burst potential of macrophages (Baeke *et al.*, 2010). It is noteworthy that mucosal inflammation downregulates VDR (VitD receptor) expression via the action of mucosal pro-inflammatory cytokines. On the other hand, increased VitD supplementation leads to upregulation of the VDR and downregulates pro-inflammatory cytokines (Autier *et al.*, 2014). Therefore, these facts imply that higher dietary levels of VitD might benefit the innate immune response to pathogens and the ability of broilers to cope with the disease. Moreover, reduced pro-inflammatory cytokine production may also improve their productivity considering the role of the latter on the distribution of nutrients away for growth

processes and the induction of anorexia (Kyriazakis *et al.*, 1998; Lochmiller and Deerenberg, 2000).

Regarding immune responses of D3- or OHD-fed broilers, Fritts *et al.* (2004) reported no significant differences on aspects of macrophage function such as nitric oxide production and cytotoxicity and cutaneous basophil hypersensitivity when feeding graded levels (125 to 4,000 IU/kg of feed) of VitD to non-infected birds either in the form of D3 or OHD. On the other hand, it has been shown that the addition of OHD to a diet containing 3000IU of D3, resulted in lower total serum IgA at d14 d of age and lower total serum IgG at 21 d of age in uninfected broilers (Chou *et al.*, 2009). However, birds infected with *Salmonella Typhimurium* fed OHD produced higher total serum IgG at 21 d of age. That study also showed that supplemental OHD improved small intestinal morphology and that these effects varied with age and section of the small intestine (Chou *et al.*, 2009). Furthermore, immune response deriving from OHD supplemented in drinking water at 0.06 ml/l whilst offering feed containing extra 5500 IU/kg D3 supplementation attenuated outbreak of bacterial chondronecrosis with osteomyelitis (BCO) induced lameness in broilers (Wideman *et al.*, 2015). All these are evidence of VitD potentials to mediate immune response in broilers.

Despite the improved performance and in contrast with the studies above, there was a significant increase in parasite load and mucosal injury in response to higher VitD activity [Chapter 3]. IFN-γ and IL-10 are key cytokines regulating the immune response to coccidiosis (Min *et al.*, 2013). *E. maxima* evoke a complex cytokine response characterised by increased production of Th1 pro-inflammatory cytokines; IL-1b, IL-6, IL-8, IL-17, and IFN-γ in the small intestine, as well as Th2 anti-inflammatory cytokines; IL-4, IL-10 (Hong *et al.*, 2006b; Min *et al.*, 2013). Increased IFN-γ mRNA has been associated with antigen-specific resistance to coccidiosis; favouring Th1 cell production, whilst inhibiting Th2 cell production (Laurent *et al.*, 2001; Cornelissen *et al.*, 2009), balanced by IL-10 (Rothwell *et al.*, 2004). On the other hand, elevated IL-10 has been associated with susceptible but not resistant broiler lines (Rothwell *et al.*, 2004). VitD has the potential to alter key cytokine responses such as IFN-γ and IL-10. 1,25D3 may support conversion of naïve T cells into T regulatory cells, which produce IL-10 and TGF-β that inhibit the expression of pro-inflammatory cytokines such as IFN-γ and IL-17 (Jeffery *et al.*, 2009) and to upregulate IL-10 production in macrophages (Baeke *et al.*, 2010; Korf *et al.*, 2012). Although differential expression of either cytokine was not confirmed in this thesis, it is possible that upregulation of IFN-γ occurred at later times pi and of IL-10 at earlier time pi in broilers receiving diets with higher vs lower dietary vitamin D activity

The only other related study on dietary VitD immune activity at the time of writing this thesis was Morris *et al.* (2015). They investigated the immunomodulatory effects of VitD (OHD metabolite) in coccidiosis-infected layer chickens. The layers received graded levels of dietary OHD; 250, 1000, 2000 and 4000IU/kg, from day-old and then were infected at 21d of age with 10^5 live oocysts (Inovocox; Zoetis), containing a mixture of *E. acervulina, E. maxima and E. tenella*. Morris *et al.* (2015) reported an increased IL-10 mRNA in the caecal tonsils of coccidiosis-infected layers receiving OHD supplemented at 250 (3.3-fold), 1000 (4.5-fold), 2000 (4.9-fold) and 4000 IU/kg (3.5-fold) in comparison to their uninfected counterparts at d6 pi. Amongst infected layers, IL-1β mRNA amount decreased in the caecal tonsils of those receiving dietary OHD supply at 1000 (1.7-fold), 2000 (4.2-fold) and 4000 (3.4-fold) in comparison to those receiving 250 IU/kg OHD at d6 pi. Also, IL-1β mRNA levels in 250 IU/kg OHD-fed layers were similar in the presence or absence of coccidia infection (Morris *et al.*, 2015). The improved feed efficiency at higher (4000 vs 1000 IU/kg) dietary VitD supply amidst significantly increased parasite load [Chapter 3] can also be associated with the decrease in IL-1β expression described by Morris *et al.* (2015).

Upon further investigations at d14 pi, Morris *et al.* (2015) revealed that the percentage of $CD4^+$ cells in cecal tonsils of 1000, 2000 and 4000 IU/kg OHD-fed layers did not differ from those of their 250 IU/kg OHD-fed counterparts. At d15 pi, infected compared to uninfected 4000 IU/kg OHD-fed layers had 17% more $CD4^+CD25^+$ in cecal tonsils (Morris *et al.*, 2015). Overall, a higher percentage of T regulatory cells, a natural source of IL-10, in the cecal tonsil amongst 4000 IU/kg OHD-fed coccidia-infected layers may have caused the upregulation of IL-10 mRNA (Morris *et al.*, 2015). Again, a surprising outcome of their experiment was that OHD-induced increase in IL-10 mRNA, as well as the percentage of $CD8^+$ cells in the cecal tonsils, did not affect oocysts output. Trout and Lillehoj (1995) demonstrated the involvement of $CD8^+$ T cells in transporting coccidia sporozoites, and their capacity to increase oocyst shedding, which partly relates to the increased parasite burden with VitD activity for broilers in this thesis [Chapter 3]. Indeed, the scientific literature holds minimal information on the immune-potentials of dietary VitD for coccidiosis-infected chickens. It is recommended that priority should be given to dissecting the mechanism underlying a possible association of higher parasite burden with increased VitD activity [Chapter 3] or none (Morris *et al.*, 2015) in a comprehensive study.

Although there is a scarcity of information on the effects of dietary VitD supply in other host-parasite systems, the few available studies in the scientific literature report variable effects depending on the parasite involved. Rajapakse *et al.* (2005) associated VitD with increased

mortality in *Toxoplasma gondii*-infected mice due to its downregulation of Th1 cytokine response. The mice received dietary 1,25D3 supplementation at 800 IU/kg of feed and survival rate reduced by 37% at d10 following infection (Rajapakse *et al.*, 2005). Contrariwise, intraperitoneal injection with VitD conferred protection against inoculation with trypomastigotes of *Trypanosoma cruzi*, with histopathology revealing diminished tissue inflammation and parasitism, in another murine infection model (Silva *et al.*, 1993). Overall, detailed research is required to elucidate the effects of VitD supply in other host-parasite systems.

6.3.3 Combination of VitD with other nutritional strategies

Since a higher VitD supply results in improved performance, despite its adverse effects on parasite replication, it might be preferable to combine it with other immunonutrition strategies which target the attenuation of parasite-induced gut damage such as dietary supplementation with nucleotide-rich yeast extracts (YN) (Leung *et al.*, 2018). The recent work by Leung *et al.* (2018) highlighted the potentials of dietary YN to attenuate gut damage during coccidiosis suggesting that YN is even more beneficial for infected than uninfected broilers. This finding was in line with previous studies, which reported that nucleotide is conditionally essential and more useful for broilers subjected to stress or health challenge (Jung and Batal, 2012; Alizadeh *et al.*, 2016).

Leung *et al.* (2018) supplemented YN at 500g/MT of feed, and it contained CP (32.7%) carbohydrates (14.3%), cell wall polysaccharides (21.6%) and a mixture of five nucleotides (1.1%); adenosine monophosphate, cytosine monophosphate, guanosine monophosphate, uridine monophosphate and inosine monophosphate. A gram of YN supplied approximately 0.1% of mixed nucleotides. Specifically, Leung *et al.* (2018) provided experimental evidence that YN supplementation was more effective in improving villi height (1.7-fold), final BW (3.3-fold), body weight gain (1.8-fold) and FCR (3.3-fold) amongst coccidiosis-infected compared to uninfected broilers. Furthermore, YN supplementation did not affect lesion scores, oocyst shedding and blood carotenoid concentration in that study, suggesting that it does not affect parasite burden (Leung *et al.*, 2018). Therefore, VitD/nucleotide co-supplementation is here recommended for further investigations as a tool to circumvent gut damage from the increased parasite burden associated with VitD nutrition during coccidiosis [Chapter 3]. Researchers may also want to explore other derivable benefits from the duo for coccidiosis-infected broilers.

6.3.4 VitD and bone mineralisation

In this thesis, the markers used to assess how dietary VitD supplementation affects coccidiosis-impaired long bone mineralisation of modern fast-growing broilers include 1) tibia and femur breaking strength relative to body weight (BW) at dissection (BS/BW), 2) tibia ash weight in proportion to BW at dissection (Ash/BW), and 3) ash percentage in dry defatted tibia bone (AP). Chapters 3 and 4 further suggested that the significantly reduced concentrations of OHD, Ca and P in the blood plasma of broilers during the acute phase of coccidia infection (d6 pi), are essential factors contributing to the significant penalties on long bone mineralisation during the recovery phase of infection, d12 or 14 pi. The thesis consistently showed that the beneficial effects of VitD supplementation on broiler bone mineralisation were attainable to a similar degree in the presence or absence of coccidiosis. The hypothesis of more pronounced effects of dietary VitD supplementation on long bone mineralisation amongst infected than uninfected broilers was accepted for femur BS at d10 pi [Chapter 3] in this thesis. High compared to low (4000 vs 1000 IU/kg) VitD supply significantly increased femur strength amongst coccidia-infected but not amongst uninfected broilers, which had similar femur BS values irrespective of VitD supply level. Furthermore, a low level of vitamin supply resulted in the lowest amount of tibia ash/BW at late stages post coccidia infection (d14 pi). Collectively these results suggest that a higher level of dietary vitD supply may confer additional benefits to infected broilers regarding long bone quality.

A major driver of chapter 4 experiment was the need to reduce P levels in broiler diets for environmental and economic benefits (Miles *et al.*, 2003; Maguire *et al.*, 2005; Huang *et al.*, 2018). However, contrary to expectations, there was no evidence of a VitD and infection status interaction suggesting a more pronounced effect on long bone mineralisation amongst the infected broilers. Tibia strength was significantly improved amongst uninfected but similar amongst infected broilers receiving adequate compared to the marginally deficient Ca/P diets. These results are in accordance with those of studies where phytase supplementation has been shown to have more limited effects in infected as opposed to non-infected broilers (Shaw *et al.*, 2011).

Supplying dietary P above the nutrition requirements did not improve tibia ash parameters in *E. acervulina* infected birds (Willis and Baker, 1981). Phytase supplementation was more effective in increasing tibia ash percentage and performance in uninfected starter control chicken compared to *E. acervulina*-infected (4 x10^5) chicken (Watson *et al.*, 2005). Phytase supplementation in grower chicks infected with *E. acervulina* (1 x 10^5) and *E. tenella* (5 x

10^3) did not improve bone breaking strength (Shaw *et al.*, 2011). Contrary to the above studies, the addition of phytases increased the absorptive capacity of the intestine in *E. acervulina* infected chicks (Mansoori *et al.*, 2010). Excess Ca supplementation did not affect tibia ash in *E. acervulina*-infected birds, whilst it reduced it in control birds (Watkins *et al.*, 1989). On the other hand, feeding Ca and nPP deficient diets decreased tibia ash percentage to a higher degree in control than in infected chicks (Watson *et al.*, 2005). Further studies should include femur relative ash weight (Ash/BW) and ash percentage based on the different mineralisation rate established between tibia and femur in chapter 5 and other studies (Applegate and Lilburn, 2002)

6.4 Growth rate effects in coccidiosis-infected modern broilers

Rapid growth rate (GR) with the associated skeletal disorders constitute a major source of concern regarding modern broilers (Knowles *et al.*, 2008). This thesis tested the effects of altering broiler GR using any of two conventional methods, i.e. genetic [Chapter 2] and nutritional or artificial [Chapter 5], on coccidiosis-impaired long bone quality. It provided novel evidence that both these GR reduction methods led to a similar degree of improvement in long bone mineralisation for modern broilers in the presence or absence of coccidiosis. Furthermore, the thesis reported a more pronounced effect of GR on femur than tibia mineralisation irrespective of the GR alteration method utilised. However, it is worth noting that nutritional GR reduction using poor vs good quality diets [Chapter 5] might elicit a higher magnitude of anorexia and weight loss post-coccidia infection amongst broilers receiving the good quality diet (see Chapter 5 discussion for details). In essence, the thesis suggests that offering adequate diets to two broiler lines with divergent genetic growth potentials [Chapter 2] or utilising a poor quality (up to 15% lignocellulose-diluted) diet to reduce the GR of the fast-growing broiler line in chapter 2 [Chapter 5] achieved similar results in the context of coccidiosis-impaired long bone quality.

6.4.1 Genetically influenced broiler growth rate

A decisive stance like that of the Dutch Organisation of retails to only sell chicken meat from slow-growing birds because of the problems associated with rapid GR may spread quickly across Europe (Burton *et al.*, 2016) and indeed globally. In the context of coccidiosis, the thesis showed no evidence that selection for improved GR compromised resistance and tolerance of broilers per se [Chapter 2]. However, this was an exception in relation to the several studies highlighting the negative consequences of the genetically increased GR of modern broilers (Knowles *et al.*, 2008; Tallentire *et al.*, 2016). Broiler breeders are advised to

incorporate robust selection schemes covering essential traits like bone quality and disease resistance alongside improved performance into their breeding programmes. Also, since femur mineralisation differed between fast- and slow-growing broilers [Chapters 2 and 5], it is advisable that the industry incorporated markers of femur mineralisation on long bone development in genetic selection schemes. Furthermore, selection-improved GR is strongly correlated with reduced activity levels, which reduce bone quality in broilers (Bizeray *et al.*, 2000; Bizeray *et al.*, 2002a; Rutten *et al.*, 2002) according to Wolff's law (1982). Wolff's law postulated that bone would adapt to loads under which it is placed, which means that deposition of bone and invariably bone strength will increase in response to imposed stress. Therefore, farmers can be encouraged to explore husbandry tools to facilitate exercise or higher activity levels amongst fast-growing broilers for beneficial effects on bone quality.

6.4.2 Nutritional or artificial reduction in growth rate

Substantial economic returns derive from genetic selection for increased GR, but skeletal and welfare disorders constitute a significant drawback in the poultry industry. It is now known that reducing early GR of modern fast-growing broilers during the second and third week or life via diet dilution has beneficial effects on the skeletal development irrespective of coccidia infection status [Chapter 5]. However, this method stands a high risk of rejection by profit-seeking commercial broiler producers due to the delays (2 to 5 days) it may cause in attaining market weight. Further investigations are, therefore, required to compare the economic and welfare implications of adding a few days to the production period, with the huge loss reported due to skeletal and other rapid GR-related disorders (Knowles *et al.*, 2008; Pines and Reshef, 2015; Pratt and Cooper, 2018). The outcome of such a study could make the method more appealing to commercial farmers, especially if reducing early GR turns out to be more profitable. Also, incentives for broiler welfare could add to the value of the product.

6.5 Scope for future research

In the course of this project, several potential research areas geared toward improving the long bone quality and the general welfare of modern fast-growing broilers in the presence or absence of coccidia infections were identified.

- Future research on the bone quality of coccidiosis infected birds could be improved by the use of Dual Energy X-Ray Absorptiometry (DEXA) Bone Scan technology (Morris, 2018). This will allow for effective monitoring and assessment of bone

development in the same chicken across the period post-infection. It will also simplify the data collection process creating time for more data to be collected and analysed.

- The concentration of specific minerals in the bone following *Eimeria* infection could be investigated to provide further knowledge on the effects of coccidiosis on the bone. Markers of bone resorption (Akbari Moghaddam Kakhki *et al.*, 2018) should also be examined alongside bone mineral content.

- Assessment of the immune response could be done over multiple time points post-infection including cytokines and CD4 CD8.

- Value could also be added to future studies by investigating using different *Eimeria* species to ascertain the ameliorative effects of Vit on the consequences of infection. A mixed infection model and the use of 1,25D3 metabolite could also be investigated.

- Gait scoring and locomotion capacity assessment could be incorporated into future studies to investigate the effect of coccidiosis on walking ability and if VitD has any beneficial effects in this regards.

- A co-supplementation of VitD with nucleotide-rich yeast extracts (Leung *et al.*, 2018) could be investigated for any beneficial effects on damaged gut morphology arising from increased parasite burden associated with VitD supply [Chapter 3].

- The economic and welfare implications of extending the production cycle of modern fast-growing broilers by few days compared to skeletal and other rapid GR-related disorders losses (Knowles *et al.*, 2008; Pines and Reshef, 2015; Pratt and Cooper, 2018) could be investigated. The outcome of this study could influence the decision of commercial farmers on employing reduction in early GR as a means of improving long bone quality of broilers.

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