The effects of broiler breeder diet supplementation of canthaxanthin and 25-hydroxyvitamin D_3 and age on offspring performance traits and innate immune function

by

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ABSTRACT

Experiments were conducted to understand the effect of dietary canthaxanthin (**CXN**) and 25-hydroxyvitamin D_3 (**25-OHD$_3$**) on broiler breeder productivity, progeny performance traits and progeny innate immune function. Beginning at 22 wk of age, breeder hens were fed either 1) Control (Vitamin D_3 at 2,760 IU/kg), 2) CXN (Control plus 6 mg/kg CXN) 3) 25-OHD_3 (Control plus 25-OHD_3 at 2,760 IU/kg replacing vitamin D_3) or 4) HC (CXN diet plus 25-OHD_3 at 2,760 IU/kg replacing vitamin D_3). Roosters were fed either the Control or HC diet. Hens were inseminated weekly, and hatching eggs collected at 25, 37, 49 and 59 wk of age. The chicks were fed a standard commercial broiler diet containing vitamin D_3 as the sole source of vitamin D activity and no CXN. Broiler body weight (**BW**), feed intake (**FI**) and feed conversion ratio (**FCR**) were determined. Broilers were processed at 43 day of age. Whole blood phagocytic index (cells engulfing at least 1 *Escherichia coli*; ***E.coli***) and phagocytic capacity (relative number of *E. coli* engulfed/cell) were measured at 1 and 4 d of age. Total and settable egg production, mid, late and total embryonic mortality, hatchability of fertile, shell thickness and infectious bursal disease (**IBD**) antibody titers were not affected by dietary treatments. The HC roosters maintained fertility after 47 wk of age longer than the Control roosters. Egg CXN contents were higher in CXN and HC hens than other treatments but there were no hen treatment effects on whole egg 25-OHD_3 content. Maternal treatment did not affect broiler BW at 42 d but FI from 0 to 42 d was lower in chicks from the 25-OHD_3 hen treatments than Control and CXN treatments; hence, FCR was lowest in broilers from 25-OHD_3 hens compared to other hen treatments.

Chicks from hens inseminated by HC roosters had increased total and lymphocyte/thrombocyte phagocytic index compared to chicks from hens inseminated by Control roosters. For further investigation of innate immunity in young chicks in the furture, a heterophil extracellular trap (**HET**) assay was developed. First, the HET method was developed in laying hens and then it was developed for use in young chicks. The second experiment was conducted to determine if the HET assay was capable of detecting sex and age differences that are expect to affect innate immunity. *E.coli* killing was greater at 1, 4 and 6 dof age than at 8 d of age. The HET release in response to *E.coli* was greater at 1 d than at any other age. Based on the second experiment, the HET assay developed is useful to measure white blood cell extracellular trap production in chicks. Attempts were made to develop a heterophil isolation assay in this study for future use in a heterophil extracellular trap assay. Complete heterophil isolation was not possible using different isolation procedures. The greatest degree of heterophil isolation was using Histopaque 1.077/1.119 with Dextran T500.

PREFACE

This thesis is an original work by Misaki Cho. M. Cho conducted experiments, lab analysis, statistical analysis and writing of this thesis. The research experiemnt, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, canthaxanthin and 25-OH vitamin D_3 in broiler chicken diets, AUP00000162 (chaper 2, 3, and 4), August 25, 2016, and development of *ex vivo* innate immune function assays, AUP00001925 (chapter 5 and 6), July 1, 2012. No part of thesis has been previously published.

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$1,25(OH)_2D_3$-1,25-dihydroxyvitamin D_3

$25\text{-}OHD_3$- 25-hydroxyvitamin D_3

ACK- Ammonium Chloride Potassium Chloride

ADFI- Average Daily Feed Intake

AnnV- Annexin V

BSA- Bovine Serum Albumin

BW - Body Weight

CXN- Canthaxanthin

d - day

DBP- Vitamin D Binding Protein

E. coli- Escherichia coli

FCR - Feed Conversion Ratio

FI - Feed Intake

HET- Heterophil Extracellular Trap

IBD- Infectious Bursal Disease Virus

IFNγ- Interferon Gamma

IL-6- Interleukin-6

IL-8- Interleukin-8

iNOS- Inducible Nitric Oxide Synthase

LPS- Lipopolysaccharide

MCHII- Major Histocompatibility Complex Class II

MPO- Myeloperoxidase

NDV- Newcastle Disease Virus

NET- Neutrophil Extracellular Trap

NF-kB- Nuclear factor kappa β

ONE- 4-Oxo-2-Nonenal

PAMP- Pathogen-Associated Molecular Pattern

PBS- Phosphate-Buffered Saline

PHA-Phytohemmagglutinin

PepT1- Peptide Transportor 1

PI- Propidium Iodide

PMA- Phorbol Myristate Acetate

PRR- Pattern Recognition Receptors

PS- Phosphatidylserine

RBC- Red Blood Cell

ROS- Reactive Oxygen Species

SEM - Standard Error of the Mean

TBARS- Thiobarbituric Acid Reactive Substances

Th- helper T cell

Th1- T helper 1

Th2- T helper 2

Th17- T helper 17

TNF-α-Tumor Necrosis Factor-α

WBC- White Blood Cell

Wk- week

1. LITERATURE REVIEW

1.1 INTRODUCTION

Broiler breeder genetics are constantly changing to meet the increasing consumer demand for broiler meat; chicken per capita consumption in Canada was 31.86 kg/person in 2015 compared to 16.88 kg/person in 1980 (Agriculture and Agri-Food Canada, 2015). Over the years, broiler strains with greater potential for breast meat yield and efficient feed conversion have been selected. A strain of broilers from the year 2001 fed a 2001 diet had higher breast meat yield (20% breast meat yield of body weight) than a 1957 broiler line fed a 1957 diet (11.6% breast meat yield of body weight; Havenstein et al., 2003). Approximately 85 to 90% of the increase in productivity has been due to genetics and the other 10 to 15 % is due to changes in nutrition (Havenstein et al., 2003). Broiler growth traits and breeder reproductive traits are negatively correlated (Tona et al., 2004). The continued genetic advancement and selection of broiler genetic lines that have high feed efficiency, body weight gain and meat yield requires constant changes in breeder management and feed to optimize fertility, hatchability and egg production. The genetic selection for broilers with high performance also requires changes in management and nutrition to meet the genetic potential of the bird.

The aim of the hatchery is to maximize hatchability of good quality chicks. Healthy chicks are important because these chicks will have a good start on the rearing period after being handled and transported; healthy chicks have low first week mortality compared to poor quality chicks (Olsen et al., 2012).

The number of fertilized eggs produced and hatched determines the number of chicks per hen housed, hence non-fertilized eggs have little value to the breeder producer. Various external factors can affect egg production and fertility, such as feed ingredients, breeder management and

breeder age (Abudabos, 2010; Rosa et al., 2012; Johnson-Dahl et al., 2017). Canthaxanthin (**CXN**) is an antioxidant feed addtitive. It can affect breeder productivity; CXN-fed broiler breeder hens increased hatchability, fertility and egg production compared to breeder hens without CXN (Rosa et al., 2012; Johnson-Dahl et al., 2017). Vitamin D is important for embryo development and regulation of the immune system (Narbaitz et al., 1987; Aslam et al., 1998). The 25-hydroxyvitamin D_3 (**25-OHD$_3$**) is a metabolite of vitamin D and it increased hatchability when fed to broiler breeder hens (Coto et al., 2010b). Dietary CXN and 25-OHD_3 can also affect broiler performance. Chicks hatched from breeder hens fed CXN (6 mg/kg) had increased *E. coli* killing compared to chicks hatched from hens fed without CXN (Johnson-Dahl et al., 2017). Furthermore, broilers fed 25-OHD_3 had increased breast meat yield and tibia bone ash compared to broilers fed dietary vitamin D_3 (Yarger et al., 1995*;* Coto et al., 2010a). Currently, few studies have been done looking at the effects of the combination of dietary CXN and 25-OHD_3 on broiler breeder production and broiler performance.

Based on the literature, dietary CXN and 25-OHD_3 can independently increase breeder production and broiler performance. Canthaxanthin and 25-OHD_3 may have synergistic actions based on their metabolic pathways; CXN working as an antioxidant and 25-OHD_3 involved in vitamin D activity. Since CXN and 25-OHD_3 can be transferred from the hen to the egg (Coto et al., 2010b; Johnson-Dahl et al., 2017) and hence to the broiler chick, it may affect broiler performance when these two compounds are fed together to the broiler breeder. More studies are needed to fully understand the effect of the combination of these two feed supplements in chickens to increase breeder production and broiler performance.

A bacteria killing mechanism, heterophil extracellular trap has been reported in adult chickens and may play an important role in protection against pathogens in young chicks

(Chaummitri et al., 2009). Heterophil extracellular trap is the functional equivalent to neutrophil extracellular trap in mammals. Neutrophils can produce extracellular fibres made from granules containing protein and chromatin that bind to and kill pathogens (Brinkmann et al., 2004; Urban et al., 2006). The feed and bacteria in the intestine of the newly hatched chick stimulate production of pro-inflammatory mediators that recruit innate cells, such as heterophils, and then later, it gradually increases recruitment of lymphocytes (Bar-Shira and Friedman, 2006). Hence, adaptive immunity may take more time to fully develop compared to the innate immunity in young chicks, and young chicks may rely on innate immunity for the first few d of their life or until the adaptive immunity is fully functional and developed. Having a clear understanding of chick bacteria killing mechanisms is important because it is vital to poultry immunology and may contribute and lead to obtaining healthy chicks in the future. Suggestions and future research for dietary CXN and 25-OHD_3 in breeders and broilers as well as immunity in young chicks will be addressed.

The objective of this literature review is to understand the mechanism and the recent finding related to the effect of dietary CXN and 25-OHD_3 on broiler breeder production and broiler performance as well as the effect of broiler age on immunity.

1.2 CAROTENOIDS

Currently, over 600 different carotenoids have been identified (Kull and Pfander, 1995) and some of them are used in the poultry industry for pigmentation of products such as egg yolk and broiler skin (Koutosos et al., 2003). Carotenoids are important for deactivating many reactive radical species and protecting cells from these harmful radicals; specific carotenoid functions is determined based on their structure and functional group.

1.2.1 Carotenoid structure and function

Carotenoids are derived from $C_{40}H_{56}$ and can be divided into two different categories based on their structure: xanthophylls and carotenes. Xanthophylls such as CXN and astaxanthin contain oxygen in their structure, while carotenes such as beta-carotene do not (Figure 1.1; Goodwin, 1986). The presence or abscense of oxygen in the carotenoid structure detrermines their orientation in the cell membrane. Since xanthophylls contain oxygen in their structure, they are oriented perpendicular to the membrane surface, with the oxygen-containing polar functional groups on the outside of the membrane. While carotenes are oriented parallel to the membrane surface and located inside the membrane (Johansson et al., 1981). An antioxidant prevents oxidation of other molecules. Compared to carotenoids, xanthophylls have greater antioxidant activity because they contain oxygen and are more structurally stable after trapping free radicals, and are less susceptible to oxidation (Terao, 1989).

1.2.2 Reactive oxygen species

Reactive oxygen species (ROS) are reactive free radicals that contain oxygen in their structure, for instance, superoxide anion radical ($O_2^{\cdot -}$) and hydroxyl radical (HO•) (Fiedor and Burda, 2014). Superoxide anion radical is one of the most abundant free radicals produced in the body; it is formed normally through metabolic activity in the body such as from complex 1 and 3 in the mitochondrial electron transport chain as well as through nicotinamide adenine dinucleotide phosphate (**NADPH**) oxidase in the cell membrane (Fiedor and Burda, 2014). High amounts of superoxide anion radicals in the human body can cause Alzheimer's disease (Aliev et al., 2014) and chronic liver disease (Novo et al., 2006), but moderate amounts of superoxide anions is essential to the body for example, by killing phagocytosed bacteria . Superoxide anion radical produced by NADPH oxidase is converted to hydrogen peroxide by the antioxidant

enzyme, superoxide dismutase (Weiss and Slivka, 1982); myeloperoxidase further converts the hydrogen peroxide to hypohalous acid, which is important for killing pathogens during phagocytosis in neutrophils and macrophages (Weiss and Slivka, 1982). Superoxide anion radical is also important for neutrophil extracellular trap (**NET**) activation in neutrophils (Kirchner et al., 2012).

1.2.3 Antioxidants

Antioxidants can be divided based on their structure: enzymatic antioxidants and non-enzymatic antioxidants (Fiedor and Burda, 2014). The major enzymatic antioxidants that break down and eliminate free radicals include superoxide dismutase, catalase, and glutathione peroxidase. Superoxide anion can be converted into oxygen and hydrogen peroxide by superoxidise dismutase, which contains metal ions such as copper, zinc and manganese; hydrogen peroxide can be converted further into water by catalase and glutathione peroxidase (Fiedor and Burda, 2014). Enzymatic antioxidant activity can vary depend on the species, breed and tissue. Ganders had higher enzymatic antioxidant activity in their sperm than roosters (Partyka et al., 2012) and Jungle fowl had higher superoxide dismutase and glutathione peroxidase activities in their feather melanocytes than White Leghorns and Barred Plymouth Rocks (Bowers et al., 1994). Non-enzymatic antioxidants such as vitamin E, ascorbic acid, beta-carotene and CXN can break the free radical chain reaction. Carotenoids are lipid soluble non-enzymatic antioxidants and therefore can scavenge free radicals and quench singlet oxygen in the cell membrane (Conn et al., 1991; Fiedor and Burda, 2014).

1.2.4 Antioxidant protection on lipid peroxidation

Lipid peroxidation is the process by which ROS causes the deterioration of lipid; under stressful conditions the rate of ROS production can increase and cause damage to lipid molecules

(Cadenas et al., 2000). Therefore, it can cause damage to phospholipids in the cell membrane and can change cell membrane composition and structure (Niki et al., 1991). The lipid peroxidation mechanism has three main steps: initiation, propagation, and termination (Ayala et al., 2014; Figure 1.2). The intiation step is when ROS, such as hydroxyl radical, removes hydrogen from fatty acid to form a lipid radical. In the propagation phase, the lipid radical will react further with oxygen to form a lipid peroxyl radical.The lipid peroxyl radical removes hydrogen from adjacent lipid molecules and creates a chain reaction (Ayala et al., 2014). In the termination phase, when there is a high concentration of initiating radicals, they can react with one another to create a non-radical damaged lipid product. Antioxidants such as vitamin E and CXN, can provide a hydrogen atom to the lipid peroxyl radical and form a non-radical product (Ayala et al., 2014). It is important to have antioxidants, such as CXN, that scavenge ROS in the cell membrane and break the chain reaction (DiMascio et al., 1990; Mortensen et al., 1997).

There are a few possible mechanisms by which carotenoids can react with free radicals. One possible mechanism is adduct formation (Burton and Ingold, 1984). Carotenoids (C) react with a lipid peroxyl radicals ($ROO^{\cdot}$) to form a carotenoid-adduct radical ($ROO\text{-}C^{\cdot}$; Figure 1.3 (1)). This can further react with another radical to form a stable product (Figure 1.3 (2)). Another possible mechanism is electron transfer (Conn et al., 1992; Surai, 2012); electrons are transferred when a carotenoid reacts with radicals and forms a carotenoid radical cation ($C^{\cdot+}$; Figure 1.3 (3)). The last possible mechanism is hydrogen abstraction (Woodall et al., 1997); hydrogen is transferred from the carotenoids (C(H)) to the radical to a form carotenoid radical ($C^{\cdot}$; Figure 1.3 (4)).

1.2.5 Dietary CXN in poultry

Canthaxanthin is fat-soluble carotenoid that is commercially available to the poultry industry. Dietary CXN is absorbed in the intestine, where the CXN is mixed with lipid to form micelles

and are transferred to intestinal mucosal cells (Parker, 1996). Because the lymphatic system in the chicken is not well developed, it will be delivered directly to the liver and other tissues through portomicrons instead of chylomicrons as in mammals (Krogdahl, 1985). Therefore, CXN can accumulate in the chicken liver and be effectively deposited into the egg when fed to breeder hens (Johnson-Dahl et al., 2017). Canthaxanthin is an effective antioxidant and hence it may protect the animal from oxidative damage in the body (Surai et al., 1998) and increase breeder production (Rosa et al., 2012) and broiler performance (Zhang et al., 2011).

1.2.5.1 Canthaxanthin antioxidant protection in broiler breeder eggs

Egg yolk is susceptible to lipid peroxidation because it is composed of 30% lipid (Ayerza and Coates, 2000), with the majority being in the form of triglycerides (Christie and Moore, 1972). Specific tissues, such as the liver and brain of newly hatched chicks, are also susceptible to lipid peroxidation since these tissues have high amounts of polyunsaturated fatty acids (Surai et al., 1998). Furthermore, newly hatched chicks can be prone to oxidative stress because oxygen consumption and heat production in the embryo increases rapidly before hatch (Hamidu et al., 2007).Therefore, antioxidant protection is important in chick embryos and newly hatched chicks to reduce lipid peroxidation and oxidative damage, and ultimately increase viability and performance.

Approximately 40% of the CXN fed to laying hens is transferred to the egg (Grashorn and Steinberg, 2002); when 2.5 mg CXN/kg diet was fed to laying hens, 1 mg CXN/kg egg yolk was deposited (Grashorn and Steinberg, 2002). Broiler breeder hens also deposit dietary CXN in a dose-dependent manner (Johnson-Dahl et al., 2017; Rosa et al., 2017). Breeder eggs enriched in CXN had reduced lipid peroxidation and increased antioxidant protection compared to eggs without CXN (Rosa et al., 2012; Rosa et al., 2017). Egg thiobarbituric acid reactive substances

(**TBARS**; a product of lipid peroxidation) decreased and total antioxidant capacity increased (Robert et al., 2007; Zhang et al., 2011; Johnson-Dahl et al., 2017) when compared to eggs without CXN. Hence, when dietary CXN is fed to broiler breeders, CXN is deposited into the egg and may provide antioxidant protection to the chick embryo.

1.2.5.2 Impact of dietary CXN on broiler breeder production

Broiler breeder hens fed CXN had increased hatchability and reduced embryonic mortality (Rosa et al., 2012). Since CXN is an antioxidant, it can be efficiently transferred to the egg yolk when fed to breeder hens. It can reduce lipid peroxidation and increase antioxidant capacity in the yolk (Johnson-Dahl et al., 2017; Rosa et al., 2017). Therefore, CXN can provide protection against lipid peroxidation in the yolk and the embryo, and may be the basis for increased hatchability and reduced embryonic mortality in CXN-fed breeder hens (Rosa et al., 2012).

When broiler breeder hens were fed CXN, fertility increased (Rosa et al., 2012). Chicken sperm contains high levels of polyunsaturated fatty acids (52.1%; Surai et al., 1998) and therefore is susceptible to lipid peroxidation. Furthermore, chicken sperm cells have lower superoxide dismutase and glutathione peroxidase activities than mammals (Mennella and Jones, 1980) and yet the hen needs to store sperm in sperm storage tubules that are located in the utero-vaginal junction for a few d to several wk (Bakst et al., 1994). This suggests that CXN may be important to provide effective lipid peroxidation protection of the sperm during storage in the tubules. This may explain the increased fertility in breeders fed CXN.

Dietary CXN increased egg production in broiler breeders (Johnson-Dahl et al., 2017) and laying hens (Cho et al., 2013). Hens fed dietary carotenoids, such as lutein, can transfer carotenoids to the liver (Leeson et al., 2007) and increase antioxidant capacity and reduce malondialdehyde (**MDA;** a product of lipid peroxidation; Gao et al., 2013). Similarly, CXN-fed

hens may transfer CXN to the liver and decrease liver lipid peroxidation and cell damage due to oxidation. Heat-stressed hens fed 500 mg/kg of vitamin E produced egg yolk precursors, such as vitellogenin and triglycerides, more efficiently from the liver, and hence increased egg production compared to hens fed 10 mg/kg of vitamin E (Bollengier-Lee et al., 1998). This may be due to the antioxidant activity of vitamin E, which protected the liver from lipid peroxidation (Bollengier-Lee et al., 1998). If increased antioxidant activity in CXN-fed hens resulted in more efficient production of egg yolk precursors in the liver than hens not fed CXN, then this may explain the increased egg production in CXN fed hens.

1.2.5.3 Effect of maternal dietary CXN on broiler performance

Maternal dietary CXN decreased broiler chick mortality during the first 21 d of age post-hatch (Zhang et al., 2011; Rosa et al., 2017). Chicks hatched from eggs containing lutein, an antioxidant, had higher macrophage infiltration and faster lymphocyte infiltration one d after phytohemagglutinin (**PHA**) was injected into the wing web when compared to chicks hatched from eggs without lutein (Koutsos et al., 2006). Hence, if CXN-fed breeder hens have chicks that have increased immune reponse, then this may result in reduced chick mortality.

1.3 VITAMIN D

Vitamin D is a fat-soluble compound which has two main forms: ergocalciferol (vitamin D_2) and cholecalciferol (Vitamin D_3; Ameenuddin et al., 1985). Vitamin D_2 is derived from plant sources and is not used efficiently in poultry. Vitamin D_3 has 15 times the biological activity of vitamin D_2 (Valinietse and Bauman, 1981). Therefore, the main form of vitamin D provided in the diet is vitamin D_3. In poultry, vitamin D_3 is obtained mostly through the feed, and deficiency of vitamin D_3 results in rickets, decreased hatchability, and growth (Ameenuddin et al., 1985). Vitamin D_3 obtained from the diet is absorbed in the small intestine and transported in the

circulation by vitamin D-binding protein (**DBP**) to the liver and is then is converted into 25-OHD_3 by the enzyme 25-hydroxylase. Twenty five-hydroxyvitmain D_3 is further converted to the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (**$1,25(OH)_2D_3$**), in the kidney by the enzyme 1α-hydroxylase (Soares et al., 1995). The $1,25(OH)_2D_3$ is important because it is involved in calcium and phosphorous regulation through mechanisms such as calcium absorption in the intestine (Wasserman et al.,1982), bone mobilization, and parathyroid hormone production (Norman and Hurwitz, 1993).

1.3.1 Absorption of 25-OHD_3 and circulating 25-OHD_3 levels in poultry

The chick intestinal absorption of dietary 25-OHD_3 (83% absorption rate) was greater than dietary vitamin D_3 (66% absorption rate; Bar et al., 1980). This could be because of increased villus length of the duodenum and jejunum in 25-OHD_3-fed broilers when compared to vitamin D_3-fed broilers (Chou et al., 2009). It could also be because 25-OHD_3 has an additional hydroxyl group in its structure compared to vitamin D_3 and therefore 25-OHD_3 is more polar than vitamin D_3 (Fraser, 1980). It has been shown that absorption of 25-OHD_3 into the portal blood of rats is greater than that of vitamin D_3 because of their difference in polarity (Maislos et al., 1981). If this is true in chickens, the more polar 25-OHD_3 would have greater absorption than the less polar vitamin D_3. Furthermore, dietary 25-OHD_3 (7% of 25-OHD_3 daily intake; 15 µg/kg 25-OHD_3 provided) was excreted to a lesser extent from the chick than dietary vitamin D_3 (20% of vitamin D_3 daily intake; 15 µg/kg of vitamin D_3 provided; Bar et al., 1980). This suggests that 25-OHD_3 has greater absorption and retention in the body than does vitamin D_3. Additionally, dietary 25-OHD_3 does not need to be hydroxylated in the liver compared to dietary vitamin D_3, which needs to be transferred and hydroxylated in the liver (Jones et al., 1998). Therefore, dietary 25-OHD_3 may have more biological activity than vitamin D_3.

The major circulating vitamin D metabolite in the chicken is 25-OHD_3; this is followed by vitamin D_3, 25,26-dihydroxyvitamin D_3, 1,25$(OH)_2D_3$ and 21,25-dihydroxyvitamin D_3 (Haussler and Rasmussen, 1972). Broiler serum contained approximately 10 ng 25-OHD_3/mL and 35 ng/mL when broiler chicks were fed without 25-OHD_3 and with 69 µg/kg 25-OHD_3, respectively; and broiler serum 25-OHD_3 has been noted to increase with increasing dietary 25-OHD_3 (Yarger et al., 1995). The higher absorption rate of dietary 25-OHD_3 compared to vitamin D_3 (Bar et al., 1980), and the longer half-life of serum 25-OHD_3 (approximately 3 wk) than vitamin D_3 (approximately1 d; Zerwekh, 2008) may explain why dietary 25-OHD_3 is more efficiently transferred from the breeder hen to the egg than is dietary vitamin D_3 (Coto et al., 2010b).

1.3.2 The effect of 25-OHD_3 in the diet on broiler breeder production

Supplementation of 25-OHD_3 in breeder hen drinking water decreased early embryonic mortality (Saunders-Blades and Korver, 2014), and breeder hens fed 25-OHD_3 had increased hatchability of fertile eggs (Saunders-Blades and Korver, 2015). Vitamin D is important for embryonic development and hatchability (Narbaitz et al., 1987). Vitamin D deficiency decreases embryonic calcium absorption from the egg shell, resulting in decreased hatchability (Narbaitz et al., 1987). This results in decreased hatchability (Narbaitz et al., 1987). Therefore, if embryos are vitamin D deficient and dietary 25-OHD_3 is more efficiency deposited into the egg than is vitamin D_3 (Coto et al., 2010b), then this may explain the increased hatchability of fertile eggs and decreased early embryonic mortality.

Dietary 25-OHD_3 increased egg shell thickness in broiler breeder hens (Coto et al., 2010b) and breeder ducks (Ren et al., 2016a). This could be because dietary vitamin D levels were low in the vitamin D_3 treatment diet (600 IU/kg) and dietary 25-OHD_3 (2,760 IU/kg) was added on

top of the vitamin D_3 diet for the 25-OHD_3 treatment diet (3,360 IU/kg; Coto et al., 2010b). Laying hens fed vitamin D_3 or 25-OHD_3 at the same level of vitamin D activity (600 or 2,760 IU/kg) had no effect on egg specific gravity (McLoughlin and Soares, 1976; Keshavarz, 2003). The level of dietary vitamin D activity may have a greater impact on egg shell quality rather than the form of vitamin D. Hens fed lower vitamin D_3 (120 IU/kg) or no vitamin D_3 had greater soft shelled egg production compared to hens fed higher vitamin D_3 (360 IU/kg; Abdulrahim et al., 1979). Furthermore, broiler chickens deprived of vitamin D_3 for 5 to 10 d had increased kidney 1α-hydroxylase activity but had decreased plasma 1,25$(OH)_2D_3$ compared to broiler chicks that received 20 IU vitamin D_3/d orally (Booth et al., 1985). Hens forming uncalcified shells synthesize less plasma 1,25$(OH)_2D_3$, duodenum and egg shell gland calbindin compared to hens forming normal shells (Bar et al., 1999). Coto et al. (2010b) vitamin D_3 treatment (600 IU/kg) was not at a vitamin D deficient level. However, if 1,25$(OH)_2D_3$ production was lower in breeders fed vitamin D_3 treatment (600 IU/kg) compared to 25-OHD_3 treatment diet (3,360 IU/kg), then synthesis of calcium binding protein and the transfer of calcium across the intestinal wall may decrease in the vitamin D_3 treatment (Feher et al., 1979), resulting in decreased egg shell thickness.

1.3.3 The impact of maternal dietary 25-OHD_3 on broiler performance

When broilers were fed 25-OHD_3, there was increased broiler breast meat yield compared to broilers fed vitamin D_3 (Yarger et al., 1995).This could be because dietary 25-OHD_3 supplementation in broiler chickens increases satellite cell activity of the broiler breast meat (Hutton et al., 2014) or stimulates the mTOR pathway activity that increases muscle growth and proliferation (Vignale et al., 2015). Chicks hatched from 25-OHD_3-fed breeder hens may have increased breast meat yield by increasing mean diameter and size of muscle fiber during

embryonic development. The 25-OHD_3 can be converted to 1,25$(OH)_2D_3$ by 1-α-hydroxylase in the skeletal muscle (Srikuea et al., 2012); therefore, 1,25$(OH)_2D_3$ can increase the expression of myogeneic regulatory factors such as antimyoblast determination (**MyoD**) and myogenin during embryonic development (Garcia et al., 2011). This may ultimately lead to increasing mean diameter and size of muscle fiber in the embryo. However, in ovo injection of 25-OHD_3 at 18 d of incubation did not affect broiler meat yield (Bello et al., 2014). The MyoD and myogenin expression in chick embryo starts from 4 and 4.5 d of incubation, respectively (Mok et al., 2015); hence, 25-OHD_3 may need to be provided before that time to see an increase in broiler meat yield.

Broilers hatched from 25-OHD_3-fed broiler breeder hens had increased tibia ash compared to vitamin D_3-fed hens (Coto et al., 2010a). However, this could be due to low maternal dietary vitamin D activity in the vitamin D_3-fed hens (600 IU/kg) compared to 25-OHD_3-fed hens (2,760 IU/kg); broiler breeder hens fed lower vitamin D_3 levels (500 IU/kg) had chicks with reduced tibia ash compared to chicks from breeder hens fed higher vitamin D_3 levels (1,000 IU/kg; Atencio et al., 2005). Recently, *in ovo* injection of 25-OHD_3 in a range of 0.2 to 1.8 µg resulted in a nearly significant increase in broiler bone ash percentage at 21 d of age (Bello et al., 2014). This could be because 1,25-dihydroxyvitamin D_3 increases calcium binding protein in the chorioallantoic membrane of the egg (Tuan and Scott, 1977). Calcium binding protein is responsible for translocating eggshell calcium into the embryo circulation (Tuan and Scott, 1977). It is unclear if higher 25-OHD_3 contents in the egg will result in greater 1,25$(OH)_2D_3$ production in the embryo kidney than embryos from eggs that have lower 25-OHD_3 content. Overall, the effect of maternal dietary 25-OHD_3 on broiler bone quality is not clear and further research is needed in this area.

1.4 THE EFFECT OF DIETARY CXN AND 25-OHD$_3$ ON BREEDER PRODUCTION AND PROGENY PERFORMANCE

Dietary CXN and 25-OHD$_3$ fed to broiler breeder hens (Duarte et al., 2015) and duck breeders (Ren et al., 2016a) increased egg yolk pigmentation. This is expected because CXN fed to breeder hens can be efficiently deposited into the egg (Johnson-Dahl et al., 2017; Rosa et al., 2017). Furthermore, dietary CXN and 25-OHD$_3$ increased hatchability in breeder hens (Duarte et al., 2015) and ducks (Ren et al., 2016a) and decreased early and late embryonic mortality in breeder hens (Duarte et al., 2015). Body weight and tibia ash content in 1-d-old ducklings increased when they were hatched from duck breeders fed with these two feed supplements (Ren et al., 2016b). This may suggest that CXN and 25-OHD$_3$ do not interfere with each other and may have synergistic actions. This could be because the two compounds exert different mechanisms of action. Canthaxanthin works as an antioxidant and 25-OHD$_3$ is involved in vitamin D activity and embryo absorption of calcium from the egg shell. Not many studies have investigated the effect of these two feed supplements on broiler breeder production and progeny performance, and therefore more studies are required to fully understand this area.

1.5 AVIAN IMMUNOLOGY

The avian immune system can be divided into two components: innate and adaptive immunity. Innate immunity acts as a first line of defense and reacts immediately or within hours after the invasion of pathogens, and involves white blood cells such as macrophages, heterophils, and dendritic cells. On the other hand, adaptive immunity is a specific response and may take days to respond to the pathogen. The white blood cells associated with adaptive immunity include T and B-lymphocytes. The bursa of Fabricius is unique in chickens and is important for

the development of B-lymphocytes because the lack of a bursa of Fabricius impairs antibody production (Glick et al., 1956).

1.5.1 Innate immune system

The innate immune system has many defense mechanisms to protect the host against pathogens. For instance, pathogens can be engulfed by heterophils or macrophages (phagocytosis; Segal, 2005), the heterophils or macrophages can produce toxic chemicals and kill pathogens (oxidative burst or nitric oxide; Segal, 2005), and heterophils can release extracellular DNA and immobilize the pathogens (heterophil extracellular trap; **HET**; Maxwell and Roberson, 1998). Oxidative burst does not correlate with the killing of bacteria in broiler chicks (Saunders-Blades and Korver, 2015). This could be because chickens do not rely much on oxidative burst to defend against pathogens due to reduced myeloperoxidase (**MPO**) activity in chickens when compared to mammals (Harmon, 1998). Myeloperoxidase converts hydrogen peroxide and halide to hypochlorite. Hypochlorite, a toxic compound, is involved in the killing of bacteria during an oxidative burst (Segal, 2005). Therefore, the lack of MPO in chickens may decrease bacteria killing activity by oxidative burst.

1.5.1.1 Phagocytosis

The mechanism of phagocytosis has been well studied. Phagocytes, such as, dendritic cells, heterophils, and macrophages, are attracted to pathogens by chemical substances, such as cytokines and bacterial products. Phagocytosis is then initiated by either Fc receptors, complement receptors, or pattern recognition receptors (**PRR**) on the phagocytes (Aderem and Underhill, 1999). The different receptors have various pathogen attachment mechanisms to activate phagocytosis; Fc receptors bind to antibody-coated pathogens, while complement receptors, specifically C3 receptors, recognize complement protein that opsonizes the pathogens

(Aderem and Underhill, 1999). There are many pattern recognition receptors, including mannose receptors and Toll-like receptors (**TLR**). The PRR recognizes the pathogen-associated molecular patterns (**PAMP**) on the pathogen. For instance, mannose receptors detect mannose-rich glycans in microbial glycoprotein and TLR detect lipopolysaccharides (**LPS**) on gram-negative bacteria (Janeway Jr. and Medzhitov, 2002). After the phagocytes detect and attach to the pathogen, the phagocytes engulf and internalize the pathogen, forming a phagosome (Flannagan et al., 2012). It is then fused with a digestive enzyme, such as lysozyme, and forms a phagolysosome (Flannagan et al., 2012). The pathogen is digested and killed internally in the phagocytes. Some phagocytes, such as macrophages, can display fragmented antigenby major histocompatibility complex class II (**MHCII**) molecules on the surface of their cells (Medzhitov, 2007).

1.5.1.2 Heterophil extracellular trap

Heterophils are the most abundant granulocyte present in the chicken and are the functional equivalent to neutrophils in mammals (Maxwell and Robertson, 1998). Heterophils are slightly bigger than eosinophils and on average heterophil size is 6.4 μm (Maxwell and Robertson, 1998). Heterophils also have on average two to three nuclear lobes and the heterophil granules are acidophilic (Maxwell and Robertson, 1998). Heterophils are associated with innate immunity and may play an important role in protection against pathogens in young birds (Swaggerty et al., 2005). Heterophils have many mechanisms of protecting the host against bacteria, including phagocytosis, degranulation and nitric oxide production. A novel neutrophil killing mechanism, NET, has been found in humans (Brinkmann et al., 2004). Neutrophils can produce extracellular fibres made from granule-containing proteins and chromatin that bind to gram-positive and gram-negative bacteria, and fungi (Brinkmann et al., 2004; Urban et al., 2006). When live neutrophil cells activate NET, neutrophils initiate cell death..This neutrophil cell death is

different from apoptosis or necrosis, since it releases NET and antimicrobial peptides, such as cathelicidins, to kill pathogens (Fuchs et al., 2007). After the discovery of NET, it was reported in chicken heterophils as HET (Chaummitri et al., 2009). Stimulation of heterophils with hydrogen peroxide or phorbol myristate acetate (**PMA**) activate HET *in vitro* (Chuammitri et al., 2009). This possibly occurs because PMA activates NADPH oxidase (Fuchs et al., 2007), which produces hydrogen peroxide and ROS (Apel and Hirt, 2004; Chew and Park, 2004). Hydrogen peroxide can be further converted to hypochlorite though MPO; this hypochlorite (Metzler et al., 2011; Kirchner et al., 2012) and ROS from NADPH oxidase activates extracellular traps (Kirchner et al., 2012). Since chickens have less MPO than mammals (Harmon, 1998), chickens may rely more on the ROS from NADPH oxidase than the hypochlorite from MPO.

1.5.2 Age and the effect on innate immune system in chickens

Age is one of the important factors that affects the innate immune system, and it is well studied in young chicks. The *E. coli* killing and hetrophil phagocytosis activity in broiler chicks increases with increasing age (Wells et al., 1998; Johnson-Dahl et al., 2017). Additionally, there are greater numbers of heterophils when chicks are younger, and yet younger chicks are more susceptible to diseases compared to older chicks (Zulkifli and Siegel, 1994). This may be because when compared to older chicks there are more immature heterophils when chicks are younger (Zulkifli and Siegel, 1994). Furthermore, adaptive immunity takes more time to fully develop compared to innate immunity in young chicks; the intestine of the newly hatch chick stimulates pro-inflammatory mediators that recruit innate cells, such as heterophils, and then later, it recruits lymphocytes after the intestine is developed (Bar-Shira and Friedman, 2006). Lymphocyte count and lymphocyte proliferation in response to PHA is lower in nestling tree swallows than adult tree swallows (Palacios et al., 2009). Hence, it is logical to think that

chickens may rely on innate immunity for the first few days of their life or until the adaptive immunity is fully functional and developed.

Not many studies have been conducted on the innate immunity response in chickens at an old age, but several studies have been conducted in other species. Human neutrophil (Wenisch et al., 2000) and mouse macrophage (Ferrandez and De La Fuente, 1999) phagocytosis decreased with increasing age because of reduced neutrophil and macrophage chemotaxis and hexose transport with increasing age. Since phagocytosis requires energy, a decrease in hexose transport or transport of glucose and fructose will decrease phagocytosis. Furthermore, aged people (69 years old) had lower NET release than young people (25 years old) due to reduced ROS from aged people (Hazeldine et al., 2014). Generally, there was reduced innate immune response at older ages compared to younger ages in mice and humans. The effect of age on innate immunity in chickens is not well known and therefore, more studies are needed to understand this area.

1.5.3 Association between innate immunity and adaptive immune response

When phagocytes, such as dendritic cells and macrophages, detect a pathogen, the pathogen is taken up by phagocytosis and then the antigen peptide is presented to the T cells on the phagocyte cell surface through the function of MHCII; this can trigger the adaptive immune response (Medzhitov, 2007). Once the T cell is stimulated by the antigen peptide, helper T cells (**Th**) differentiate to different types of effector Th; this includes T helper 1 (**Th1**), T helper 2 (**Th2**), and T helper 17 (**Th17**; Medzhitov, 2007). The Th1 cells stimulate a pro-inflammatory response by producing interferon gamma (**IFNγ**) and, therefore, activates macrophages and cytotoxic T cells; Th2 cells enhance an anti-inflammatory response by producing cytokines such as interleukin 4, interleukin 5 and interleukin 13 (Medzhitov, 2007). These cytokines activate B

cells and stimulate antibody production. The Th17 cells produce interleukin 17, which recruits heterophils to the infected cells (Medzhitov, 2007).

1.6 IMMUNOMODULATION USING DIETARY CANTHAXANTHIN AND 25-OHD$_3$

Canthaxanthin is an antioxidant and a free radical scavenger (Conn et al., 1991), therefore, when CXN is fed to broiler breeders, it may modulate the immune function of the breeder hen. Canthaxanthin-fed breeder hens efficiently deposit CXN into the egg (Johnson-Dahl et al., 2017; Rosa et al., 2017) and hence CXN may have influence on chick immune function. Vitamin D is important for regulating part of the immune system (Aslam et al., 1998). When fed to hens, dietary 25-OHD$_3$ is better absorbed and retained in the body compared to vitamin D$_3$ (Bar et al., 1980); dietary 25-OHD$_3$ is deposited with greater efficiency into a hen's egg than vitamin D$_3$ (Coto et al., 2010b). Therefore, dietary 25-OHD$_3$ may modulate immune function of the breeder hen and the progeny.

1.6.1 Interaction between CXN and immunity

Antibody titres against infectious bronchitis virus increased in laying hens fed lutein, a carotenoid antioxidant (Bedecarrats and Leeson, 2006). Lutein has two polar groups at the end of its structure that may increase cell membrane rigidity and strength at the site of antigen presentation; this will increase T cell activation resulting in increased antibody production (Lazrak et al., 1987). Similarly, CXN is also a carotenoid antioxidant with two polar groups in its structure (Figure 1); therefore, it may have a similar effect to lutein and may increase antibody titers in breeders.

Addition of lutein to broiler chicken macrophage cell culture increased inducible nitric oxide synthase (**iNOS**) expression compared to macrophages cultured without lutein *in vitro* (Selvaraj and Klasing, 2006); dietary CXN can increase the nuclear receptor, Retinoid X receptor (Nikawa

et al., 1995) and increase iNOS gene expression in the macrophage through the stimulation of nuclear factor kappa B (**NF-kB**; Selvaraj and Klasing, 2006). Therefore, CXN may increase nitric oxide production due to increased iNOS expression. This may partly explain a greater *E. coli* killing *ex vivo* in chicks from breeders fed CXN compared to breeders fed without CXN (Johnson-Dahl et al., 2017).

1.6.2 Relationship of 25-OHD$_3$ and immunity

Compared to vitamin D_3, chicks hatched from broiler breeders fed dietary 25-OHD_3 had increased body weight through 7 d of age (Coto et al., 2010a). This could be because eggs from 25-OHD_3-fed breeder hens (2,720 IU/kg) had higher egg 25-OHD_3 contents than eggs from breeder hens fed low levels of dietary vitamin D_3 (600 IU/kg; Coto et al., 2010b); chicks from breeder hens fed low vitamin D_3 levels (500 IU/kg) had lower body weight than chicks from breeder hens fed high levels of vitamin D_3 (2,000 IU/kg; Atencio et al., 2005). Furthermore, maternal dietary 25-OHD_3 had no effect on broiler body weight compared to maternal dietary vitamin D_3 at the same level of vitamin D activity (Saunders-Blades and Korver, 2015). Hen dietary vitamin D levels has a greater impact on chick body weight rather than the form and structure of vitamin D. However, when compared to vitamin D_3, broilers fed dietary 25-OHD_3 had increased body weight gain during inflammation (Morris et al., 2014). The intestinal absorption of dietary 25-OHD_3 is greater than dietary vitamin D_3 (Bar et al., 1980). Therefore, if broilers are vitamin D deficient, then 1,25$(OH)_2D_3$ may be produced more efficiently. The 1,25$(OH)_2D_3$ can suppress Th1 lymphocytes that enhance inflammation and stimulate Th2 lymphocytes; Th2 lymphocytes increase immunoglobulin production by inhibiting interlukin-12, which is a cytokine that regulates Th1 and Th2 lymphocytes, produced by antigen-presenting cells (D'Ambrosio et al., 1998). Therefore, dietary 25-OHD_3 -fed broilers may have reduced

inflammation during LPS challenge, and ultimately use the energy for growth and body weight rather than inflammation (Mireles et al., 2005; Morris et al., 2014).

When compared to vitamin D_3-fed broilers, antibody titres increased against *Salmonella typhimurium* in 25-OHD_3-fed broilers (Chou et al., 2009). Furthermore, *E. coli* killing, which measures the overall innate immunity against *E. coli* (Millet et al., 2007), increased in broiler chicks when breeders were fed 25-OHD_3 compared to breeders not fed 25-OHD_3 (Saunders-Blades and Korver, 2015). This could be because 25-OHD_3 in the antigen-presenting cells, such as dendritic cells and macrophages, is converted to 1,25$(OH)_2D_3$ and binds to vitamin D_3 response elements (Van Etten and Mathieu, 2005); ultimately increasing the expression of antibody production (Cantorna et al., 1998) and canthelicidin antimicrobial peptide production (Wang et al., 2004). Furthermore, vitamin D_3-deficient broilers had decreased phagocytosis compared to vitamin D_3-sufficient broilers (Aslam et al., 1998), and phagocytic activity against *Salmonella typhimurium* was higher in chickens supplemented with dietary 25-OHD_3 compared to diets without 25-OHD_3 (Chou et al., 2009). These studies indicate that vitamin D is important for phagocytosis and dietary 25-OHD_3 increases phagocytosis in broilers that is possibly due to 25-OHD_3 having a greater intestinal absorption rate than vitamin D_3 (Bar et al., 1980). Increased white blood cell numbers in response to 25-OHD_3 may have also increased phagocytosis in chickens as has been reported in pigs (Konowalchuk et al., 2013).

1.7 OBJECTIVES AND HYPOTHESES

Based on the literature review, the main hypotheses were that feeding breeder hens CXN and 25-OHD_3 will decrease embryonic mortality and will increase hatchability and antibody titers. Dietary 25-OHD_3 would also increase eggshell thickness in broiler breeders. The objective of the

first study was to investigate the effect of dietary CXN and 25-OHD_3 on broiler breeders throughout their production cycle (Chapter 2).

In the second study, the main hypotheses were that dietary CXN and 25-OHD_3 fed to breeder hens are transferred to the egg and therefore, CXN and 25-OHD_3 will increase bone ash in broiler chickens as seen in ducks (Ren et al., 2016b). Increased breast meat yield in chicks hatched from 25-OHD_3-fed hens will also be found. The objective of the second study was to investigate the effects of parental dietary CXN and 25-OHD_3 on broiler performance (Chapter 3).

In the third study, the main hypotheses were that when these two compounds are fed to broiler breeders, there will be increased innate immunity such as phagocytosis in chicks as well as increased antioxidant capacity in the broiler chicks hatched from CXN hens compared to hens with no dietary CXN. The objective of the third study was to investigate the parental dietary effects of CXN and 25-OHD_3 on broiler innate immunity and antioxidant capacity (Chapter 4).

In the fourth study, the main hypotheses were that young chicks will have decreased innate immunity compared to older chickens. The objective of this study was to develop the HET method in young chicks using laying hens as a model first, and then a second experiment was conducted in young broiler chicks to determine if the HET assay was capable of detecting sex and age differences that are expect to affect innate immunity (Chapter 5).

In the last study, the main hypotheses were that heterophil isolation was possible using various isolation procedures. The objective of the last study is to develop a heterophil isolation method in laying hens to allow for an improved HET method for use in future studies.

1.8 FIGURES

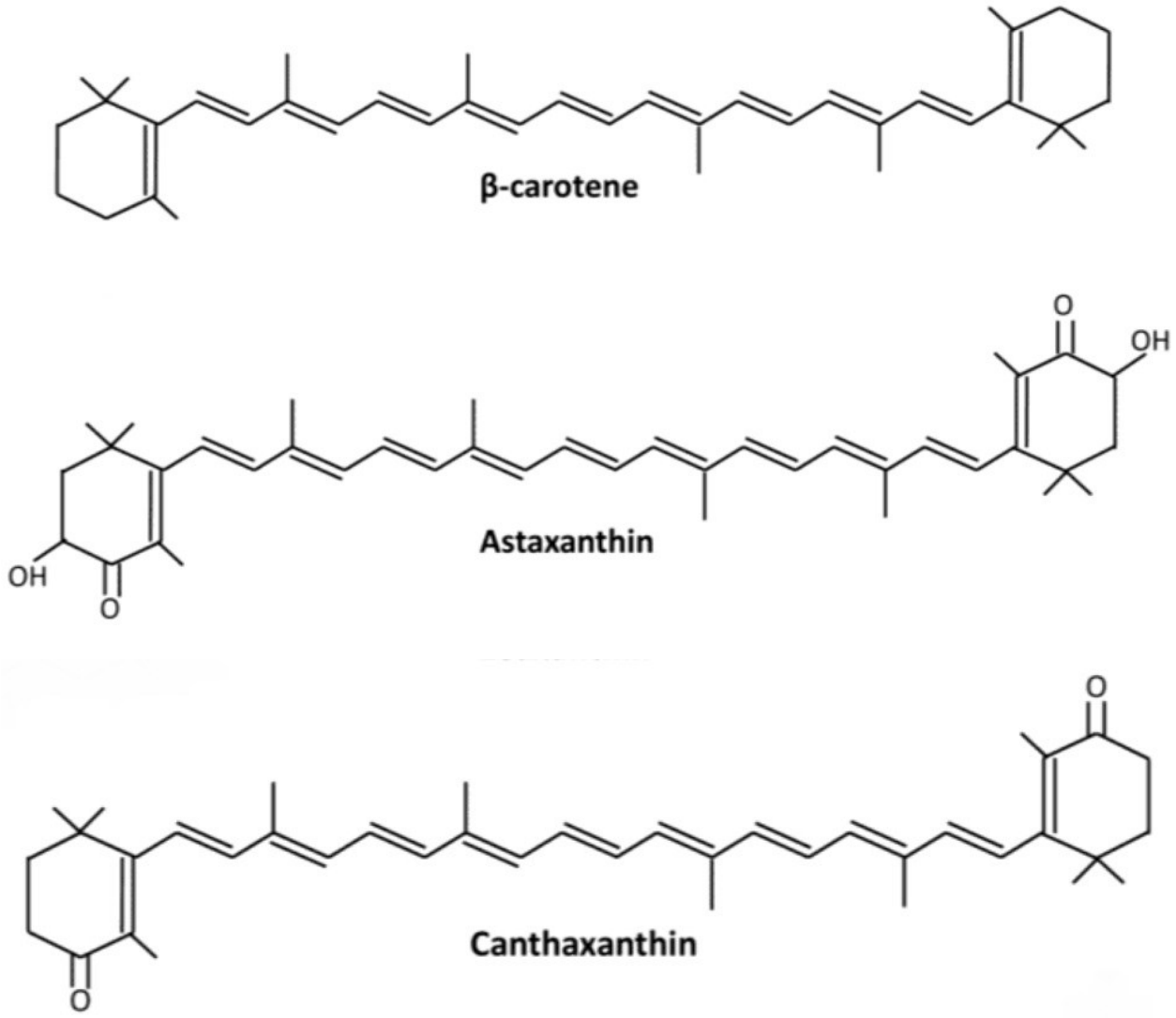

Figure 1.1 β-carotene, astaxanthin, canthaxanthin structure (Lui et al., 2014)

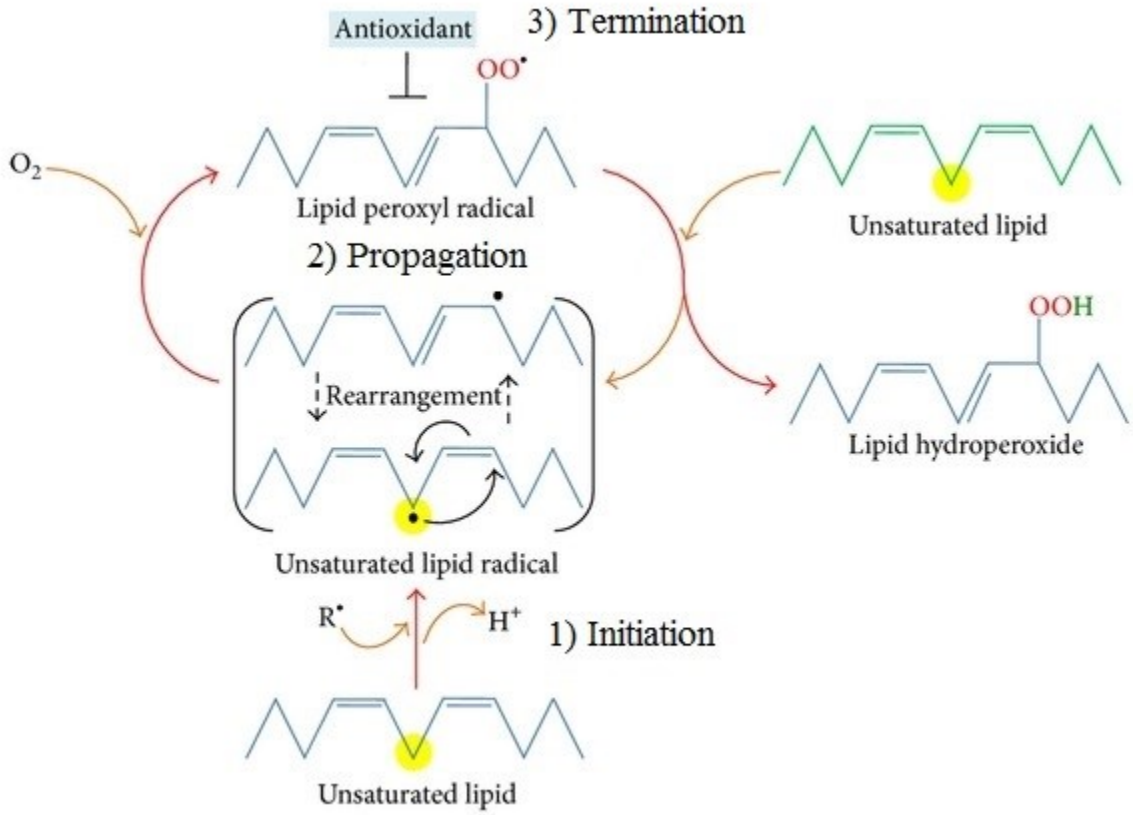

Figure 1.2 Lipid peroxidation process (adapted from Ayala et al., 2014).The lipid peroxidation mechanism has three main steps: initiation (1), propagation (2), and termination (3).

$$ROO^{\cdot\,[1]} + C^{[2]} = ROO\text{-}C^{\cdot\,[3]} \quad (1)$$

$$ROO\text{-}C^{\cdot} + ROO^{\cdot} = ROO\text{-}C\text{-}ROO \quad (2)$$

$$ROO^{\cdot} + C = ROO^{-} + C^{\cdot\,+[4]} \quad (3)$$

$$ROO^{\cdot} + C(H) = ROOH + C^{\cdot\,[5]} \quad (4)$$

[1] Lipid peroxyl radicals

[2] Carotenoid

[3] Carotenoid-adduct radicals

[4] Carotenoid radical cation

[5] Carotenoid radical

Figure 1.3 Mechanisms of antioxidants (adapted from Surai et al., 2012). Different antioxidant mechanisms include adduct formation (1 and 2), electron transfer (3) and hydrogen abstraction (4).

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2. THE EFFECT OF THE DIETARY CANTHAXANTHIN AND 25-HYDROXYVITAMIN D_3 ON BROILER BREEDER PRODUCTIVITY

ABSTRACT

Broiler breeder diets with 25-hydroxyvitamin D_3 (**25-OHD$_3$**) increase egg shell thickness and antibody titers; dietary canthaxanthin (**CXN**) increase antibody titers and hatchability. The objective was to examine the effects of these products in Ross 308 broiler breeder hens and Ross 544 roosters. At 22 wk of age, individually-caged hens were fed either 1) Control (Vitamin D_3 at 2,760 IU/kg), 2) CXN (Control plus 6 mg/kg CXN) 3) 25-OHD_3 (Control plus 25-OHD_3 at 2,760 IU/kg replacing vitamin D_3) or 4) HC (CXN diet plus 25-OHD_3 at 2,760 IU/kg replacing vitamin D_3). Individually-caged roosters were fed either the Control or HC diet. Hens were inseminated weekly. Fertile eggs from each hen were identified, stored for up to one wk (16 to 18°C; 70 to 80% relative humidity) and sent to a commercial hatchery weekly from 33 to 56 wk of age. Fertility, hatchability and embryonic mortality were determined. Egg, yolk, albumin, and shell weight, and shell thickness were measured at 27, 37, 48 and 58 wk of age and egg CXN and 25-OHD_3 contents were determined at 25, 37, 49 and 59 wk of age. Newcastle disease virus (**NDV**) and infectious bursal disease virus (**IBD**) antibody titers following vaccination (NDV at 18, 28, 70, 98, and 126 d; IBD at 126 d) were determined at 29, 32, 47 and 57 wk of age. Total egg production, total embryonic mortality, hatchability of fertile, shell thickness and IBD antibody titers were not affected by hen or rooster dietary treatments. The HC roosters maintained fertility longer than Control roosters. Egg CXN contents were higher in CXN and HC hens than in other treatments, but there were no hen treatment effect on egg 25-OHD_3 contents. Dietary CXN and 25-OHD_3 did not affect breeder productivity, egg components nor antibody titers when vitamin

D activity was provided over the breeder hen requirement and antioxidants provided at adequate levels, but dietary CXN and 25-OHD_3 helped to maintain fertility to a greater age in roosters.

Key words: canthaxanthin, 25-hydroxyvitamin D_3, broiler breeder production, egg quality, antibody titer

2.1 INTRODUCTION

Twenty five-hydroxyvitamin D_3 (**25-OHD_3**) and canthaxanthin (**CXN**) are commercially-available feed supplements used in poultry diets. Each of these fat-soluble compounds are efficiently transferred to the egg when fed to the hens (Coto et al., 2010; Johnson-Dahl et al., 2017). Vitamin D is important for embryo growth and development, and a deficiency of this vitamin in laying hens will decrease hatchability (Narbaitz et al., 1987). Twenty five-hydroxyvitamin D_3 has higher biological activity relative to vitamin D_3, possibly due to greater intestinal absorption (Bar et al., 1980), and unlike dietary vitamin D_3, dietary 25-OHD_3 does not need to be hydroxylated in the liver (Jones et al., 1998). If embyos were vitamin D deficient, then breeder hen dietary 25-OHD_3 supplementation may increase hatchability (Saunders-Blades and Korver, 2015). Dietary 25-OHD_3 has greater intestinal absorption than dietary vitamin D_3 in the broiler chicken (Bar et al., 1980). If dietary 25-OHD_3 increased production of 1,25$(OH)_2D_3$, the active form of vitamin D in comparison to vitamin D_3, then 1,25$(OH)_2D_3$ may increase synthesis of calcium binding protein and the transfer of calcium across the intestinal wall in breeders (Feher et al., 1979). This may result in increased egg shell thickness in broiler breeder hens when fed 25-OHD_3 compared to vitamin D_3 (Coto et al., 2010). One, twenty five-dihydroxyvitamin D_3 can increase antibody production as well (Kuhn et al., 1991; Kongsbak et al., 2014) and hence, dietary 25-OHD_3 supplementation in breeders may increase antibody titers.

The carotenoid pigment, CXN, is a singlet oxygen and free radical scavenger (Mortensen et al. 1997). Embryo oxygen consumption and heat production increases rapidly just before hatch (Hamidu et al., 2007) and hence embryos are prone to oxidative stress during that time. Antioxidant activity of CXN may protect the embryo from lipid peroxidation and oxidative stress and decrease embryonic mortality and increase hatchability in eggs from breeders (Rosa et al., 2012). Infectious bronchitis virus antibody titers increased in laying hens fed the antioxidant lutein (Bedecarrats and Leeson, 2006). This could have happened because lutein has two polar groups at the end of its structure, which could in turn increase cell membrane strength at the site of antigen presentation (Lazrak et al., 1987). This would lead to increased T cell activation and ultimately increased antibody production (Lazrak et al., 1987). Similarly, CXN has two polar groups in its structure and is also an antioxidant. Therefore, CXN may have a similar effect to lutein and may increase antibody titers in breeders.

Although the supplementation of dietary CXN and 25-OHD_3 in duck breeders was found to increase egg shell thickness (Ren et al., 2016), the effect of supplementing CXN and 25-OHD_3 to broiler breeders has not been well studied. We hypothesized that the combination of the two may result in a synergistic effect and therefore, breeder hens fed dietary CXN and 25-OHD_3 would have decreased embryonic mortality, and increased hatchability and antibody titers. Dietary 25-OHD_3 would also increase eggshell thickness in broiler breeders. Since roosters do not transfer nutrients to the egg, we hypothesized that rooster supplementation of dietary CXN and 25-OHD_3 would not affect embryonic mortality, hatchability nor egg components. To confirm these hypotheses, the objective of this research was to investigate the effect of dietary CXN and 25-OHD_3 on broiler breeders throughout the production cycle.

2.2 MATERIALS AND METHODS

2.2.1 Experimental diets and animals

Protocols for experiments were approved by the Animal Care and Use Committee: Livestock of University of Alberta, and birds were reared and cared for according to the Canadian Council of Animal Care (2009). Breeder pullets (Ross 308; n=375) were randomly assigned to one of 6 floor pens (62 to 63 pullets/pen) and cockerels (Ross 544; n=83) were randomly allocated to one of 2 floor pens (41 to 42 cockerel/pen) and raised until 21 wk of age. From the larger population of breeders, 288 hens and 60 roosters were randomly selected and allocated to individual cages (45 x 48 x 42 cm and 42 x 34 x 57 cm, respectively). Hens and roosters were fed the experimental diets from 22 to 60 wk of age (Table 2.1). The breeder hens were fed one of 4 experimental diets (n=72/treatment): Control (Vitamin D_3 at 2,760 IU/kg feed), 25-OHD_3 (Control diet plus vitamin D_3 replaced by 25-OHD_3 at 2,760 IU/kg feed), CXN (Control diet plus CXN added at 6 mg/kg feed), HC (Vitamin D_3 replaced by 25-OHD_3 at 2,760 IU/kg plus CXN added at 6 mg/kg feed). Breeder roosters (n=30/treatment) were fed with either the Control or the HC diet. All other nutritional specifications met or exceeded the breeder nutrient guide (Aviagen, 2007b). The birds were fed to maintain the target BW based on the Ross 308 breeder guide (Aviagen, 2007a). The lighting program followed the Ross 308 breeder guide (Aviagen, 2007a) and breeders were photostimulated at 22 wk of age. The BW of each hen was measured weekly to determine the average BW for each hen treatment to calculate the daily feed allocation based on the target BW from the management guide (Aviagen, 2007a). The hens were artificially inseminated weekly with 0.5 mL pooled semen from either the Control or HC treatment roosters. The eggs from each hen were individually collected. The total egg production and total settable egg production (eggs with no cracks, soft shells, shell-less, double yolk, or abnormal shape) for

each bird was recorded daily and hen-housed egg production was calculated. The fertile eggs from each hen were identified and stored in a cooler (16 to 18˚C at 70 to 80% relative humidity) for up to one wk before the eggs were sent to a commercial hatchery (Lilydale Hatchery, Edmonton, AB, Canada) each wk. Hatchability of fertile was determined as a percentage of chicks hatched from fertile eggs. Hatchability of fertile, fertility (percentage of fertile eggs from total number of eggs incubated), dead (percent dead-in-shell chicks of fertile eggs at hatch), cull (percent cull chicks of fertile eggs at hatch; includes cull due to weak chicks, chicks with physical abnormalities such as distorted beaks and weak limbs, and omphalities), and early (0 to 7 d), mid (8 to 14 d), late (15 to 21 d) and total (0 to 21 d) embryonic mortality data were collected every other wk from 33 to 56 wk of age by examination of hatch residue after collection from the commercial hatchery. Missing data in tables were due to fertile eggs being collected and hatched at the University of Alberta for use in broiler studies (Chapters 3 & 4).

2.2.1.1 Egg composition

At 27 wk of age, 20 randomly-selected eggs per dietary treatment were collected. The whole egg weight, albumin weight, yolk weight, dry shell weight and shell thickness were measured. Shell thickness (mm) was measured using a digital thickness gauge. The process was repeated on eggs from the same breeder hens at 37, 48 and 58 wk of age.

2.2.1.2 Canthaxanthin content in the egg and feed

The amount of CXN in the egg (15 eggs/treatment) and feed was determined using HPLC at 25, 37, 49 and 59 wk of age as described by Johnson et al. (2017). The CXN contents in the whole egg (μg/g) and feed (mg/kg) were determined by comparing with a CXN standard (Sigma-Aldrich Canada Co., Oakville, ON, Canada). Canthaxanthin (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ) at 6 mg/kg feed was supplemented in CXN and HC diet. Average

analyzed CXN content in feed for Control, 25-OHD_3, CXN and HC diet was 0, 0, 6.91, and 6.72 mg/kg, respectively.

2.2.1.3 25-hydroxyvitamin D_3 contents in the egg

Whole egg 25-OHD_3 contents were determined at 25, 37, 49 and 59 wk of age (15 eggs/ treatment). Ten g of homogenized egg was weighed in 50 mL glass tube. Then, 1.5 mL of methanol and 2 mL of methanolic KOH was added and mixed vigorously. It was then saponified overnight at 70 °C. It was centrifuged at 430 x *g* for 5 min and an aliquot of the saponified mixture was transferred to a glass tube. A mixture of acetone and chloroform (3:7, v/v) was added and then centrifuged at 430 x *g* for 15 min. The bottom organic layer was transferred to a glass test tube. It was dried under a nitrogen stream in a 40 °C water bath. Methanol was added to the samples and then transferred to a microcentrifuge tube. It was centrifuged at 215 x *g* for 5 min and then the organic layer was transferred to an HPLC vial. Fifty µL was injected and the samples were analyzed and determined using HPLC and UV detector with a Supelcosil LC-18 column (3 *u*m x 4.6 mm x 150 mm; Agilent, Mississauga, ON, Canada). Methanol was used as a solvent and the flow rate was 1 mL/min. Retention time and quantity of 25-OHD_3 was determined by comparing to a 25-OHD_3 standard (Sigma-Aldrich Canada Co., Oakville, ON, Canada). The 25-OHD_3 in whole egg was quantified as ng/mg.

2.2.1.4 Vitamin D_3 and 25-OHD_3 contents in the feed

Feed samples were sent to DSM Nutritional Products Canada Inc. (Ayr, ON, Canada) to analyze and determine the amounts of vitamin D_3 (IU/kg) and 25-OHD3 (µg/kg) in the feed using HPLC. Twenty five-hydroxyvitamin D_3 (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) at 69 µg/kg feed was supplemented in 25-OHD_3 and HC diet. Average analyzed 25-OHD_3 content in feed for Control, 25-OHD_3, CXN and HC diet was 0, 50.44, 0, and 47.31

µg/kg, respectively. Vitamin D_3 (Rovimix 500, DSM Nutritional Products Ltd., Parsippany, NJ) at 2,760 IU /kg feed was supplemented in Control and CXN diet. Average analyzed vitamin D_3 content in feed for Control, 25-OHD_3, CXN and HC diet was 2,655, 154, 1,584, and 264 IU/kg, respectively.

2.2.1.5 Antibody titers

All birds were water-vaccinated with Newcastle disease virus **(NDV)** vaccine at 18 (B1B1 Mass/Con, Merial inc., Duluth, GA), 28 (B1B1 Mass/Con, Merial inc., Duluth, GA), and 98 d (AviPro, Elanco Animal Health, Greenfield, IN). Hens were vaccinated (Breedervac-Reo-Plus, Merck Animal Health Products, Madison, NJ) against NDV and infectious bursal disease virus **(IBD)** at 70 d of age though subcutaneous injection. Furthermore, both hens and roosters were vaccinated against IBD and NDV though subcutaneous injection (Breedervac-IV-Plus, Merck Animal Health Products, Madison, NJ) at 126 d. At 29 wk of age, randomly-selected breeder hens (n=16/ dietary treatment) were bled by brachial veinipuncture. The same birds were bled at 32, 47 and 57 wk of age, and plasma samples were sent to Poultry Health Services Ltd. (Airdrie, AB, Canada) for determination of NDV and IBD antibody titers using ELISA.

2.2.2 Statistical analysis

Data were tested for normality using Proc Univariate of SAS version 9.2 (SAS Institute, 2001) and then analyzed using Proc Mixed as a 3-way ANOVA (SAS Institute, 2001) for broiler breeder productivity (4 hen treatments x 2 rooster treatments x 10 breeder ages). The experimental unit was individual cage (n=72/hen treatment and n=30/rooster treatment). Total egg production, total settable eggs and hen-housed production data were analyzed as a 2-way ANOVA (4 hen treatments x 2 rooster treatments). Egg CXN and 25-OHD_3 contents, egg composition and antibody titers were analyzed as a 2-way ANOVA (4 hen treatment x 4 breeder

ages). Means were separated using LSMeans comparison and differences were considered significant when $P \leq 0.05$.

2.3 RESULTS

2.3.1 Breeder production

There were no differences between hen treatments on BW at any breeder ages (Figure 2.1). The BW of the hens were 2.5% to 5.7% lighter than the recommended BW (Aviagen, 2007a) from 26 to 32 wk of age, but followed the recommend BW (ranging from 1.3 % lighter to 1.6 % heavier compared to recommended BW) for all the other breeder ages. Hen and rooster mortality was 2.4% and 6.6%, respectively.

No hen or rooster treatment effects on total egg production, total settable egg or hen-housed production were found (Table 2.2). Embryos from CXN and HC hens had higher early embryonic mortality (P=0.004; Table 2.3) than those from the Control hen treatment. Early embryonic mortality decreased at 39 wk of age relative to 33 and 35 weeks of age and then remained the same for the rest of the breeder ages. There were no rooster treatment effects for early, mid, late, dead, total embryonic mortality or hatchability of fertile. No hen treatment effects for mid embryonic mortality were observed; however mid embryonic mortality decreased slightly at 53 wk of age, increased at 55 wk and decreased again at 56 wk of age (P=0.038). There were no hen treatment or breeder age effects on late embryonic mortality. The highest proportion of dead chicks at hatch was seen at 33 wk of age but there were no hen treatment effects. At 33 wk of age, Control hens inseminated by Control roosters had a higher proportion of cull chicks compared to when inseminated by HC roosters (P= 0.047; Figure 2.2). At 41 wk of age, CXN hens inseminated by Control roosters had a higher proportion of cull chicks compared to CXN hens inseminated by HC roosters. There was no interaction of rooster and hen treatments

at any other breeder ages. No hen treatment effects on total embryonic mortality were found but total embryonic mortality was highest at 33 wk of age. Fertility was similar between rooster treatments from 33 to 39, and from 43 to 45 wk of age ($P<0.001$; Figure 2.3). Fertility of Control roosters declined after 45 wk of age, and decreased further after 53 wk of age. However, HC roosters maintained a consistent level of fertility from 43 wk of age to the end of the trial. Fertility was not affected by hen treatments. Hatchability of fertile peaked at 43 wk of age, decreased at 47 wk of age and then slightly increased at 53 wk of age ($P<0.001$; Table 2.3). Hatchability of fertile was also not affected by hen treatments.

2.3.2 Egg composition

Eggs from HC and CXN hens had higher CXN contents than from Control and 25-OHD_3 hens ($P=0.001$; Figure 2.4). At 59 wk of age, HC and CXN hens had higher egg CXN contents than at other breeder ages. There was almost no CXN detected in the eggs from Control and 25-OHD_3 hens at any breeder age. There were no hen treatment ($P=0.478$) or breeder age ($P=0.214$) effects on whole egg 25-OHD_3 contents (data not shown). The analyzed 25-OHD_3 contents in whole egg from the Control, 25-OHD_3, CXN and HC hens were 133, 118, 95, and 135 ng/mg, respectively.

At 48 wk of age, Control and CXN hens had higher egg weights than HC hens ($P=0.013$; Table 2.4). At 58 wk of age, CXN hens had higher egg weights than the other hen treatments, but there were no hen treatment effects at other breeder ages. The albumin weight increased with increasing breeder ages ($P<0.001$), but there were no hen treatment effects. The CXN hens laid eggs with higher yolk weights than all other hen treatments at 58 wk of age ($P=0.003$). No hen treatment effects were observed for other ages. Hen treatment did not affect dry shell weight nor shell thickness, however there was increased dry shell weight at 48 and 58 wk of age compared to 27 and 37 wk of age. Shell thickness decreased with increasing breeder age.

2.3.3 Plasma antibody titers

No significant hen treatment effects on IBD antibody titers were found but NDV antibody titers were lower in the HC fed hens compared to all the other treatments (P=0.047). The IBD antibody titers decreased with increasing age until 47 wk of age and then increased at 57 wk of age (P<0.001; Table 2.5). Lower NDV antibody titers were seen at 59 wk of age compared to all the other breeder ages (P<0.0001).

2.4 DISCUSSION

2.4.1 Breeder production

No differences between hen treatments on total egg production, total settable eggs, hen housed egg production, mid, late or total embryonic mortality, fertility or hatchability of fertile were found. Vitamin D_3 contents in the CXN diet (1,584 IU/kg) and 25-OHD_3 contents in the 25-OHD_3 (2,017 IU/kg) and HC (1,892 IU/kg) diet were lower than expected (2,760 IU/kg). All treatment diets were above the NRC (1994) vitamin D activity requirement (300 IU/kg); hatchability was not affected when broiler breeders were fed 25-OHD_3 at 2,760 IU/kg, or vitamin D_3 at either 1,200 or 2,400 IU/kg (Coto et al., 2010). This indicates that 25-OHD_3 and vitamin D_3 contents at levels over 1,584 IU/kg may meet the nutritional requirements of broiler breeders, and therefore we did not see any hen treatment effects in breeder productivity. The lack of CXN effects on breeder productivity may be because breeders had adequate antioxidant contents other than CXN (50 IU/kg vitamin E; 0.3 mg/kg selenium); when breeders were provided with 6 mg/kg CXN, hatchability and fertility increased when other antioxidant contents were low (Rosa et al., 2012; 7.6 IU/kg vitamin E; 0.108 mg/kg selenium). This suggests that when other antioxidant contents such as vitamin E and selenium are low, CXN may provide antioxidant protection and increase breeder productivity. Breeders under high stocking density and high

oxidative stress may need antioxidant supplementation. This is because concentration of glutathione, an important antioxidant, in the liver and heart is lower under high stocking density as seen in broilers (Simitzis et al., 2012). Furthermore, breeder productivity increased when breeders were fed with CXN and were housed at high stocking density (Rosa et al., 2012; 0.117 m^2 per bird). Therefore, the lack of CXN effects on breeder productivity in the current study could also be because breeders were housed at low stocking density (stocking density of 0.216 m^2 per bird) and low oxidative stress.

Dietary CXN increased early embryonic mortality, however Rosa et al. (2012), found decreased early embryonic mortality in eggs from CXN-fed breeders. Most of the triglyceride in the egg yolk is oleic acid (C18:1; Noble and Cocchi, 1990), which is important for embryo survival and development (Tullet, 1990). Furthermore, a ratio of stearic acid (C18:0) to oleic acid greater than 0.25 decreased embryo membrane permeability and function (Noble and Cocchi, 1990), increases mortality and decreases hatchability (Tullet, 1990). Yolk is synthesized in the hen liver; Δ9 desaturase converts stearic acid to oleic acid (Noble and Cocchi, 1990). Antioxidants may decrease liver Δ9 desaturase activity and therefore, increase the ratio of stearic acid to oleic acid in the egg yolk (Hayat et al., 2009). In laying hens fed 3% canola oil, dietary vitamin E at 100 IU/kg resulted in a yolk stearic acid to oleic acid ratio of 0.186, whereas hens fed 12 IU/kg had a ratio of 0.163 (Mazalli et al., 2004). In the current study, canola oil was added at 1.70%, therefore de novo synthesis of fatty acids by the breeders would likely have been greater than in the previous study. Since the main fatty acid synthesized by the hen is oleic acid, low levels of dietary fat inclusion might result in increased oleic acid deposition (Gül et al., 2012). If CXN further increased yolk oleic acid and decreased stearic acid, the resultant increase

in the ratio of these fatty acids might explain the increase in embryonic mortality in this treatment group.

The HC rooster treatment maintained fertility longer than Control roosters after 47 wk of age, possibly due to the effects of CXN. Chicken sperm contain high levels of polyunsaturated fatty acid (52.1%; Surai et al., 1998a) and are therefore, susceptible to lipid peroxidation. Dietary vitamin E decreased lipid peroxidation in rooster sperm (Surai et al., 1998b) and increased the proportion of motile sperm (Cerolini et al., 2005). The antioxidant activity of CXN may have scavenged free radicals and therefore protecting the sperm from oxidative damage, and thus maintaining fertility in the HC roosters. Semen from older roosters has decreased glutathione peroxidase activity compared to semen from younger roosters (Kelso et al., 1996). Therefore, the antioxidant activity of CXN may have offered greater protection from peroxidative damage in the CXN roosters at the older ages.

The low hatchability observed in this experiment from young breeders under 35 wk of age was due to the high total embryonic mortality, specifically the early embryonic mortality. It could be due to the production of small eggs with thicker shells in young breeders, which have lower water vapor conductance and pore concentration compared to older breeders (Peebles and Brake, 1987). This may have limited embryo development and caused increased early embryonic mortality (Peebles and Brake, 1987).

The proportion of dead chicks at hatch was highest at 33 wk of breeder age relative to other ages. Embryo temperature is important for development and high embryo temperature can cause increased proportion of dead chicks (Ande and Wilson, 1981). Embryos from 29-wk-old breeders produce more heat than embryos from 40-wk-old breeders during incubation (Hamidu et al., 2007). Therefore, higher embryo heat production may have caused increased proportion of

dead chicks from young breeders. Embryos from breeders over 45 wk of age produced heat similar to 29-wk-old breeders (Hamidu et al., 2007), but overall the proportion of dead chicks at hatch after 45 wk of age in our study did not increase. Hence, increased the proportion of dead chicks at hatch from 33wk of breeder age may not have been due to high embryo heat production. Young breeders lay eggs with lower yolk fat contents and hence have reduced embryo yolk mobilization compared to older breeders (Noble et al., 1986; Yadgary et al., 2010). Therefore, embryos may not have been receiving sufficient energy and nutrients to hatch, resulting in increased dead chicks in young breeders.

2.4.2 Egg composition

Dietary CXN is absorbed in the intestine (Parker, 1996), delivered directly to the liver through portomicrons, and is deposited into the yolk (Krogdahl, 1985). Therefore, CXN was efficiently deposited into the egg when it was provided to the hens. Egg CXN content in breeders at 59 wks of age was higher compared to other breeder ages when fed HC and CXN. Feed intake was greater in breeders at 59 wk of age (137g/d/bird) compared to 25, 35 and 49 wk of age (121, 135 and 133 g/d/bird, respectively); hence, CXN intake was greater in 59-wk-old breeders (0.82 mg/d/bird) than 25-, 35- and 49-wk-old breeders (0.73, 0.81 and 0.79 mg/d/bird, respectively). Breeders at 59 wk of age did not lay as many eggs (52.4% production) compared to 35 and 49 wk of age (85.6% and 63.7% production, respectively) except for at 25 wk of age (12.6% production). Therefore, egg CXN contents in 59 wk of age was higher (0.008 mg/g) and efficiency of CXN transfer from the feed to the egg was greater (37%) compared to 25, 35, and 49 wk of age (4.34, 3.27 and 5.13 μg/g, respectively; 5, 21 and 28%, respectively).

Although the diets were formulated to contain 2,760 IU/kg of vitamin D activity, no hen treatment effects were found on whole egg 25-OHD_3 contents possibly because vitamin D activity was relatively close between different treatments (1,584 IU/kg to 2,655 IU/kg). Breeders fed 300 IU/kg or 2,400 IU/kg vitamin D_3 had greater differences between the egg yolk 25-OHD_3 contents (4 µg/kg and 13 µg/kg egg yolk, respectively) compared to when vitamin D_3 levels were fed at similar levels (1,200 IU/kg or 2,400 IU/kg vitamin D_3; 8 µg/kg and 13 µg/kg egg yolk, respectively; Coto et al., 2010). Therefore, vitamin D activity in a range between 1,584 IU/kg to 2,655 IU/kg did not affect the whole egg 25-OHD_3 content.

There were no hen treatment effects for egg shell thickness, possibly due to the high vitamin D activity (over 1,584 IU/kg); egg shell quality, measured as specific gravity, was not different between layers supplemented 25-OHD_3 or vitamin D_3 at 2,760 IU/kg (3.3% calcium; Keshavarz, 2003). However, higher egg shell thickness was reported in 62-wk-old laying hens fed 600 IU/kg of 25-OHD_3 compared to 600 IU/kg of vitamin D_3 (3.5% calcium; McLoughlin and Soares, 1976). Therefore, vitamin D activity over 1,584 IU/kg did not affect egg shell thickness in this study.

The reason for overall larger breeder eggs and egg components, measured as egg, albumin, yolk and shell weight, in older breeders is because older breeders are heavier (Summers and Leeson, 1983) and also have larger oviducts than young breeders (Joyner et al., 1987). The thinner shells in older breeders compared to young breeders are due to the shell glands; older breeders are inefficient at removing calcium from circulation and depositing it to the egg (Joyner et al., 1987). Furthermore, old hens produce larger eggs but deposit egg shell at the same rate as young hens (Roland, 1979), which may also explain why older breeders have thinner shells.

2.4.3 Plasma antibody titers

The lack of CXN hen treatment effect on IBD antibody titer may be due to high contents of antioxidant in all hen diets (50 IU/kg of vitamin E). Dietary vitamin E from 0 to 20 IU/kg increased breeder IBD antibody titers but over 40 IU/kg had no effect on IBD antibody titers (Lin and Change, 2006). Twenty-five hydroxyvitamin D_3 can be converted to 1,25-dihydroxyvitamin D_3 in helper T cells (CD4+; Kongsbak et al., 2014) and increase the expression of interleukin 4 (IL-4; Cantorna et al., 1998). Interleukin 4 promotes T helper 2 **(Th2)** cell development as well as B cell differentiation; most will become plasma cells that will produce antibodies (Kuhn et al., 1991). Furthermore, Th2 cells are more important for protection against IBD (Van den Berg et al., 1991). T helper 1 cells **(Th1)** involved in the cellular immune response are important for protection against NDV (Mariono and Hanson, 1987). Therefore, we expected increased IBD antibody titer from 25-OHD_3 hens and the reason for lack of 25-OHD_3 hen treatment effects on IBD antibody titers is unknown. The different hen antibody response between NDV and IBD could be due to their different effects on T cell function.

The reason for decreased IBD and NDV antibody titers with increasing breeder ages may be due to reduced helper T cell function as reported in old mice (Eaten et al., 2003), which will lead to reduced B cell function (Callard and Basten, 1978) and ultimately decreased antibody production.

In conclusion, breeder hens fed 25-OHD_3 or vitamin D_3 provided above the requirement levels as well as breeder hens provided adequate levels, of antioxidants were not affected in terms of breeder productivity, egg components and antibody titers. On the other hand, older roosters fed CXN and 25-OHD_3 had increased fertility relative to the Control roosters. Dietary antioxidant, specifically CXN may have a potential role in increasing fertility of old roosters,

possibly by increasing sperm quality. When breeder hens were fed CXN, it was efficiently transferred to the egg and therefore, it may increase the viability and performance of the chick early in life.

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2.6 TABLES

Table 2.1 Experimental diet ingredient and calculated nutrient composition.

Ingredient	% of diet (as fed)
Corn	36.46
Soybean meal	15.93
Wheat	34.87
Calcium carbonate	7.93
Dicalcium phosphate	1.29
Salt	0.37
L-lysine HCl	0.027
Methionine hydroxy analogue, 95%[1]	0.18
Vitamin D-free vitamin-mineral premix[2]	1.00
Canola oil	1.70
Avizyme 1302[3]	0.045
Canthaxanthin premix [4]	0.006
25-hydroxyvitamin D_3 premix [5]	0.05
Vitamin D_3 premix [6]	0.00055
Calculated Nutrient Profile	
ME (kcal/kg)	2,882
CP (%)	15.00
Calcium (%)	3.00
Available P (%)	0.37
Lysine (%)	0.73
Sodium (%)	0.19

[1]Novus International, Inc., Saint Charles, MO.

[2]Vitamin/mineral premix supplied the following per kg of feed: vitamin A, 12,500 IU; vitamin E 50 IU; vitamin K, 2.5 mg; niacin, 37.5 mg; D-pantothenic acid, 12.5 mg; riboflavin, 7.5 mg; pyridoxine, 5 mg; thiamine, 2.55 mg; folic acid, 0.9 mg; biotin, 0.15 mg; vitamin B_{12}, 0.019 mg; iron, 80 mg; zinc, 110 mg; manganese, 120 mg; copper, 20 mg; iodine, 2 mg; choline, 2.7 mg; selenium, 0.3 mg.

[3]Danisco Animal Nutrition, Marlborough, Wiltshire, United Kingdom.

[4]Canthaxanthin premix was supplemented as Carophyll-Red (DSM Nutritional Products Ltd., Parsippany, NJ) at 6 mg/kg feed in CXN and HC treatment. Analyzed CXN content in feed for Control, 25-OHD_3, CXN and HC diet was 0, 0, 6.91, and 6.72 mg/kg, respectively.

[5]25-hydroxyvitamin D_3 premix was supplemented as HyD (DSM Nutritional Products Ltd., Parsippany, NJ) at 69 µg/kg feed in 25-OHD_3 and HC treatment. Analyzed 25-OHD_3 content in feed for Control, 25-OHD_3, CXN and HC diet was 0, 50.44, 0, and 47.31 µg/kg, respectively.

[6]Vitamin D_3 premix was supplemented as Rovimix 500 (DSM Nutritional Products Ltd., Parsippany, NJ) at 2,760 IU /kg feed in Control and CXN treatment. Analyzed vitamin D_3 content in feed for Control, 25-OHD_3, CXN and HC diet was 2,655, 154, 1,584, and 264 IU/kg, respectively.

Table 2.2 Effects of hen diet and rooster diet on egg production from 24 to 58 wk of age.

Hen treatment	Rooster treatment	n	Total egg production	Total settable egg	Hen-housed production
			----------Number of eggs----------		----------%----------
Control[1]		72	175.82	168.64	68.15
25-OHD_3 [2]		72	177.93	170.88	68.97
CXN[3]		72	174.16	167.31	67.50
HC[4]		72	176.99	170.24	68.60
SEM			3.63	4.05	1.39
	Control[5]	144	175.01	167.44	67.83
	HC[6]	144	177.44	171.09	68.78
SEM			3.30	3.73	1.28
			----------Probabilities----------		
Hen treatment			0.657	0.703	0.657
Rooster treatment			0.277	0.123	0.277
Hen x Rooster			0.664	0.790	0.664

[1]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3/kg feed.
[2]25-OHD_3 hens were fed the Control diet plus 69 μg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.
[3]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[4]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[5]Control roosters were fed the same diet as the Control hens.
[6]HC roosters were fed the same diet as the HC hens.

Table 2.3 Effects of hen diet, rooster diet and breeder flock age on fertility, hatchability and embryonic mortality.

Hen treatment	Rooster treatment	Breeder age (wk)[1]	n	Early (0-7 d)	Middle (8-14 d)	Late (15-21 d)	Dead[2]	Cull[3]	Total embryonic mortality[4]	Fertility	Hatchability of fertile
				----------%----------							
Control[5]			720	3.30[c]	1.04	3.67	1.06	0.55	8.81	88.38	88.96
25-OHD$_3$ [6]			720	4.11[bc]	1.67	3.49	0.76	0.45	9.61	88.36	87.90
CXN[7]			720	5.18[ab]	1.39	4.21	1.01	0.65	11.44	87.80	85.83
HC[8]			720	5.69[a]	1.40	3.34	0.56	0.48	10.71	87.23	86.39
SEM			720	0.50	0.41	0.65	0.19	0.23	1.53	1.18	1.49
	Control[9]		1,440	4.66	1.38	3.96	0.73	0.60	10.37	86.38[b]	86.51
	HC[10]		1,440	4.48	1.37	3.39	0.96	0.46	9.91	89.50[a]	88.03
SEM				0.35	0.35	0.46	0.13	0.19	1.02	0.83	1.04
		33	288	6.51[ab]	1.50[abc]	4.20	2.57[a]	1.18[a]	14.44[a]	88.41[bc]	81.77[d]
		35	288	6.90[a]	2.05[a]	3.05	0.74[bc]	0.38[bcd]	12.42[ab]	92.42[a]	85.56[c]
		39	288	5.15[bc]	1.67[ab]	2.51	0.50[bc]	0.40[bcd]	9.48[cde]	93.83[a]	89.31[a]
		41	288	3.52[c]	1.50[abc]	3.56	0.77[bc]	0.58[bcd]	9.02[cde]	91.27[ab]	88.79[ab]
		43	288	3.55[c]	1.25[abc]	2.82	0.45[bc]	0.31[bcd]	7.74[e]	88.61[bc]	90.72[a]
		45	288	4.22[c]	1.27[abc]	3.44	0.73[bc]	0.21[d]	9.32[cde]	89.02[bc]	88.81[ab]
		47	288	4.11[c]	1.00[bc]	5.04	1.06[b]	0.75[abc]	10.93[bc]	86.47[cd]	85.40[c]
		53	288	3.77[c]	0.73[c]	3.71	0.31[c]	0.79[ab]	8.22[de]	84.08[de]	88.82[ab]
		55	288	4.17[c]	1.96[a]	4.24	0.56[bc]	0.47[bcd]	10.62[bcd]	82.01[e]	85.78[bc]
		56	288	3.79[c]	0.82[bc]	4.20	0.74[bc]	0.25[cd]	9.25[cde]	83.28[e]	87.75[abc]
SEM				0.64	0.45	0.63	0.26	0.26	0.96	1.00	1.29
				----------Probabilities----------							
Hen treatment				0.004	0.344	0.791	0.202	0.669	0.251	0.904	0.421
Rooster treatment				0.708	0.980	0.380	0.220	0.263	0.538	0.013	0.296
Breeder age				<0.001	0.038	0.059	<0.001	0.007	<0.001	<0.001	<0.001

Hen x Rooster	0.911	0.286	0.186	0.875	0.691	0.348	0.819	0.430
Hen x Breeder age	0.205	0.445	0.586	0.854	0.204	0.322	0.573	0.417
Rooster x Breeder age	0.120	0.629	0.348	0.950	0.568	0.550	<0.001	0.856
Hen x Rooster x Breeder age	0.328	0.231	0.114	0.526	0.047	0.084	0.621	0.651

[a-e]Means with no common letters within the same column are significantly different ($P<0.05$).

[1]Eggs collected during wk 32, 37, 49 and 58 were set at the University of Alberta for immune function assays on the resulting chicks

[2]Percent dead chicks of fertile eggs at hatch includes dead-in-shell embryos.

[3]Percent cull chicks of fertile eggs at hatch includes culls from weak chicks, chicks with physical abnormalities such as distorted beaks and weak limbs, and omphalitis.

[4]Percent total embryonic mortality of fertile eggs includes embryonic mortality from 0 to 21 d.

[5]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.

[6]25-OHD_3 hens were fed the Control diet plus 69 μg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[7]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[8]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[9]Control roosters were fed the same diet as the Control hens.

[10]HC roosters were fed the same diet as the HC hens.

Table 2.4 Effects of hen diet and breeder age on breeder egg composition.

Hen treatment	Breeder age (wk)	n	Egg weight (g)	Albumin (g)	Yolk (g)	Dry shell weight (g)	Shell thickness (mm)
Control[1]		288	64.24	35.61	20.07	5.36	0.34
25-OHD_3[2]		288	63.45	34.74	20.03	5.45	0.35
CXN[3]		288	65.10	35.73	20.50	5.42	0.33
HC[4]		288	62.34	34.26	19.78	5.34	0.34
SEM			0.89	0.70	0.29	0.12	0.005
	27	288	53.88^{d}	31.15^{d}	15.11^{d}	5.17^{b}	0.37^{a}
	37	288	62.86^{c}	35.04^{c}	19.66^{c}	5.18^{b}	0.34^{b}
	48	288	68.04^{b}	36.57^{b}	22.18^{b}	5.63^{a}	0.33^{c}
	58	288	70.34^{a}	37.58^{a}	23.44^{a}	5.59^{a}	0.32^{c}
SEM			0.54	0.42	0.20	0.07	0.004
Control	27	72	54.79^{g}	31.88	15.21^{e}	5.17	0.36
Control	37	72	63.80^{ef}	35.59	19.92^{d}	5.29	0.34
Control	48	72	69.05^{bc}	37.64	22.31^{bc}	5.59	0.32
Control	58	72	69.33^{bc}	37.34	22.86^{bc}	5.40	0.32
25-OHD_3	27	72	53.67^{g}	30.53	15.02^{e}	5.15	0.38
25-OHD_3	37	72	62.50^{f}	34.66	19.71^{d}	5.19	0.34
25-OHD_3	48	72	67.40^{cd}	36.08	22.26^{bc}	5.70	0.34
25-OHD_3	58	72	70.24^{b}	37.70	23.14^{b}	5.75	0.33
CXN	27	72	54.05^{g}	31.65	15.21^{e}	5.16	0.36
CXN	37	72	63.00^{f}	35.42	19.43^{d}	5.04	0.34
CXN	48	72	69.71^{bc}	37.14	22.35^{bc}	5.81	0.33
CXN	58	72	73.64^{a}	38.73	25.00^{a}	5.68	0.31
HC	27	72	53.03^{g}	30.55	15.00^{e}	5.20	0.37
HC	37	72	62.14^{f}	34.51	19.58^{d}	5.22	0.34
HC	48	72	66.02^{de}	35.42	21.79^{c}	5.41	0.32
HC	58	72	68.17^{bc}	36.57	22.76^{b}	5.54	0.33
SEM			1.10	0.83	0.40	0.15	0.007
			----------Probabilities----------				
Hen treatment			0.162	0.386	0.391	0.911	0.251
Breeder age			<0.001	<0.001	<0.001	<0.001	<0.001
Hen x Breeder age			0.013	0.686	0.003	0.069	0.862

[a-g]Means with no common letters within the same column are significantly different (P<0.05).

[1]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.

[2]25-OHD_3 hens were fed the Control diet plus 69 μg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[3]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[4]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

Table 2.5 Main effects and interaction of hen diet and breeder age on infectious bursal disease virus (IBD) and Newcastle disease virus (NDV) plasma antibody titer in broiler breeder.

Hen treatment	Breeder age (wk)	n	IBD antibody titer	NDV antibody titer
Control[1]		72	15,253	8,902[a]
25-OHD_3 [2]		72	16,585	9,109[a]
CXN[3]		72	15,606	9,398[a]
HC[4]		72	15,451	6,620[b]
SEM			554	735
	29	288	16,625[a]	8,944[a]
	32	288	15,625[b]	8,880[a]
	47	288	14,904[c]	8,683[a]
	57	288	15,741[b]	7,523[b]
SEM			313	403
			----------Probabilities----------	
Hen treatment			0.349	0.047
Breeder age			<0.001	<0.001
Hen x Breeder age			0.678	0.813

[a-c]Means with no common letters within the same column are significantly different (P<0.05).
[1]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.
[2]25-OHD_3 hens were fed the Control diet plus 69 μg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.
[3]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[4]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

2.7 FIGURES

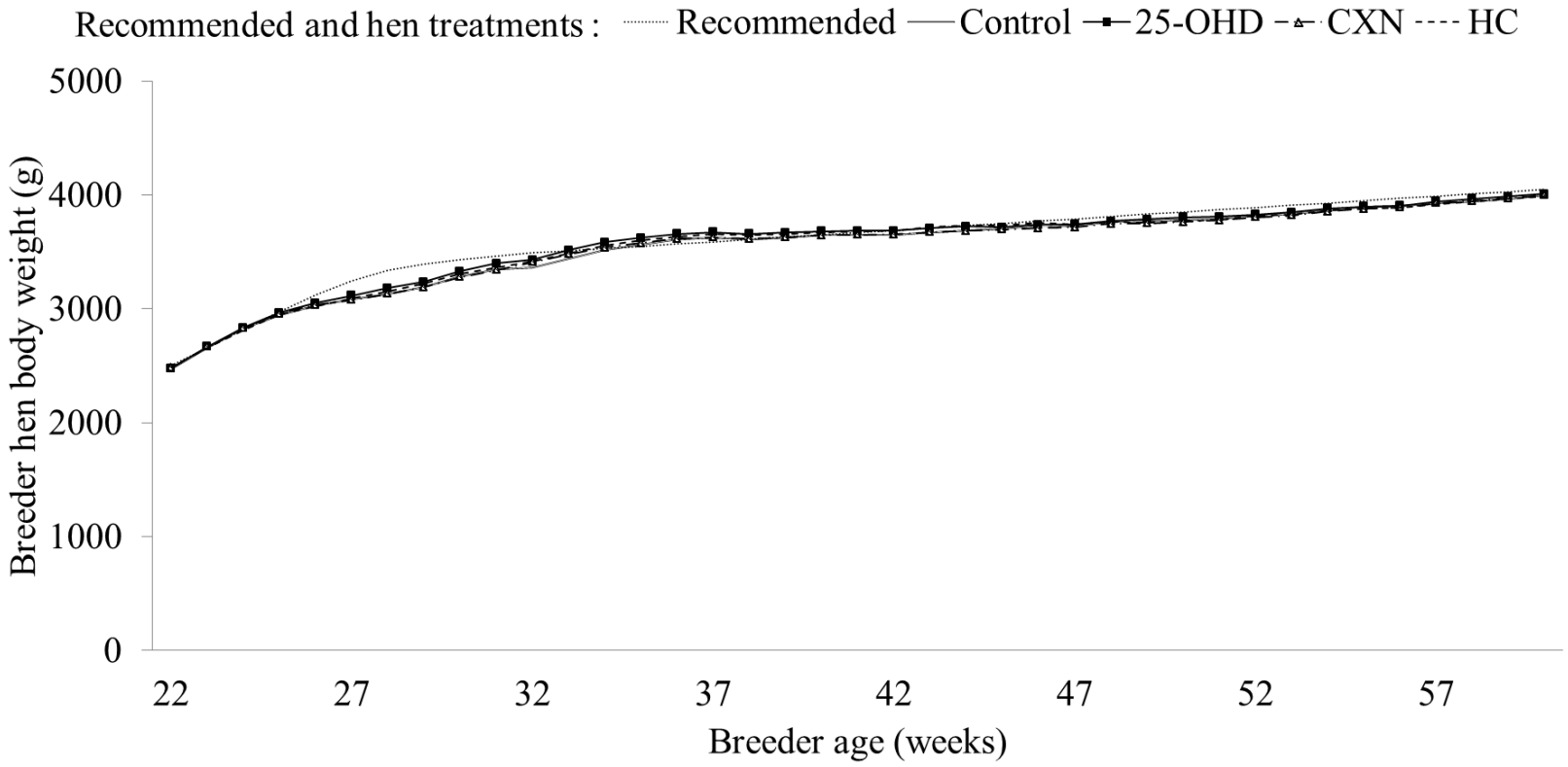

Figure 2.1 Breeder hen body weight from 22 to 60 weeks (P=0.807; CV=10.3862%). Recommended body weight based on Aviagen guideline (Aviagen 2007a). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental Vitamin D_3. 25-OHD_3 hens were fed the basal diet with 2,760 IU 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd.,

Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). Control Roosters were fed the same diet as the Control hens. HC roosters were fed the same diet as the HC hens.

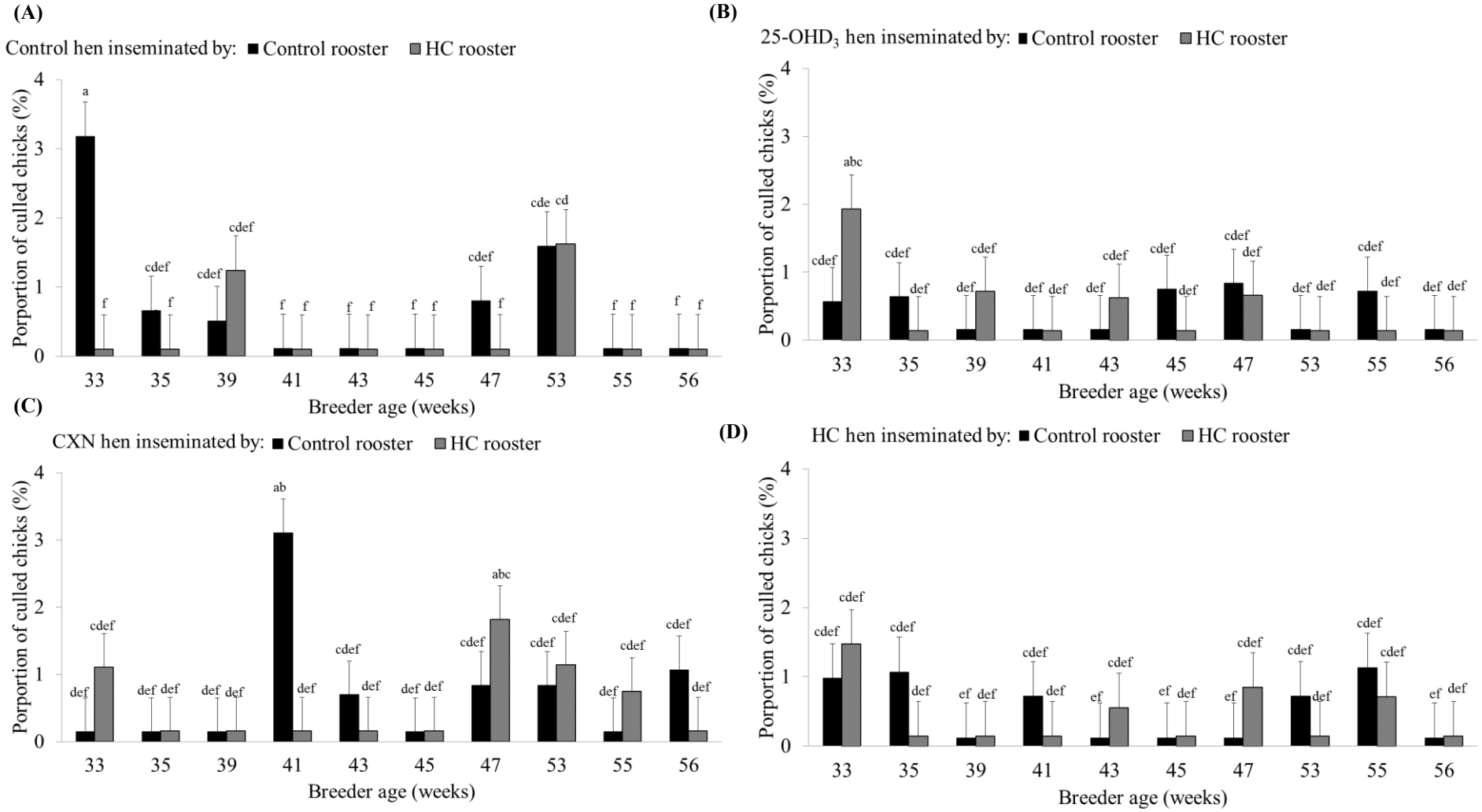

Figure 2.2 Significant three-way interaction between breeder age, rooster treatment and hen treatment on proportion of culled chicks

(P=0.047; n=36). (A) Control, (B) 25-OHD_3, (C) CXN, and (D) HC hen treatment with different rooster treatments and breeder ages. Means with no common letters are significantly different ($P<0.05$). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 2,760 IU 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). Control roosters were fed the same diet as the Control hens. HC roosters were fed the same diet as the HC hens.

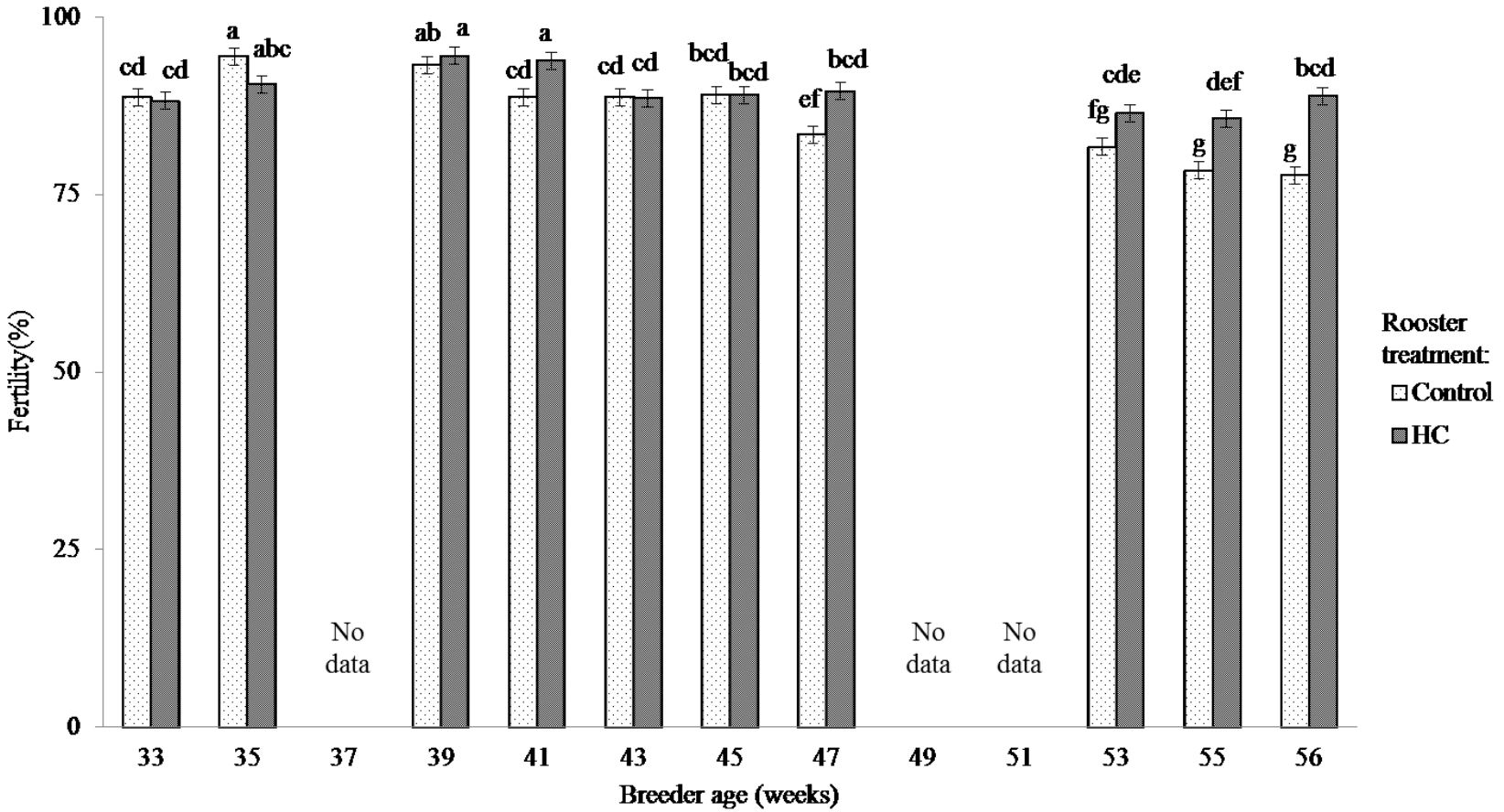

Figure 2.3 The interaction between breeder age and rooster treatment for fertility ($P<0.001$; n=144). Means with no common letters are significantly different ($P<0.05$). Control roosters were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. HC roosters were fed with 2,760 IU 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3 plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

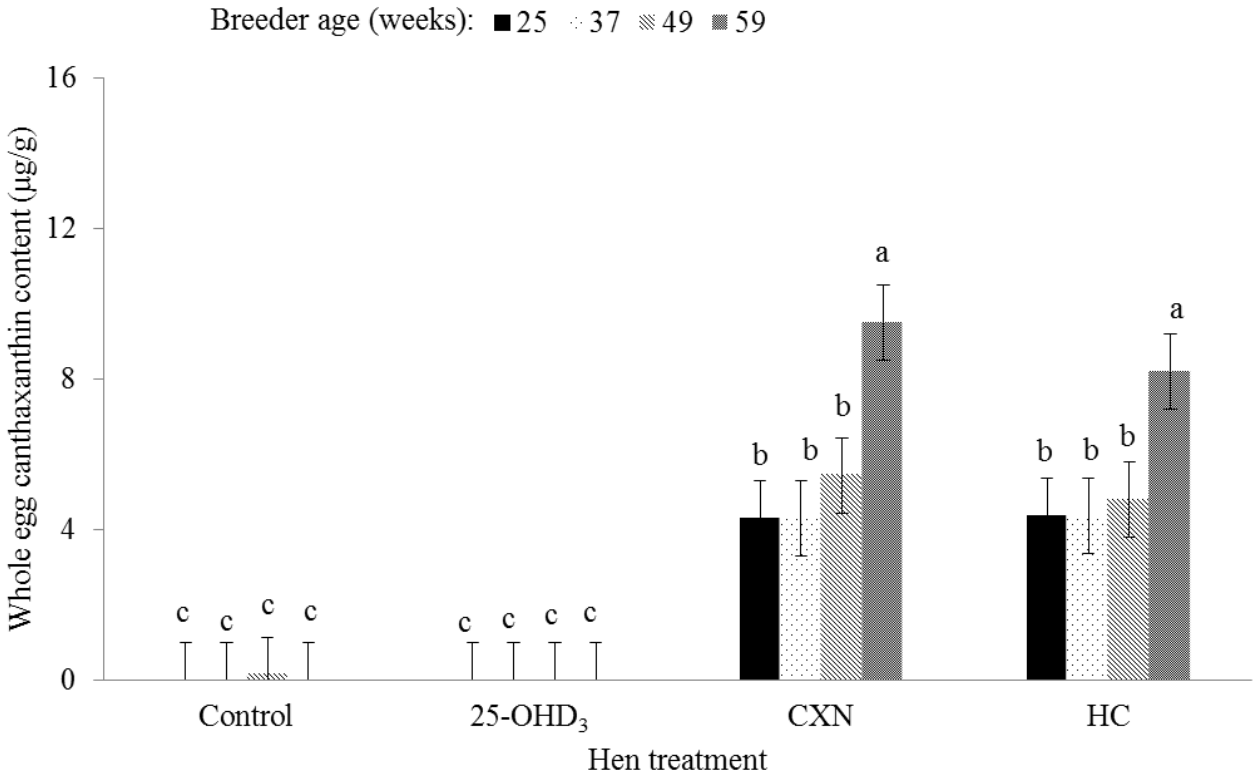

Figure 2.4 Interaction between breeder age and hen treatment on whole egg CXN content (P=0.015; n=72). Means with no common letters are significantly different (P<0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the Control diet plus 2,760 IU 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

3. THE EFFECT OF PARENTAL DIETARY CANTHAXANTHIN AND 25-HYDROXYVITAMIN D_3 ON PERFORMANCE OF BROILER CHICKENS

ABSTRACT

In previous studies, maternal dietary canthaxanthin **(CXN)** and 25-hydroxyvitamin D_3 **(25-OHD_3)** were efficiently transferred to the egg and increased broiler growth and performance. The objective was to investigate the effects of parental dietary CXN and 25-OHD_3 on broiler performance. Individually-caged Ross 308 hens were fed diets as either 1) Control (Vitamin D_3 at 2,760 IU/kg feed; no CXN), 2) CXN (Control with 6 mg/kg CXN) 3) 25-OHD_3 (Control with 25-OHD_3 at 2,760 IU/kg feed replacing vitamin D_3) and 4) HC (CXN diet with 25-OHD_3 at 2,760 IU/kg feed replacing vitamin D_3). Individually-caged Ross 544 males were fed either the Control or the HC diet. Hens were inseminated weekly, and hatching eggs collected at 25, 37, 49 and 59 wk of age. The resulting chicks were allocated to one of 32 pens based on parental treatments and were fed a standard commercial broiler diet containing vitamin D_3 as the sole source of vitamin D activity and no CXN. Broiler BW **(**0, 10, 24 and 42 d of age), feed intake (**FI**; 0 to 10 d, 11 to 24 d, 25 to 42 d, 0 to 42 d) and feed conversion ratio (**FCR**; 0 to 10 d, 11 to 24 d, 25 to 42 d, 0 to 42 d) were determined. Broilers were processed at 43 d of age. Broiler femur mineral density and cross-sectional area at 30% and 50% of femur length was measured at 14, 28 and 42 d of age. Maternal treatment did not affect broiler BW at 42 d of age but FI from 0 to 42 d of age was lower in chicks from maternal 25-OHD_3 than Control and CXN treatments. Broiler FCR was lowest from maternal 25-OHD_3 compared to other maternal treatments. Broiler trabecular density decreased from maternal CXN and HC compared to maternal Control and 25-OHD_3 at 50% femur length. There were only small effects of parental dietary treatment on broiler performance traits, possibly because broilers were fed a diet without 25-OHD_3 and CXN.

Key words: canthaxanthin, 25-hydroxyvitamin D_3, broiler breeder, broiler performance, bone quality

3.1 INTRODUCTION

Canthaxanthin **(CXN)** and 25-hydroxyvitamin D_3 **(25-OHD$_3$)** are commercially-available dietary supplements used by the poultry industry. Broiler intestinal absorption of 25-OHD_3 is higher than vitamin D_3 (Bar et al., 1980) and 25-OHD_3 can directly go to the kidney to be converted to the active form of vitamin D, 1,25-dihydroxyvitamin D_3. Therefore, 25-OHD_3-fed broilers can have higher BW, tibia bone ash and breast meat yield compared to vitamin D_3-fed chickens (Yarger et al., 1995; Fritts and Waldroup, 2003). Breeder hens fed 25-OHD_3 can effectively transfer and deposit 25-OHD_3 into the egg (Coto et al., 2010). Broilers fed 25-OHD_3 had greater villus length and crypt depth than broilers fed vitamin D_3 (Chou et al., 2009). Therefore, broilers from 25-OHD_3-fed breeder hens may have increased intestinal nutrient absorption, and this may ultimately result in greater BW compared to broilers from hens not fed with 25-OHD_3. Calcium absorption from the eggshell is induced by 1,25-dihydroxyvitamin D_3 (Tuan and Scott, 1977) and therefore, broilers from 25-OHD_3-fed breeder hens may have greater bone quality than broilers from breeder hens fed without dietary 25-OHD_3.

Canthaxanthin is an antioxidant and a fat-soluble carotenoid pigment (Kull and Pfander, 1995) that is widely used for pigmentation of animal products such as egg yolk and broiler skin (Koutsos et al., 2003). Lutein contains oxygen in its structure and is an antioxidant similar to CXN (Terao, 1989). Broilers fed lutein had increased BW at 21 d of age compared to broilers not fed lutein (Koutsos et al., 2006). Low antioxidant status can increase reactive oxygen species in the chicken mitochondria and damage lipid, DNA and protein (Akbarian et al., 2016). Therefore, this can reduce energy generation and ultimately decrease chicken BW (Akbarian et al., 2016).

Since CXN can be efficiently deposited in the egg when incorporated in the hen's feed (Koutsos et al., 2003; Surai et al., 2003; Zhang et al., 2011), broilers hatched from CXN-fed hens may be capable of increased growth rate.

Few studies have investigated the transfer of CXN and 25-OHD_3 from the hen to the chick and subsequent effects on broiler performance. Therefore, the objective of this research was to investigate the effects of parental dietary CXN and 25-OHD_3 on broiler performance. We hypothesized that CXN and 25-OHD_3 fed to the breeder hens are transferred to the egg and therefore, it would increase BW and decreased feed conversion ratio (**FCR**) in the broiler chickens hatched from hens fed CXN and 25-OHD_3. Increased bone quality and meat yield in broilers hatched from 25-OHD_3-fed hens would be observed. Rooster treatment would not affect broiler performance traits, meat yield and bone quality because roosters do not transfer nutrients to the egg.

3.2 MATERIALS AND METHODS

3.2.1 Experimental diets and animals

Experimental protocols were approved by the Animal Care and Use Committee: Livestock of the University of Alberta and all chickens were cared for according to the guidelines of the Canadian Council of Animal Care (2009). Individually-caged broiler breeders (n=288 Ross 308 breeder hens; n=60 Ross 544 roosters) were fed experimental diets from 22 to 60 wk of age. The 4 experimental hen diets were Control (vitamin D_3 at 2,760 IU/kg feed), 25-OHD_3 (vitamin D_3 replaced with 25-OHD_3 at 2,760 IU/kg feed), CXN (Control diet with CXN added at 6 mg/kg feed), HC (25-OHD_3 diet with CXN added at 6 mg/kg feed). Individually-caged roosters (n=30/treatment) were fed either the Control or the HC hen diet. All diets met or exceeded the primary breeder nutrient recommendations (Aviagen, 2007c). Each week, the hens were

artificially inseminated with 0.5 mL of pooled semen from one of the two rooster treatment groups. Each hen was mated with pooled semen from only one rooster treatment group throughout the trial (n=30/rooster treatment of pooled semen). From each hen, fertile eggs were identified and collected at 25, 37, 49 and 59 wk of age. Fertile eggs were then stored in a cooler (16 to 18°C at 70 to 80% relative humidity) for up to one week and were incubated (37.5°C and 56% relative humidity) and hatched at University of Alberta (Edmonton, Canada). The resulting chicks were feather-sexed.

The birds were allocated to one of 32 pens (232 x 549 cm; 7 to16 chicks/pen, depending on the number of chicks that hatched) based on parental dietary treatments (4 replications/parental treatment). Each pen had softwood shavings litter, a hanging tube feeder and nipple drinkers; the temperature, lighting program and light intensity followed the management guide (Aviagen, 2007a). All broilers were fed a starter diet (0 to 14 d; 3,067 ME kcal/kg, 23% CP, 4,000 IU/kg vitamin D_3, 10,000 IU/kg vitamin A, and 35 IU/kg vitamin E), a grower diet (15 to 28 d; 3,152 ME kcal/kg, 20% CP, 4,000 IU/kg vitamin D_3, 10,000 IU/kg vitamin A, and 35 IU/kg vitamin E) and a finisher diet (15 to 42 d; 3,196 ME kcal/kg,19% CP, 4,000 IU/kg vitamin D_3, 10,000 IU/kg vitamin A, and 35 IU/kg vitamin E). Each diet met or exceeded the broiler nutrition recommendations (Aviagen, 2007b). Broiler BW, feed intake **(FI)**, and FCR were measured at 0, 10, 25 and 42 d. Between 244 to 324 broilers (61 to 81 birds/dietary treatment) were processed at 43 d of age for each of the hatches. Approximately 12 hours before processing broilers, feed and water were withdrawn. The broilers were processed by electrically stunning the birds and then bleeding the birds for 2 min. After bleeding, the birds were scalded for 45 s (52 to 53°C). Carcasses were then mechanically defeathered, manually eviscerated, and portion yields were measured. Carcass and portion weights were determined by weighing the individual carcass,

pectoralis major, pectoralis minor, wings, and drums. Total breast meat weight was calculated by adding pectoralis major and pectoralis minor together. Carcass yield and yield for each carcass component was calculated as mentioned below.

Carcass yield (%) = (carcass weight / live BW) × 100.

Each carcass component yield (%) = (carcass component weight/eviscerated carcass weight) × 100.

3.2.1.1 Broiler femur mineral density and cross-sectional area

Broiler femur bone mineral density (mg/cm^3) and cross-sectional area (mm^2) were determined using Quantitative Computed Tomography with a Stratec Norland XCT Research scanner (Norland Medical Systems Inc., Fort Atkinson, WI) as described by Jendral et al. (2008), and Saunders-Blades and Korver (2015). The right femur of broilers hatched from 25, 37, 49 and 60 wk old breeders were collected at 14, 28 and 42 d (n=32/breeder age). The bone mineral density and cross-sectional area at 30% and 50% of femur total length from the proximal epiphysis was measured. Bone mineral density was multiplied by the cross-sectional area to calculate the bone mineral content in a 1-mm linear section scanned in the bone as outlined by Saunders-Blades et al. (2009).

3.2.2 Statistical analysis

Data were tested for normality using Proc Univariate of SAS version 9.2 (SAS Institute, 2001) and then analyzed using Proc Mixed as a 3-way ANOVA (SAS Institute, 2001) for broiler performance, and carcass trait (4 maternal treatments x 2 rooster treatments x 4 breeder ages); 4-way ANOVA for bone mineral density and cross-sectional area (4 maternal treatments x 2 rooster treatments x 4 breeder ages x 4 broiler ages). The experimental unit was the broiler pen

(4 replication/parental treatment). Least-squares means of comparison was used for mean separation and differences were considered significant when $P < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Broiler performance

Chicks from Control hens inseminated by HC roosters had heavier 0 d BW than chicks from 25-OHD_3 hen inseminated by HC roosters (Table 3.1). Higher 0 d BW was found in chicks from Control hens inseminated by HC roosters compared to chicks from Control hens inseminated by Control roosters (Table 3.1). This was due to a heavier egg weight from Control hens inseminated by HC roosters (65.57 g ± 0.46) than eggs from Control hens inseminated by Control roosters (62.56 g ± 0.46). Egg weight is correlated to hatching chick weight (Pinchasov, 1991); chicks from heavier eggs have heavier hatching weight than chicks from light eggs. No other parental treatment effect on 0 d BW was found. At 37 wk of breeder age, 25-OHD_3 hens inseminated by Control roosters had 10-d-old chick BW greater than those from hens inseminated by HC roosters (Figure 3.1). At 49 wk of breeder age, the opposite effect was seen. Rooster treatment had no effect on 10 d chick BW for any other hen treatment group, at any breeder age. No hen treatment effects on 24 d BW were found but chicks from hens inseminated by Control roosters had higher BW than chicks from HC roosters at 24 d of age. Parental dietary effects were not observed on broiler BW at 42 d of age ($P=0.066$).

As expected, broiler BW increased at 0, 10, 24 and 42 d of age with increasing breeder age because older breeders laid heavier eggs (Table 3.1; Chapter 2). Older breeders tend to be heavier (Summers and Leeson, 1983) and have a larger preovulatory follicle and oviduct than young breeders (Joyner et al., 1987) and therefore, laid heavier eggs. Since chick weight at hatch is greater from heavier eggs (Pinchasov, 1991), chicks from older breeders were heavier at hatch

than from young breeders (Ulmer-Franco et al., 2010). Furthermore, heavier chicks at hatch can have greater final BW because these chicks have greater muscle mass with increased satellite cell proliferative activities compared to lighter weight chicks at hatch (Sklan et al., 2003). Broiler BW may have been also greater with increasing breeder age because broiler feed consumption was higher in chicks from older breeder ages.

Broiler FI was not affected by paternal dietary treatments from 0 to 24 d (Table 3.2). From 25 to 42 d of age, chicks from CXN hens had higher FI than those from 25-OHD_3 hens at 25 wk of breeder age (Figure 3.2); and FI was greater for chicks from CXN and Control hens compared to 25-OHD_3 and HC hens at 59 wk of breeder age. No differences due to the hen treatments at 37 and 49 wk of breeder age were seen. From 25 to 42 d, FI increased in chicks from hens inseminated by HC roosters compared to those inseminated by the Control roosters at 37 wk of breeder age (Figure 3.3). The opposite was seen at 59 wk of breeder age; FI was higher in chicks from Control roosters compared to those from HC roosters. No differences were seen between the rooster treatments in FI at 25 and 49 wk of age. The increased FI in chicks hatched from hens inseminated by HC roosters at 37 wk of age resulted in decreased broiler FCR from 25 to 42 d from Control and HC hens inseminated by HC roosters compared to Control roosters. Broilers from the Control and CXN hens had higher FI than 25-OHD_3 hens from 0 to 42 d of age (Table 3.2). Broiler FI for all chick ages increased with increasing breeder age.

Similar to FI, broiler FCR was unaffected by parental treatments from 0 to 24 d of age (Table 3.3). From 25 to 42 d, 25-OHD_3 hens inseminated by Control roosters had chicks with decreased broiler FCR compared to Control and HC hens inseminated by Control roosters. No hen treatment effects on broiler 42 d BW was observed, but broiler FI from 0 to 42 d of age decreased from 25-OHD_3 maternal treatment compared to CXN and Control maternal treatment.

This resulted in broilers from CXN hens having higher FCR than 25-OHD_3 hens. Putrescine, a polyamine, is important in the development, growth and function of the small intestine (Grant et al., 1990). The active form of vitamin D, 1,25-dihydroxyvitamin D_3, can increase the activity of enzymes that produce putrescine, and increase the intestinal development (Takahashi et al., 1982; Shinki, 1985). The decreased small intestine weight observed in broilers fed 25-OHD_3 may lead to reduced energy requirement relative to birds fed vitamin D_3 (Chou et al., 2009); 25-OHD_3-fed broilers also had greater villus length and crypt depth than vitamin D_3-fed broilers, which may increase nutrient absorption in the intestine (Chou et al., 2009). Since no maternal treatment effects on the whole egg 25-OHD_3 contents were found (Chapter 2) and the broilers were not fed with 25-OHD_3, it is unlikely that the 25-OHD_3 effect on FI and FCR would come from changes in intestinal morphology. Epigenetics is the study of heritable changes in gene expression not due to changes in the gene sequence. The maternal 25-OHD_3 effect in our study could be due to epigenetic mechanisms such as DNA methylation. The DNA methylation is the addition of methyl groups to a DNA strand to modify DNA expression (Fetahu et al., 2014); DNA methylation is common on unmethylated CpG islands, which is a DNA region that has cytosine and guanine located next to each other (Fetahu et al., 2014). The peptide transporter 1 (**PepT1**) is located in the chicken intestinal epithelial cell and is associated with absorption and transportation of peptides (Mott et al., 2008). The PepT1 gene is regulated by transcription factors such as caudal-related homeobox 2 (Shimakura et al., 2006). Maternal dietary methyl donor supplementation increased methylation on PepT1 promotor CpG islands and PepT1 expression in jejunum of the new born pigs compared to pigs without maternal methyl donor supplementation (Mou et al., 2017). The PepT1 expression increased when methylation occurred on PepT1 promotor CpG islands because methylation-sensitive transcription factors attached to

methylated PepT1 CpG regions and induced PepT1 gene transcription and expression (Mou et al., 2017). Breeders fed dietary 25-OHD_3 had increased plasma 25-OHD_3 content (Coto et al., 2010); plasma 25-OHD_3 content is positively correlated with vitamin D receptor gene methylation in humans (Beckett et al., 2016). We do not know if plasma 25-OHD_3 content is positively correlated with methylation of PepT1 or other nutrient transporters such as glucose transporter gene in chickens but if it did, 25-OHD_3-fed breeder hens might increase plasma 25-OHD_3 and therefore increase methylation of PepT1 or other nutrient transporter promotor CpG islands. That could pass onto the broiler chick and increase PepT1 or other nutrient transporter expression in the broiler intestine. This may result in increased nutrient absorption and ultimately reduced broiler FI and FCR from 25-OHD_3 hens.

From 25 to 42 d FCR of broilers from Control and HC hens inseminated by Control roosters were higher than of those from Control and HC hens inseminated by HC roosters. No other differences were observed due to hen or rooster treatments. At 0 to 10 d of age, greater broiler FCR at 25 wk of breeder age compared to other breeder ages was found. At 11 to 24 d of age, increased broiler FCR at 59 wk of breeder age compared to other ages were observed. At 25 to 42 d of age, broilers from 37 and 49 wk of breeder age had greater FCR than from 25 and 59 wk of breeder ages but FCR were not affected by breeder age from 0 to 42 d of age.

The overall lack of maternal diet effects on broiler BW may be due to the absence of 25-OHD_3 and CXN in the broiler feed; BW increased when 25-OHD_3 (Yarger et al., 1995; Fritts and Waldroup, 2003) or marigold flower extract (containing lutein; Rajput et al., 2012) was fed to broilers consistently. Similar to CXN, lutein is a xanthophyll, a carotenoid that contains oxygen in its structure, and is also an antioxidant (Terao, 1989). Therefore, if CXN has the same effect as lutein, we would have expected increased BW when CXN was fed to broilers. The absence of

a maternal 25-OHD_3 effect on broiler BW maybe due to the lack of maternal dietary treatment effect on the amount of 25-OHD_3 in the egg (Chapter 2). Glutathione is an important antioxidant in the body; reduced state glutathione can donate an electron to reactive oxygen species to neutralize them (Quintana-Cabrera and Bolaños, 2012). Broilers housed under high stocking density (16 birds/m^2) decreased glutathione concentration in the heart and spleen compared to broilers housed under low stocking density (10 birds/m^2; Sun et al., 2013). Overproduction of reactive oxygen species in the chicken mitochondria due to low glutathione and other antioxidants can damage lipid, DNA and protein in the mitochondria (Akbarian et al., 2016). This would lead to reduced ATP synthesis and energy generation efficiency (Akbarian et al., 2016), and ultimately may result in decreased BW. Excess reactive oxygen species in the body can damage intestinal cells and cause impairment of intestinal tissues (Wang et al., 2008). This could lead to reduced nutrient digestibility in broiler chickens (Wang et al., 2008). Hence, low antioxidant protection would result in decreased BW gain and FCR in broilers as seen in broilers under high stocking density compared to broilers housed under low stocking density (Simitzis et al., 2012). Since CXN is an antioxidant, CXN may maintain broiler growth when glutathione concentration is low in the chicken's body. Broilers were reared in pens under low stocking density in this study (9.4 birds/m^2) and therefore, antioxidant mechanisms were not likely impaired. Adding more antioxidant or CXN into the system that is not lacking antioxidant protection would not be expected to affect broiler BW.

3.3.2 Broiler carcass traits

Chicks from hens inseminated by Control roosters had increased absolute broiler carcass and wing weight compared to those from HC roosters (Table 3.4). There were no other parental treatment effects on carcass or wing weights. Pectoralis major weight was increased in chicks

from HC hens inseminated by Control roosters compared to those from HC hens inseminated by HC roosters. No other interactions of parental treatments on pectoralis major weight were observed. Parental effects were not seen in pectoralis minor and total breast weight. Broiler drum weight of chicks from CXN and HC hens inseminated by Control roosters increased compared to those inseminated by HC roosters. No other parental effects were found on broiler drum weight. Carcass, total breast and drum weights increased with increasing breeder age. Additionally, a nearly significant breeder age effect was found in pectoralis minor weight (P=0.061), which showed the same pattern as carcass, total breast and drum weight. Higher wing weight was seen at older breeder ages (49 and 59 wk of age) compared to younger ages (25 and 37 wk of age). Breeder age did not affect pectoralis major weight. The overall increase of carcass and portion weight in broilers with increasing breeder age was expected because older breeder hens lay larger eggs than young breeders (Chapter 2) and therefore have heavier chicks with greater final BW (Pinchasov, 1991; Sklan et al., 2003).

Parental treatment did not affect broiler carcass and portion yield except for drum yield. Broilers from CXN hens inseminated by HC roosters had decreased drum yield, and numerically higher breast yields relative to those from CXN hens inseminated by Control roosters (Table 3.5). A large proportion of the male broiler chicks were sampled shortly after hatch for use in innate immune functional assays (Chapter 4). As a result, there were 107 female and only 41 males from CXN hens inseminated by HC roosters, but 85 females and 45 males from CXN hens inseminated by Control roosters. The skewing of male-female distribution may explain the difference in yield, since female broilers have increased breast yield and decreased drum yield relative to male broilers when broilers were processed between 37 and 51 d of age (Young et al., 2001).

Similar to the broiler performance results, the lack of maternal treatment effects on processing traits could be because whole egg 25-OHD_3 contents were not different across the hen treatments (Chapter 2). Broiler breast meat yield was not affected when either 25-OHD_3 or vitamin D_3 were fed at the same vitamin D activity (2,760 IU/kg; Lozano, 2014). Furthermore, broilers fed different levels of vitamin D_3 above 2,500 IU/kg had similar broiler breast meat yield (Khan et al., 2010). Therefore, the lack of maternal effect on broiler breast meat yield in the current study could be because broilers were fed vitamin D activity over 2,500 IU/kg (vitamin D_3 fed at 4,000 IU/kg) and whole egg 25-OHD_3 contents were not different between the different maternal treatments. Fetuses from gilts fed diets supplemented with 25-OHD_3 (500 IU/kg vitamin D_3 plus 2,000 IU/kg 25-OHD_3) had increased skeletal muscle fibre number, myoblast number and myoblast proliferation compared to fetuses from gilts fed only vitamin D_3 (2,500 IU/kg vitamin D_3; Hines et al., 2013). Currently, no study had looked at the effect of maternal dietary 25-OHD_3 on meat yield in broiler chickens. Egg 25-OHD_3 contents from 25-OHD_3-fed hens have been reported to be greater than that from vitamin D_3-fed hens (Coto et al., 2010). One-α-hydroxylase is present in the skeletal muscle and hence, 25-OHD_3 can be converted to 1,25-dihydroxyvitamin D_3 (Srikuea et al., 2012). One, twenty-five-dihydroxyvitamin D_3 can increase myogenic regulatory factor expression such as antimyoblast determination and myogenic factor 5 during embryonic development (Garcia et al., 2011; Mok et al., 2015). It is unknown whether greater 25-OHD_3 supply to the chick embryo would increase 1,25-dihydroxyvitamin D_3 production and therefore, increase myogenic regulatory factor expression and skeletal muscle fibre number during embryonic development. However, if it did, an increase in egg 25-OHD_3 could result in greater broiler skeletal muscle fibre number and meat yield compared to broilers from breeders fed vitamin D_3.

Carcass yield was lowest from chicks hatched from hens at 49 wk of age compared to all the other ages. The pectoralis major and total breast meat yield was lowest at 37 wk of age compared to 49 and 59 wk of age. There was a nearly significant male treatment effect (P=0.052), in which chicks from hens inseminated by HC roosters had higher pectoralis major yield compared to Control roosters. The highest broiler drum yield was seen at 59 wk, and the lowest was seen at 37 wk of breeder age. Broilers from breeders at 49 wk of age had the highest wing meat yield and the lowest was seen at 37 wk of breeder age. Overall, the breeder age effects on broiler meat yield showed inconsistent patterns and the reason for this is unclear.

3.3.3 Broiler femur mineral density and cross-sectional area

Both trabecular and cortical bone are important for bone strength and integrity in chickens (Passi and Gefen, 2005; Dacke et al., 2015). There is more trabecular bone at 30% of bone length than at the mid-point, and a greater amount of cortical bone is found at the midpoint than at 30% of bone length (Passi and Gefen, 2005; Dacke et al., 2015). Trabecular cross-sectional area was greater at 30% of femur length in male broilers at 19 d of age when fed combination of 25-OHD_3 and CXN compared to when fed 25-OHD_3 and CXN seperately or fed solely vitamin D_3 (Lozano, 2014). However, this effect was not observed at 50% femur length in male broilers at 19 d of age (Lozano, 2014). Hence, bone mineral density and cross-sectional area at 30% and 50% of femur total length from the proximal epiphysis were measured in this study because parental treatment, and broiler age may affect the two regions of the bone differently. The femur was used in this study, since the femur may be more sensitive to dietary changes than the tibia. Femur had reduced bone mineral density at proximal and distal epiphysis when fed low dietary phosphorous while tibia bone mineral density did not change when fed the same low dietary phosphorous (Moran and Todd, 1994).

No maternal treatment effects on femur bone mineral density, cross-sectional area nor bone mineral content at 30% of bone length were found except for trabecular area (Table 3.6). At 49 wk of breeder age, chicks from HC hens inseminated by HC roosters had high trabecular cross sectional area than HC hens inseminated by Control roosters (P=0.036; Figure 3.4). No other parental treatment and breeder age interaction were found for trabecular cross-sectional area. Fourteen- and 42-d-old broilers from hens inseminated by Control roosters had increased trabecular cross-sectional area compared to HC roosters but no rooster treatment effect was seen at 28 d of age (Table 3.6). Total cross-sectional area increased in broilers from hens inseminated by HC roosters compared to Control roosters. At 49 and 59 wk of age, broiler trabecular cross-sectional area increased from hens inseminated by HC roosters compared to Control roosters. No differences between paternal treatments were found at 25 and 37 wk of age. Broiler trabecular cross-sectional area increased in chicks from hens inseminated by HC roosters compared to Control roosters at 42 d age, but no paternal treatment effect was found in 14 or 28 d of age. No other parental treatment effect was observed in femur bone mineral density, cross-sectional area nor bone mineral content.

Total and trabecular mineral density at 30% of femur length decreased with increasing broiler age at all breeder ages. The opposite pattern was seen for cortical mineral density; cortical mineral density increased with increasing broiler age at all breeder ages. Total cross-sectional area increased at 28 d of age and then decreased at 42 d of age (P<0.0001). Cortical cross-sectional area decreased with increasing broiler age (P<0.0001). Breeder age did not affect total nor cortical cross-sectional area. Trabecular cross-sectional area increased with increasing broiler age at 25 and 59 wk of age (P=0.047). At 37 and 49 wk of age, trabecular area increased up to 28 d of age and then remained the same until 42 d of age. Total and trabecular bone mineral content

decreased after 28 d of age at 25, 37, 49 wk of age. At 59 wk of age, total and trabecular bone mineral content decreased with decreasing broiler age.

Parental treatment affected femur total mineral density, trabecular mineral density and trabecular bone mineral content at 50% of bone length but no other parental treatment effects were observed on mineral density, cross-sectional area and bone mineral content (Table 3.7). At 49 wk of breeder age, broiler from 25-OHD_3 hens inseminated by Control roosters had higher total mineral density compared to CXN and HC hens inseminated by Control roosters (P=0.023; Figure 3.5). Broilers from 25-OHD_3 and HC hens had increased total mineral density at 59 wk of breeder age compared to Control hens inseminated by HC roosters. No other parental treatment and breeder age effects were found on total mineral density. Fourteen-d-old broiler trabecular mineral density from CXN hens decreased compared to other hen treatments at 25 wk of age (P=0.005; Figure 3.6). At 37 wk, broiler chicks from CXN hens had lower trabecular mineral density than Control and HC hens. No hen treatment, breeder age nor broiler age effects were seen at 49 wk of age but at 59 wk of age, 25-OHD_3 and HC hens had higher trabecular mineral density than Control hens. Increased 28 d trabecular mineral density from Control and 25-OHD_3 hens compared to CXN and HC hens was observed at 37 wk of age. Broiler trabecular mineral density was higher from 25-OHD_3, CXN and Control hens than HC hens at 59 wk of age. No treatment, breeder age and broiler age effects were found at 25 and 49 wk of age. Forty-two d trabecular mineral density decreased from HC hens compared to other hen treatments at 25 wk of age. At 49 wk of age, broiler from Control and 25-OHD_3 hens had higher trabecular mineral density compared to HC hens. At 59 wk of age, broilers from Control and 25-OHD_3 hens had greater trabecular mineral density than CXN hens and there were no differences between hen treatments, broiler nor breeder ages were seen on trabecular mineral density at 37 wk of age.

Trabecular bone mineral content was higher from 25-OHD_3 hens compared to other treatments inseminated by Control roosters at 49 wk of age (P=0.007; Figure 3.7). Broilers from Control hens inseminated by HC roosters had higher trabecular bone mineral content than 25-OHD_3 and CXN hens inseminated by HC roosters at 49 wk of age. At 59 wk of age, broilers from 25-OHD_3 and HC hens had higher trabecular bone mineral content compared to Control hen inseminated by HC roosters. Broiler trabecular bone mineral content also increased from HC hens inseminated by HC roosters compared to Control roosters at 59 wk of age. There were no parental treatment nor breeder age effects at 25 and 37 wk of age. The 42 d trabecular bone mineral content decreased from 25-OHD_3 and HC hens compared to CXN hens at 49 wk of age (P=0.005; Figure 3.8). At 59 wk of age, 42 d broiler trabecular bone mineral content increased from HC hens than other hen treatments. No other hen treatment, broiler age and breeder age effects were found.

Overall, reduced total mineral density at 49 wk of age and trabecular density at all broiler ages from CXN and HC hens compared to Control and 25-OHD_3 hens were found at 50% femur length. Broiler chicks hatched from breeder hens fed CXN at 6 mg/kg had greater α-tocopherol in the liver and plasma at 1 d of age than breeder hens not fed with CXN (Surai et al., 2003). The antioxidant activity of CXN may have spared vitamin E, ultimately leading to increased liver and plasma vitamin E concentration (Surai et al., 2003). Broilers fed high dietary vitamin E (5,000 IU/kg) and low dietary of vitamin D_3 (500 IU/kg) had decreased tibia bone ash at 16 d of age compared to broilers fed low dietary vitamin E (10 IU/kg) and vitamin D_3 (500 IU/kg; Aburto and Britton, 1998). Vitamin E and D were fed at industry-relevant levels in the current study (vitamin E at 50 IU/kg and vitamin D activity at 2,760 IU/kg for broiler breeder; vitamin E at 35 IU/kg and vitamin D activity at 4,000 IU/kg for broilers). Therefore, vitamin E coming from the

maternal CXN and HC diets resulting in an interference with vitamin D absorption and subsequently reducing broiler tibia bone ash is unlikely. Osteoclasts break down and resorb bone mineral (Yagi et al., 2005). Osteoclasts can fuse and form a large cell; this osteoclast fusion stimulates reabsorption in the bone (Yagi et al., 2005). Osteoclasts can fuse and form a large cell; this osteoclast fusion stimulates reabsorption in the bone (Yagi et al., 2005). *In vitro* α-tocopherol at 20 μM stimulates osteoclast fusion by increasing the dendritic cell-specific transmembrane protein expression, which is important for the osteoclast fusion process, and hence α-tocopherol can decrease bone density (Yagi et al., 2005; Fujita et al., 2012). If CXN increased α-tocopherol content in the chick's body and stimulated osteoclast fusion in the bone, it may explain why chicks from CXN and HC hens had decreased femur bone density.

Generally, maternal 25-OHD_3 effects on broiler femur mineral density, cross-sectional area, and bone mineral content were not found. Similar to the broiler growth performance, the overall lack of 25-OHD_3 effects on broiler mineral density and cross-sectional area in our study could be due to the lack of breeder hen treatment effects on egg 25-OHD_3 contents (Chapter 2). Increasing *in ovo* inection of 25-OHD_3 from 0 to 48 IU/kg tended to increase 21 d broiler tibia ash (P=0.06; Bello et al., 2014). Although egg 25-OHD_3 content was not reported by Bello et al. (2014), higher embryo plasma 25-OHD_3 (33.8 ng/mL) was observed when 25-OHD_3 at 24 IU/kg was injected into breeder eggs *in ovo* compared to that of diluent-injected control (13.7 ng/mL; Bello et al., 2013). In breeder hens fed 3,000 IU/kg vitamin D_3, supplementation of 34.5 μg 25-OHD_3/L of water did not affect broiler femur mineral density and cross-sectional area at 41 d of age (Saunders-Blades and Korver, 2014). In the current study, broiler plasma 25-OHD_3 was not measured. However, if similar egg 25-OHD_3 contents between different treatments resulted in similar broiler plasma 25-OHD_3, then it may explain lack of 25-OHD_3 effects on broiler mineral

density and cross-sectional area. Furthermore, broilers were not fed 25-OHD_3 in this study. Tibia ash increased when broilers were fed dietary 25-OHD_3 compared to vitamin D_3 (Fritts and Waldroup, 2003). The breeders and the broilers in our study were fed vitamin D activity contents (2,760 IU/kg and 4,000 IU/kg, respectively) well above the NRC requirement (300 IU/kg for breeder and 200 IU/kg for broiler chickens; 1994). No differences in broiler bone quality between maternal dietary 25-OHD_3 and vitamin D_3 were found when Cobb breeder hens were fed vitamin D activity over 3,000 IU/kg (breeders fed 3,000 IU/kg vitamin D_3 or 3,000 IU/kg vitamin D_3 supplemented with 34.5 μg 25-OHD_3/L of water) and broilers were fed vitamin D activity at 2,500 IU/kg (Saunders-Blades and Korver, 2014).

Total femur cross-sectional area was higher from hens inseminated by HC roosters compared to Control roosters at 30% bone length. At 50% bone length, trabecular bone mineral content increased from HC hens inseminated by HC roosters compared to Control roosters at 49 wk of age. The CYP27B gene, which is important for synthesis of 1α-hydroxylase, can be expressed in human sperm (Jensen et al., 2010). Since the sperm cell membrane is mainly composed of polyunsaturated fatty acids (Surai et al., 1998), it is susceptible to lipid peroxidation. Reactive electrophiles, such as 4-oxo-2-nonenal **(ONE)**, are produced by lipid peroxidation (Galligan et al., 2014). This ONE can react with lysine contained in the histone and form an irreversible adduct and ultimately causing disruption of gene transcription and chromosome replication (Galligan et al., 2014). If CYP27B gene transcription is disrupted by irreversible adduct, this may decrease 1,25-dihydroxyvitamin D_3 production in the broiler chicks and decrease bone mineral density and cross-sectional area. Canthaxanthin is an antioxidant and therefore it may have prevented the lipid peroxidation in the sperm and reduced ONE production; ultimately maintaining CYP27B gene expression and 1,25-dihydroxyvitamin D_3

production in the broiler chick. Hypermethylation of CYP27B1 promoter CpG islands were found in human breast cancer cells (Shi et al., 2002). Oxidative stress increases DNA damage and genome instability which can result in cancer initiation and progression (Visconti and Grieco, 2009). Therefore, if hypermethylation on CYP27B1 promoter CpG islands occurred due to lipid peroxidation in the sperm, this may lead to the transcriptional inactivation of the CYP27B1 gene. This may reduce 1,25-dihydroxyvitamin D_3 production and decrease bone mineral density and cross-sectional area in the broiler chicks. Canthaxanthin may have reduced lipid peroxidation in the sperm and decrease methylation on CYP27B CpG islands and increased 1,25-dihydroxyvitamin D_3 production and bone mineral density and cross-sectional area in the broiler chicks. However, if 1,25-dihydroxyvitamin D_3 production increased due to epigenetic alternations in the sperm, we might expect to observe increased broiler performance and meat yield traits in chicks from hens inseminated by HC roosters compared to Control roosters. We overall did not observe parental dietary treatment effect on broiler performance and meat yield traits in this study, hence increased 1,25-dihydroxyvitamin D_3 production from epigenetic alternations in the sperm is not likely the cause of increased mineral density and cross-sectional area. The cause of the increased bone mineral density and cross-sectional area in chicks from hens inseminated by HC roosters compared to Control roosters is unknown.

Total mineral density decreased with increasing broiler age at 50% of femur bone length, and the opposite pattern was seen for cortical mineral density. Cortical mineral density decreased with increasing broiler age (Table 3.7). Cortical mineral density remained the same up to 37 wk of age and then increased at 49 wk of age and decreased at 59 wk of age. Total and cortical cross-sectional area decreased after 28 d of age; trabecular cross-section area increased after 28

d. Total and cortical bone mineral content remained the same until 28 d of age and then decreased until 42 d of age for all breeder ages.

Total and trabecular bone mineral content decreased with increasing broiler age at 30% and 50% of femur length. This could be because femur density and area is the highest between 14 and 28 d of age; tibia bone ash (%) increased and was the highest at 20 d of age and then gradually decreased until 43 d of age (Rath et al., 2000). This could be explained by increased transfer of minerals such as calcium to the bone tissue in the broiler up to 21 d of age (Barreiro et al., 2011).

Generally, broiler total mineral density, area and bone mineral content did not change with breeder age at 30% and 50% of femur length. Day-old broilers from 32- to 35-wk-old breeders had lower tibia mineral density compared to those from 56- to 48-wk old breeders (Yalcin et al., 2001). Furthermore, broiler tibia strength was greater from breeders at 65 wk of age relative to 25 wk of age (Shaw et al., 2010). Therefore, we expected increased bone mineral density with increasing breeder age and the reason for lack of breeder age effect is unknown.

Chicks from breeder hens fed 25-OHD_3 had lower FI and therefore lower FCR than chicks from CXN and Control hens. This could be due to dietary 25-OHD_3 induced epigenetic change such as DNA methylation and histone modification. Overall, small or no differences between the parental treatments in broiler performance, carcass traits, and bone mineral density and cross-sectional area were found. The broiler diets did not include CXN and 25-OHD_3 and hen treatments did not affect egg 25-OHD_3 contents.

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3.5 TABLES

Table 3.1 Effects of hen diet, rooster diet and broiler chick age on broiler chick BW.

Hen treatment	Rooster treatment	Breeder age (wk)	n	Broiler BW(g)			
				0 d	10 d	24 d	42 d
Control[1]			32	44.6	280.2	1,309.8	3,250.4
25-OHD_3[2]			32	44.2	280.4	1,293.8	3,194.2
CXN[3]			32	44.5	284.1	1,308.1	3,200.8
HC[4]			32	44.1	281.4	1,317.9	3,185.8
SEM				0.58	4.60	10.63	39.71
	Control[5]		64	44.2	282.0	1,322.1 ^{a}	3,213.9
	HC[6]		64	44.5	281.1	1,292.7 ^{b}	3,201.8
SEM				0.55	4.29	7.52	35.87
		25	32	38.8^{c}	247.9^{d}	1,195.0^{c}	3,110.9^{c}
		37	32	43.6^{b}	276.8^{c}	1,305.3^{b}	3,118.3^{c}
		49	32	47.2^{a}	292.9^{b}	1,356.2^{a}	3,243.8^{b}
		59	32	47.9^{a}	308.5^{a}	1,373.0^{a}	3,358.3^{a}
SEM				0.57	4.50	10.63	38.93
Control	Control		16	44.0^{b}	280.3	1,322.3	3,218.5
Control	HC		16	45.2^{a}	280.0	1,297.3	3,282.4
25-OHD_3	Control		16	44.6^{ab}	279.6	1,297.2	3,208.3
25-OHD_3	HC		16	43.8^{b}	281.3	1,290.3	3,180.2
CXN	Control		16	44.4^{ab}	288.6	1,324.7	3,231.5
CXN	HC		16	44.6^{ab}	279.6	1,291.5	3,170.2
HC	Control		16	43.6^{b}	279.3	1,344.3	3,197.4
HC	HC		16	44.6^{ab}	283.4	1,291.5	3,174.3
SEM				0.65	5.25	15.03	45.95
				Probability			
Hen				0.543	0.626	0.447	0.234
Rooster				0.139	0.696	0.007	0.617
Breeder age				<0.001	<0.001	<0.001	<0.001
Hen x Rooster				0.029	0.249	0.496	0.305
Hen x Breeder age				0.743	0.026	0.131	0.066
Rooster x Breeder age				0.602	0.588	0.835	0.232
Hen x Rooster x Breeder age				0.719	0.039	0.369	0.335

[a,b,c]Means with no common letters within the same column are significantly different (P<0.05).

[1]Control hens were fed a nutritionally complete basal diet containing 2,760 IU of supplemental vitamin D_3.

[2]25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[3]CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[4]HC hens were fed the 25-OHD_3 diet and 6 mg CXN in per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[5]Control roosters were fed the same diet as the Control hens.

[6]HC roosters were fed the same diet as the HC hens.

Table 3.2 Effects of hen diet, rooster diet and broiler chick age on broiler chick average daily feed inatke.

Hen treatment	Rooster treatment	Breeder age (wk)	n	Average daily feed intake (g/bird)			
				0-10 d	11-24 d	25-42 d	0-42 d
Control[1]			32	28.40	108.28	206.10[a]	123.41[a]
25-OHD_3[2]			32	25.87	103.86	197.69[c]	118.68[c]
CXN[3]			32	28.72	108.57	204.21[ab]	123.04[ab]
HC[4]			32	28.82	108.37	199.60[bc]	121.38[bc]
SEM				0.33	1.52	4.06	2.03
	Control[5]		64	28.79	108.08	201.50	121.10
	HC[6]		64	28.46	106.46	202.30	122.16
SEM				0.23	1.07	3.83	1.87
		25	32	27.54[c]	99.72 [c]	189.03[d]	116.25[c]
		37	32	27.50[c]	105.20 [b]	196.90[c]	119.63[b]
		49	32	28.95[b]	107.80 [b]	202.79[b]	122.29[b]
		59	32	30.50[a]	116.35 [a]	218.88[a]	128.34[a]
SEM				0.33	1.52	3.96	1.98
				Probability			
Hen				0.815	0.087	0.008	0.012
Rooster				0.318	0.289	0.675	0.331
Breeder age				<0.001	<0.001	<0.001	<0.001
Hen x Rooster				0.470	0.973	0.252	0.298
Hen x Breeder age				0.553	0.518	0.026	0.072
Rooster x Breeder age				0.201	0.631	0.005	0.492
Hen x Rooster x Breeder age				0.188	0.792	0.761	0.829

[a,b,c,d]Means with no common letters within the same column are significantly different ($P<0.05$).

[1]Control hens were fed a nutritionally complete basal diet containing 2,760 IU of supplemental vitamin D_3.

[2]25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[3]CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[4]HC hens were fed the 25-OHD_3 diet and 6 mg CXN in per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[5]Control roosters were fed the same diet as the Control hens.

[6]HC roosters were fed the same diet as the HC hens.

Table 3.3 Effects of hen diet, rooster diet and broiler chick age on broiler chick feed conversion ratio.

Hen treatment	Rooster treatment	Breeder age (wk)	n	Feed conversion ratio (g feed/g gain)			
				0-10 d	11-24 d	25-42 d	0-42 d
Control[1]			32	1.22	1.56	1.84	1.63[ab]
25-OHD_3[2]			32	1.22	1.52	1.82	1.59[b]
CXN[3]			32	1.22	1.57	1.84	1.65[a]
HC[4]			32	1.24	1.56	1.86	1.63[ab]
SEM				0.02	0.03	0.04	0.01
	Control[5]		64	1.23	1.55	1.86	1.62
	HC[6]		64	1.22	1.56	1.83	1.64
SEM				0.01	0.02	0.03	0.01
		25	32	1.34[a]	1.49[b]	1.80[b]	1.60
		37	32	1.19[b]	1.55[b]	1.88[a]	1.65
		49	32	1.19[b]	1.53[b]	1.88[a]	1.62
		59	32	1.18[b]	1.65[a]	1.79[b]	1.64
SEM				0.02	0.03	0.04	0.01
Control	Control		16	1.23	1.56	1.88[ab]	1.64
Control	HC		16	1.21	1.57	1.80[c]	1.61
25-OHD_3	Control		16	1.23	1.52	1.79[c]	1.56
25-OHD_3	HC		16	1.22	1.52	1.84[abc]	1.63
CXN	Control		16	1.22	1.58	1.82[bc]	1.64
CXN	HC		16	1.22	1.57	1.86[abc]	1.66
HC	Control		16	1.26	1.54	1.90[a]	1.63
HC	HC		16	1.22	1.58	1.81[bc]	1.64
SEM				0.02	0.04	0.04	0.02
				Probability			
Hen				0.718	0.430	0.613	0.037
Rooster				0.355	0.709	0.227	0.175
Breeder age				<0.001	<0.001	<0.001	0.072
Hen x Rooster				0.856	0.917	0.016	0.069
Hen x Breeder age				0.052	0.139	0.439	0.404
Rooster x Breeder age				0.393	0.479	0.671	0.922
Hen x Rooster x Breeder age				0.577	0.761	0.397	0.533

[a,b,c,d]Means with no common letters within the same column are significantly different (P<0.05).

[1]Control hens were fed a nutritionally complete basal diet containing 2,760 IU of supplemental vitamin D_3.

[2]25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[3]CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[4]HC hens were fed the 25-OHD_3 diet and 6 mg CXN in per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[5]Control roosters were fed the same diet as the Control hens.
[6]HC roosters were fed the same diet as the HC hens.

Table 3.4 Effects of hen diet, rooster diet and breeder age on carcass and portion (absolute weight) of broilers.

Hen treatment	Rooster treatment	Breeder age (wk)	n	Carcass	Pectoralis major	Pectoralis minor	Total breast	Drums	Wings
				g					
Control[1]			32	2,176.18	554.93	120.52	674.90	679.79	234.58
25-OHD_3[2]			32	2,163.33	551.95	121.77	673.22	675.04	234.69
CXN[3]			32	2,181.98	555.36	121.94	676.74	680.49	233.74
HC[4]			32	2,171.35	550.84	119.61	669.91	675.51	231.44
SEM				144.57	16.60	1.67	17.29	60.86	19.04
	Control[5]		64	2,190.68^{a}	557.26	120.92	677.61	687.27^{a}	236.56^{a}
	HC[6]		64	2,155.74^{b}	549.28	121.00	669.78	668.15^{b}	230.67^{b}
SEM				143.92	16.28	1.25	16.90	60.79	18.98
		25	32	2,111.5^{c}	536.53	118.18	654.19^{c}	660.86^{c}	228.15^{b}
		37	32	2,162.32^{b}	544.28	121.64	665.39^{bc}	658.70^{c}	225.09^{b}
		49	32	2,161.95^{b}	551.42	119.44	670.30^{b}	676.66^{b}	241.07^{a}
		59	32	2,257.08^{a}	580.85	124.58	704.90^{a}	714.60^{a}	240.14^{a}
SEM				144.25	16.61	1.55	18.15	60.84	19.04
Control	Control		16	2,177.89	554.20^{a}	120.30	673.93	678.22^{ab}	235.00
Control	HC		16	2,174.48	555.66^{a}	120.74	675.87	681.36^{ab}	234.16
25-OHD_3	Control		16	2,174.12	551.98^{ab}	121.94	673.41	682.69^{ab}	238.59
25-OHD_3	HC		16	2,152.55	551.92^{ab}	121.60	673.03	667.40^{bc}	230.79
CXN	Control		16	2,199.84	555.71^{a}	121.11	676.31	695.27^{a}	237.92
CXN	HC		16	2,164.12	555.00^{a}	122.77	677.18	665.70^{bc}	229.55
HC	Control		16	2,210.89	567.15^{a}	120.32	686.77	692.89^{a}	234.72
HC	HC		16	2,131.82	534.53^{b}	118.90	653.05	658.13^{c}	228.16
SEM				146.20	17.18	2.46	17.08	61.05	19.18

	Probability					
Hen	0.831	0.880	0.742	0.848	0.765	0.730
Rooster	0.016	0.089	0.959	0.163	<0.001	0.010
Breeder age	<0.001	<0.001	0.062	<0.001	<0.001	<0.001
Hen x Rooster	0.342	0.039	0.932	0.093	0.015	0.615
Hen x Breeder age	0.848	0.894	0.592	0.901	0.340	0.445
Rooster x Breeder age	0.550	0.175	0.234	0.110	0.179	0.539
Hen x Rooster x Breeder age	0.153	0.406	0.918	0.633	0.378	0.306

[a,b,c]Means with no common letters within the same column are significantly different ($P<0.05$).

[1]Control hens were fed a nutritionally complete basal diet containing 2,760 IU of supplemental vitamin D_3.

[2]25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[3]CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[4]HC hens were fed the 25-OHD_3 diet and 6 mg CXN in per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[5]Control roosters were fed the same diet as the Control hens.

[6]HC roosters were fed the same diet as the HC hens.

Table 3.5 Effects of hen diet, rooster diet and breeder age on carcass (% of live weight) and portion yield (% of carcass weight) of broilers.

Hen treatment	Rooster treatment	Breeder age (wk)	n	Carcass[7]	Pectoralis major[8]	Pectoralis minor[8]	Total breast[8]	Drums[8]	Wings[8]
				----------%----------					
Control[1]			32	65.88	25.48	5.59	31.04	31.31	10.76
25-OHD_3[2]			32	66.29	25.52	5.65	31.14	31.32	10.82
CXN[3]			32	66.18	25.45	5.64	31.06	31.27	10.67
HC[4]			32	66.42	25.39	5.56	30.92	31.32	10.66
SEM				0.52	1.00	0.37	1.36	0.82	0.18
	Control[5]		64	66.26	25.44	5.55	30.96	31.49 ^{a}	10.75
	HC[6]		64	66.13	25.48	5.67	31.12	31.11 ^{b}	10.70
SEM				0.49	1.00	0.37	1.36	0.81	0.17
		25	32	66.34^{a}	25.41^{ab}	5.65	31.03^{ab}	31.38^{b}	10.79^{b}
		37	32	66.53^{a}	25.12^{b}	5.64	30.73^{b}	30.59^{c}	10.39^{c}
		49	32	65.26^{b}	25.57^{a}	5.59	31.13^{a}	31.49^{ab}	11.11^{a}
		59	32	66.65^{a}	25.74^{a}	5.56	31.27^{a}	31.76^{a}	10.61^{b}
SEM				0.52	1.00	0.37	1.36	0.82	0.18
Control	Control		16	65.76	25.43	5.58	30.98	31.21^{abc}	10.75
Control	HC		16	66.00	25.53	5.61	31.10	31.40^{ab}	10.76
25-OHD_3	Control		16	66.49	25.42	5.61	30.99	31.56^{ab}	10.91
25-OHD_3	HC		16	66.09	25.62	5.70	31.29	31.08^{bc}	10.73
CXN	Control		16	66.09	25.27	5.56	30.79	31.70^{a}	10.75
CXN	HC		16	66.27	25.63	5.73	31.33	30.84^{c}	10.59
HC	Control		16	66.70	25.64	5.48	31.08	31.49^{ab}	10.57
HC	HC		16	66.14	25.15	5.64	30.77	31.14^{bc}	10.74
SEM				0.58	1.00	0.38	1.37	0.83	0.19
				----------Probability----------					
Hen				0.418	0.914	0.649	0.717	0.993	0.244

Table 3.5 Effects of hen diet, rooster diet and breeder age on carcass (% of live weight) and portion yield (% of carcass weight) of broilers.

Hen treatment	Rooster treatment	Breeder age (wk)	n	Carcass[7]	Pectoralis major[8]	Pectoralis minor[8]	Total breast[8]	Drums[8]	Wings[8]
Rooster				0.578	0.717	0.052	0.227	0.003	0.507
Breeder age				<0.001	0.002	0.666	0.024	<0.001	<0.001
Hen x Rooster				0.535	0.087	0.802	0.169	0.029	0.195
Hen x Breeder age				0.099	0.644	0.156	0.378	0.004	0.681
Rooster x Breeder age				0.450	0.156	0.284	0.152	0.545	0.277
Hen x Rooster x Breeder age				0.562	0.381	0.751	0.440	0.145	0.957

[a,b,c]Means with no common letters within the same column are significantly different ($P<0.05$).
[1]Control hens were fed a nutritionally complete basal diet containing 2,760 IU of supplemental vitamin D_3.
[2]25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.
[3]CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[4]HC hens were fed the 25-OHD_3 diet and 6 mg CXN in per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[5]Control roosters were fed the same diet as the Control hens.
[6]HC roosters were fed the same diet as the HC hens.
[7]Eviscerated carcass weight as a percentage of live weight.
[8]Portion weight as a percent of eviscerated carcass weight.

Table 3.6 Effects of hen diet, rooster diet, broiler age and breeder age on right femur bone mineralization and cross-sectional area at 30% of femur bone length from the proximal epiphysis of broilers.

Hen treatment	Rooster treatment	Breeder age (wk)	Broiler age (d)	n	----------Density (mg/cm^3)-------			----------Area (mm^2)--------			Bone mineral content[1] (mg/mm)		
					Total	Cortical	Trabecular	Total	Cortical	Trabecular	Total	Cortical	Trabecular
Control[2]				96	389.87	776.67	88.72	56.91	21.67	31.06	21.45	17.18	2.53
25-OHD_3[3]				96	392.20	777.51	86.27	56.88	21.91	30.79	21.51	17.36	2.45
CXN[4]				96	393.79	778.99	88.42	56.04	21.82	30.12	21.49	17.33	2.47
HC[5]				96	390.66	776.48	79.20	56.93	21.79	30.93	21.35	17.24	2.37
SEM					4.32	3.38	3.12	0.67	0.31	0.54	0.28	0.26	0.09
	Control[6]			192	398.89[a]	780.32	88.16	55.92[b]	21.88	29.94[b]	21.55	17.42	2.43
	HC[7]			192	384.38[b]	774.51	83.15	57.46[a]	21.71	31.51[a]	21.35	17.13	2.48
SEM					3.02	2.39	5.04	0.47	0.22	0.38	0.20	0.18	0.06
		25		96	395.82[ab]	773.01	91.07[a]	55.95	21.51	30.15	21.41	17.01	2.61[a]
		37		96	400.93[a]	779.32	95.79[a]	56.79	22.11	30.33	22.11	17.55	2.73[a]
		49		96	382.28[c]	778.46	76.70[b]	57.09	21.68	31.32	21.14	17.20	2.28[b]
		59		96	387.50[bc]	778.85	79.04[b]	56.94	21.89	31.10	21.15	17.35	2.19[b]
SEM					4.26	3.40	3.14	0.67	0.31	0.54	0.28	0.26	0.09
			14	128	463.7[a]	695.43[c]	121.3[a]	55.69[b]	25.93[a]	22.68[c]	25.10[a]	19.10[a]	3.09[a]
			28	128	393.57[b]	784.47[b]	92.21[b]	60.51[a]	23.70[b]	31.88[b]	23.65[a]	18.62[a]	2.99[a]
			42	128	317.61[c]	852.33[a]	43.43[c]	53.87[b]	15.76[c]	37.61[a]	15.61[b]	14.11[b]	1.29[b]
SEM					11.97	9.39	8.70	1.86	0.86	1.51	0.79	0.23	0.26
	Control	25		48	397.28	770.59	93.81	56.46	21.78	30.42[bc]	21.56	17.14	2.64
	Control	37		48	405.13	779.19	97.89	56.41	21.91	30.11[c]	22.05	17.43	2.74
	Control	49		48	398.47	787.91	79.08	55.19	21.88	29.42[c]	21.29	17.58	2.09
	Control	59		48	394.68	783.57	81.86	55.63	21.95	29.79[c]	21.32	17.53	2.24

Table 3.6 Effects of hen diet, rooster diet, broiler age and breeder age on right femur bone mineralization and cross-sectional area at 30% of femur bone length from the proximal epiphysis of broilers.

Hen treatment	Rooster treatment	Breeder age (wk)	Broiler age (d)	n	----------Density (mg/cm^3)-------			----------Area (mm^2)--------			Bone mineral content[1] (mg/mm)		
					Total	Cortical	Trabecular	Total	Cortical	Trabecular	Total	Cortical	Trabecular
	HC	25		48	394.37	775.43	88.34	55.43	21.24	29.89^{c}	21.25	16.88	2.58
	HC	37		48	396.72	779.44	93.69	57.17	22.31	30.54^{bc}	22.18	17.67	2.71
	HC	49		48	366.08	769.02	74.33	58.98	21.49	33.22^{a}	20.99	16.82	2.47
	HC	59		48	380.32	774.13	76.23	58.25	21.82	32.40^{ab}	20.98	17.17	2.14
SEM					6.28	4.76	4.50	0.95	0.43	0.76	0.39	0.37	0.13
	Control		14	64	476.15^{a}	695.95	127.71	55.55	26.09	22.32^{d}	25.32	19.22	3.11
	Control		28	64	393.41^{c}	786.44	92.08	60.12	23.65	31.61^{c}	23.55	18.61	2.95
	Control		42	64	327.11^{d}	858.55	44.69	52.10	15.91	35.88^{b}	15.79	14.43	1.23
	HC		14	64	451.27^{b}	694.91	114.92	55.83	25.77	23.05^{d}	24.87	18.98	3.07
	HC		28	64	393.74^{c}	782.49	92.35	60.90	23.75	32.15^{bc}	23.75	18.63	3.03
	HC		42	64	308.11^{e}	846.11	42.17	55.64	15.61	39.34^{a}	15.43	13.79	1.34
SEM					12.50	9.85	10.10	1.95	0.97	1.58	0.82	0.82	0.27
		25	14	32	452.04^{b}	691.20^{f}	121.31^{a}	53.70	24.85	22.87^{e}	24.02^{bc}	18.23	3.09^{a}
		25	28	32	421.37^{c}	785.54^{c}	102.81^{bc}	58.67	24.25	31.67^{d}	24.45^{ab}	19.26	3.08^{a}
		25	42	32	314.06^{ef}	853.27^{b}	49.10^{ef}	55.47	15.44	38.63^{a}	15.74^{de}	13.52	1.66^{c}
		37	14	32	473.73^{a}	694.42^{ef}	132.02^{a}	55.97	26.04	23.07^{e}	25.44^{a}	19.21	3.19^{a}
		37	28	32	397.21^{d}	787.41^{cd}	103.77^{b}	61.08	23.75	31.63^{c}	24.16^{ab}	18.62	3.38^{a}
		37	42	32	331.83^{e}	850.70^{ab}	51.60^{de}	53.32	16.54	37.65^{abc}	16.73^{d}	14.81	1.61^{c}
		49	14	32	455.38^{ab}	697.86^{e}	105.93^{b}	55.89	26.07	22.68^{e}	24.99^{ab}	19.27	2.92^{ab}
		49	28	32	376.63^{d}	782.23^{d}	88.89^{c}	62.30	23.38	32.17^{bc}	23.34^{bc}	18.14	3.09^{a}
		49	42	32	314.82^{ef}	856.87^{a}	35.28^{f}	53.07	15.59	35.53^{ab}	15.10^{e}	14.18	0.84d
		59	14	32	473.69^{a}	698.25^{ef}	125.99^{a}	57.21	26.76	22.10^{e}	25.93^{a}	19.67	3.15^{a}
		59	28	32	379.08^{d}	782.69^{cd}	73.39^{d}	59.99	23.42	32.06^{c}	22.64^{c}	18.47	2.41^{b}
		59	42	32	309.73^{f}	848.50^{ab}	37.75^{ef}	53.63	15.48	38.63^{a}	14.87^{e}	13.92	1.02^{d}

Table 3.6 Effects of hen diet, rooster diet, broiler age and breeder age on right femur bone mineralization and cross-sectional area at 30% of femur bone length from the proximal epiphysis of broilers.

Hen treatment	Rooster treatment	Breeder age (wk)	Broiler age (d)	n	Density (mg/cm^3)			Area (mm^2)			Bone mineral content[1] (mg/mm)		
					Total	Cortical	Trabecular	Total	Cortical	Trabecular	Total	Cortical	Trabecular
SEM					7.55	10.53	10.04	2.25	0.98	0.93	0.92	0.86	0.15
					Probability								
Hen					0.921	0.951	0.111	0.738	0.956	0.622	0.980	0.957	0.650
Rooster					0.001	0.086	0.109	0.023	0.585	0.004	0.466	0.279	0.587
Breeder age					0.014	0.511	<0.0001	0.631	0.554	0.365	0.058	0.507	<0.0001
Broiler age					<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Hen x Rooster					0.507	0.998	0.505	0.670	0.193	0.573	0.051	0.178	0.142
Hen x Breeder age					0.553	0.153	0.411	0.740	0.483	0.869	0.405	0.383	0.821
Hen x Broiler age					0.525	0.891	0.389	0.803	0.317	0.389	0.433	0.266	0.901
Rooster x Breeder age					0.087	0.064	0.998	0.060	0.725	0.020	0.925	0.601	0.227
Rooster x Broiler age					0.042	0.365	0.202	0.105	0.823	0.052	0.590	0.598	0.782
Breeder age x Broiler age					0.001	0.023	0.030	0.093	0.169	0.047	0.008	0.101	0.005
Hen x Rooster x Breeder age					0.188	0.471	0.795	0.261	0.306	0.126	0.579	0.268	0.292
Hen x Rooster x Broiler age					0.055	0.609	0.981	0.290	0.356	0.036	0.344	0.264	0.624
Hen x Breeder age x Broiler age					0.403	0.063	0.387	0.067	0.985	0.076	0.883	0.961	0.090
Rooster x Breeder age x Broiler age					0.123	0.073	0.159	0.374	0.649	0.242	0.419	0.479	0.226
Hen x Rooster x Breeder age x Broiler age					0.086	0.424	0.376	0.325	0.365	0.262	0.284	0.333	0.157

[a-f]Means with no common letters within the same column are significantly different ($P<0.05$).

[1]Bone mineral density was multiplied by the cross-sectional area to calculate the amount of bone mineral in the 1-mm linear scanned section of the bone.

[2]Control hens were fed a nutritionally complete basal diet containing 2,760 IU of supplemental vitamin D_3.

[3]25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[4]CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[5]HC hens were fed the 25-OHD_3 diet and 6 mg CXN in per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[6]Control roosters were fed the same diet as the Control hens.

[7]HC roosters were fed the same diet as the HC hens.

Table 3.7 Effects of hen diet, rooster diet, broiler age and on right femur bone mineralization and cross-sectional area at 50% of femur bone length from the proximal epiphysis of broilers [1].

Hen treatment	Rooster treatment	Breeder age (wk)	Broiler age (d)	n	----------Density (mg/cm^3)-------			----------Area (mm^2)----------			Bone mineral content[2] (mg/mm)		
					Total	Cortical	Trabecular	Total	Cortical	Trabecular	Total	Cortical	Trabecular
Control[3]				96	557.30	884.78	69.52	49.25	26.36	20.22	26.10	23.71	1.39
25-[4]				96	562.69	878.55	69.57	48.36	26.34	19.23	26.10	23.54	1.42
CXN[5]				96	561.84	881.42	70.51	48.97	26.46	19.77	26.29	23.74	1.48
HC[6]				96	556.60	877.25	61.69	50.22	26.64	20.84	26.34	23.72	1.53
SEM					4.52	3.03	17.61	0.71	0.33	0.50	0.31	0.28	0.007
	Control[7]			192	559.41	880.79	68.60	49.58	26.59	20.22	26.36	23.81	1.46
	HC[8]			192	559.81	880.20	67.05	48.82	26.31	19.81	26.05	23.55	1.45
SEM					3.20	2.14	15.21	0.50	0.23	0.35	0.22	0.20	0.05
		25		96	562.54	878.73^{b}	79.75^{a}	48.50	26.17	19.47	26.29	23.50	1.67^{a}
		37		96	558.49	876.50^{b}	78.28^{a}	50.15	26.54	20.50	26.64	23.60	1.81^{a}
		49		96	561.22	887.83^{a}	54.58^{b}	48.33	26.29	19.61	25.86	23.72	1.20^{b}
		59		96	556.18	878.93^{b}	58.68^{b}	49.81	26.80	20.47	26.03	23.88	1.14^{b}
SEM					4.53	3.06	14.05	0.72	0.34	0.50	0.32	0.29	0.07
			14	128	630.66^{a}	780.64^{c}	104.54^{a}	54.25^{a}	31.90^{a}	17.09^{b}	30.61^{a}	26.34^{a}	2.19^{a}
			28	128	574.59^{b}	883.89^{b}	77.69^{b}	53.17^{a}	30.78^{a}	19.13^{b}	30.22^{a}	27.20^{a}	1.78^{b}
			42	128	473.57^{c}	976.96^{a}	21.24^{c}	40.19^{b}	16.68^{b}	23.83^{a}	17.79^{b}	17.49^{b}	0.40^{c}
SEM					12.52	8.40	14.36	2.20	1.02	1.54	0.96	0.78	0.22
		25	14	32	611.03^{bc}	770.70	111.56^{abc}	53.04	30.69	17.42	29.43^{b}	25.25^{b}	2.22^{a}
		25	28	32	583.01^{cd}	885.56	93.55^{bc}	53.14	30.50	19.09	30.38^{ab}	26.99^{ab}	2.04^{a}

Table 3.7 Effects of hen diet, rooster diet, broiler age and on right femur bone mineralization and cross-sectional area at 50% of femur bone length from the proximal epiphysis of broilers [1].

Hen treatment	Rooster treatment	Breeder age (wk)	Broiler age (d)	n	----------Density (mg/cm^3)-------			----------Area (mm^2)----------			Bone mineral content[2] (mg/mm)		
					Total	Cortical	Trabecular	Total	Cortical	Trabecular	Total	Cortical	Trabecular
		25	42	32	493.58^{e}	979.94	34.14^{g}	39.33	17.33	21.91	19.07^{c}	18.27^{c}	0.74^{c}
		37	14	32	632.43^{ab}	780.59	105.50^{ab}	54.75	32.08	17.24	30.88^{ab}	26.47^{ab}	2.24^{a}
		37	28	32	565.41^{d}	878.78	91.46^{bc}	54.13	30.43	20.01	30.31^{ab}	26.79^{ab}	2.11^{a}
		37	42	32	477.64^{ef}	970.12	37.87^{fg}	41.59	17.12	24.25	18.74^{c}	17.55^{cd}	1.08^{bc}
		49	14	32	631.58^{ab}	790.37	85.00^{cd}	53.88	31.71	17.02	30.45^{ab}	26.32^{ab}	2.10^{a}
		49	28	32	583.19^{cd}	892.34	66.71^{de}	51.85	30.90	18.18	30.04^{ab}	27.54^{a}	1.45^{b}
		49	42	32	468.88^{fg}	980.79	12.04^{h}	39.26	16.28	23.65	17.11^{d}	17.30^{cd}	0.05^{d}
		59	14	32	647.61^{a}	780.90	116.11^{a}	55.33	33.11	16.69	31.69^{a}	27.32^{a}	2.17^{a}
		59	28	32	566.74^{d}	878.90	59.04^{ef}	53.54	31.28	19.23	30.16^{ab}	27.47^{a}	1.51^{b}
		59	42	32	454.18^{g}	976.99	0.89^{h}	40.57	16.01	25.50	16.24^{d}	16.86^{d}	0.00^{d}
SEM					14.22	10.86	14.62	2.54	1.19	1.78	1.12	1.00	0.26
					----------Probability----------								
Hen					0.698	0.302	0.388	0.310	0.913	0.128	0.926	0.958	0.496
Rooster					0.929	0.845	0.673	0.281	0.403	0.407	0.314	0.355	0.798
Breeder age					0.751	0.047	<0.001	0.171	0.544	0.300	0.331	0.802	<0.001
Broiler age					<0.001	<0.001	<0.001	<0.001	<0.001	0.016	<0.001	<0.001	<0.001
Hen x Rooster					0.690	0.393	0.135	0.695	0.962	0.448	0.994	0.973	0.592
Hen x Breeder age					0.462	0.519	0.160	0.204	0.061	0.545	0.063	0.057	0.443
Hen x Broiler age					0.219	0.736	0.855	0.286	0.176	0.108	0.190	0.154	0.967
Rooster x Breeder age					0.779	0.619	0.906	0.420	0.336	0.657	0.345	0.391	0.563
Rooster x Broiler age					0.774	0.764	0.580	0.611	0.622	0.630	0.543	0.705	0.454
Breeder age x Broiler age					<0.001	0.424	<0.001	0.972	0.058	0.224	0.001	0.028	<0.001
Hen x Rooster x Breeder age					0.023	0.219	0.424	0.196	0.207	0.191	0.168	0.305	0.007
Hen x Rooster x Broiler age					0.744	0.304	0.119	0.873	0.849	0.787	0.888	0.940	0.478

Table 3.7 Effects of hen diet, rooster diet, broiler age and on right femur bone mineralization and cross-sectional area at 50% of femur bone length from the proximal epiphysis of broilers [1].

Hen treatment	Rooster treatment	Breeder age (wk)	Broiler age (d)	n	----------Density (mg/cm^3)-------			----------Area (mm^2)----------			Bone mineral content[2] (mg/mm)		
					Total	Cortical	Trabecular	Total	Cortical	Trabecular	Total	Cortical	Trabecular
Hen x Breeder age x Broiler age					0.426	0.204	0.005	0.776	0.877	0.904	0.671	0.848	0.005
Rooster x Breeder age x Broiler age					0.994	0.558	0.935	0.507	0.427	0.854	0.597	0.630	0.854
Hen x Rooster x Breeder age x Broiler age					0.683	0.610	0.058	0.716	0.523	0.863	0.351	0.624	0.050

[a-g]Means with no common letters within the same column are significantly different ($P<0.05$).

[1]Eggs collected during wk 25 to 59.

[2]Bone mineral density was multiplied by the cross-sectional area to calculate the amount of bone mineral in the 1-mm linear scanned section of the bone.

[3]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.

[4]25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[5]CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[6]HC hens were fed the 25-OHD_3 diet and 6 mg CXN in per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[7]Control roosters were fed the same diet as the Control hens.

[8]HC roosters were fed the same diet as the HC hens.

3.6 FIGURES

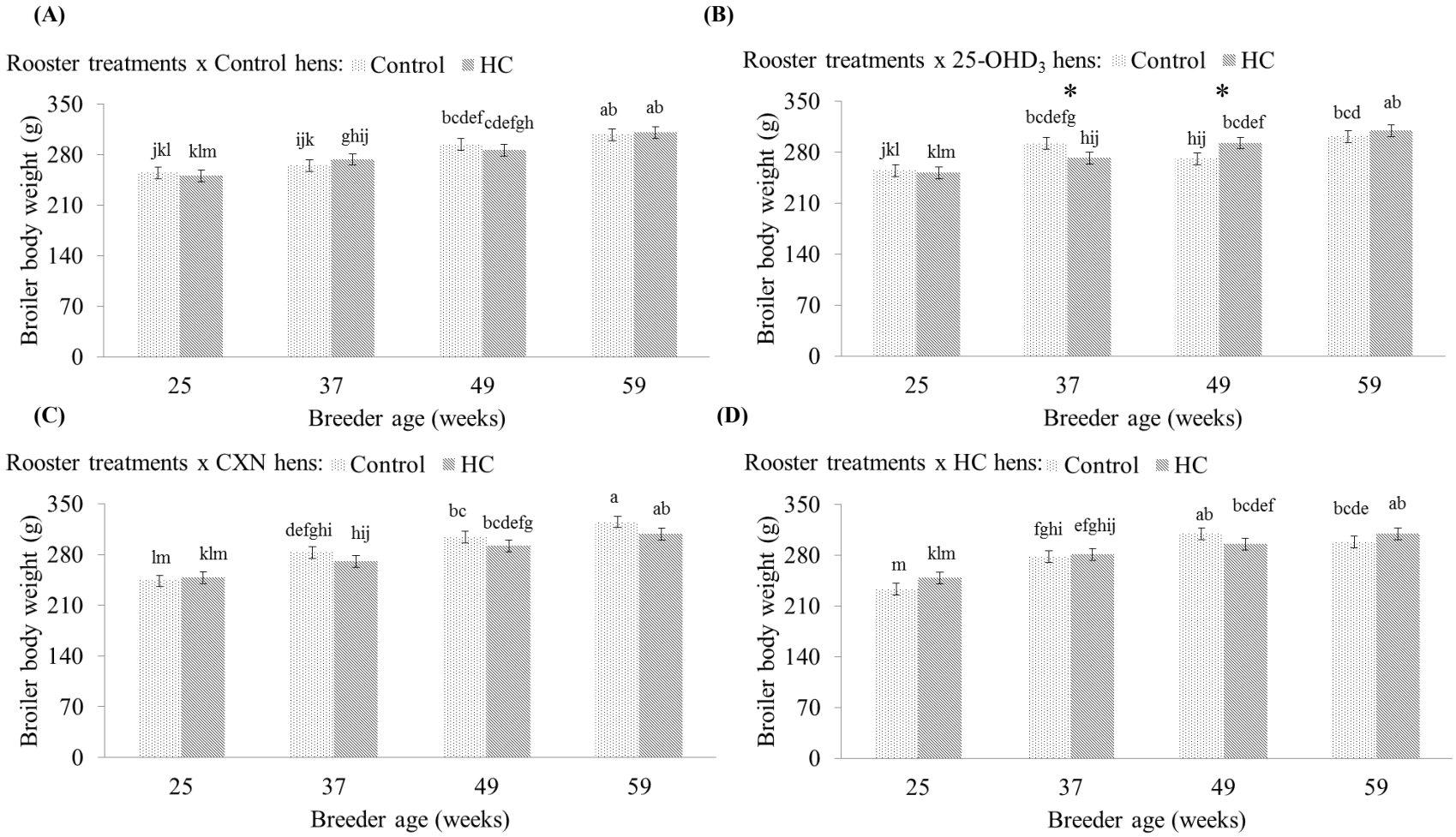

Figure 3.1 Interaction of hen treatments, rooster treatments and breeder ages on 10 d old broiler chick BW (P=0.039; n=4). (A) Control, (B) 25-OHD_3, (C) CXN, and (D) HC hen treatment with different rooster treatments and breeder ages. Means within all panels with a no common letters are significantly different (P<0.05) and are indicated with an asterisk. Control hens were fed a

nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). Control roosters were fed the same diet as the Control hens. HC roosters were fed the same diet as the HC hens.

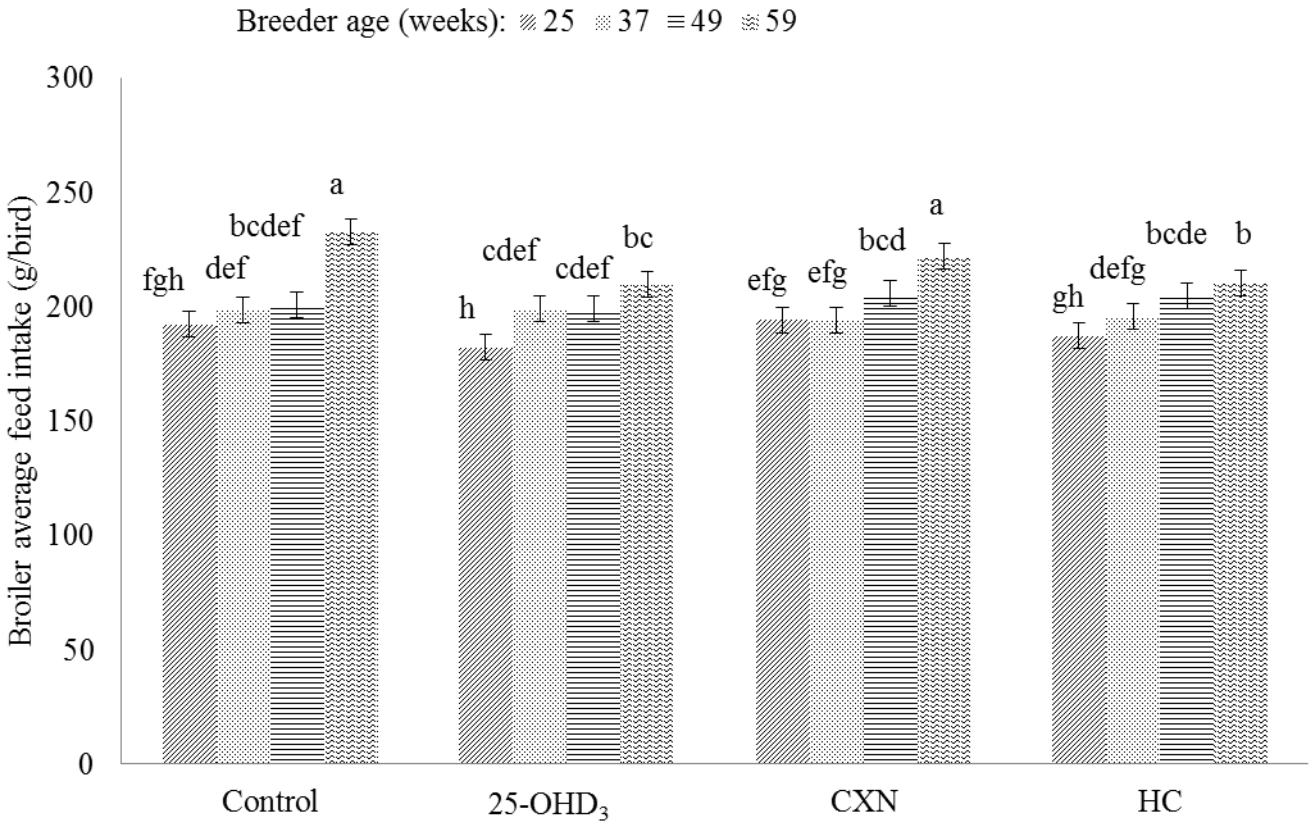

Figure 3.2 Interaction between hen treatments and breeder ages on broiler feed intake from 25 to 42 d of age (P=0.026; n=8). Means with no common letters are significantly different (P≤0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 69 µg 25-OH D_3 per kg of feed in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

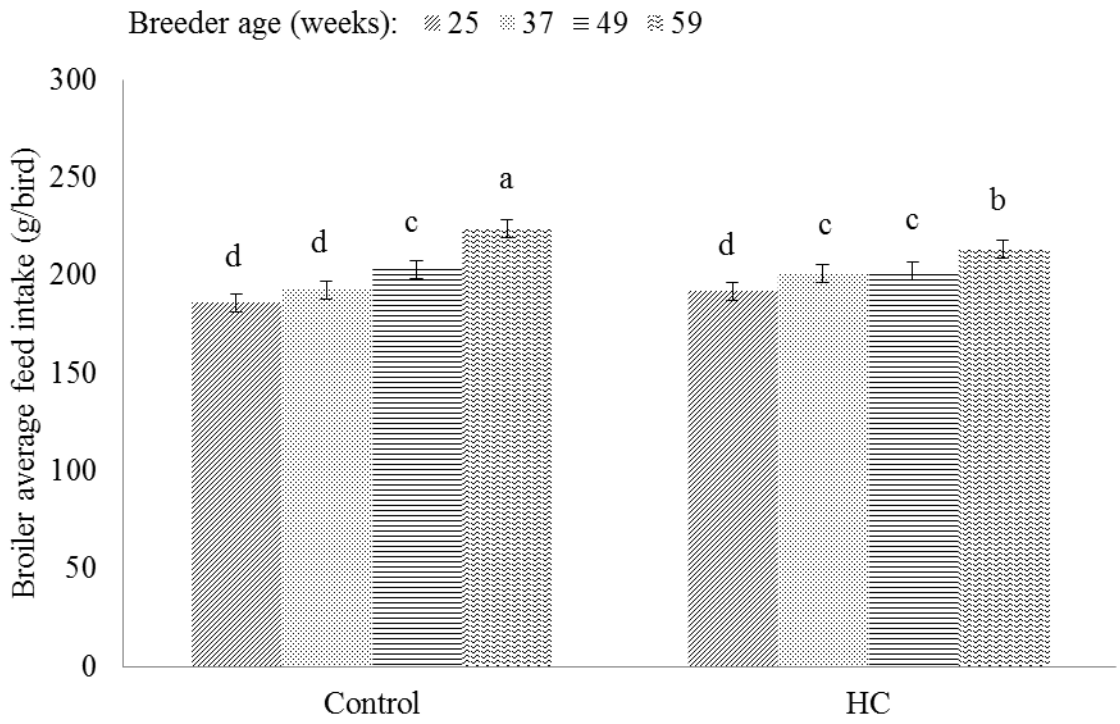

Figure 3.3 Interaction between rooster treatments and breeder ages on broiler feed intake from 25 to 42 d of age (P=0.005; n=16). Means with no common letters are significantly different (P≤0.05). Control roosters were fed a nutritionally complete diet containing 2,760 IU of supplemental Vitamin D_3. HC roosters were fed 69 µg 25-OH D_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3 with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

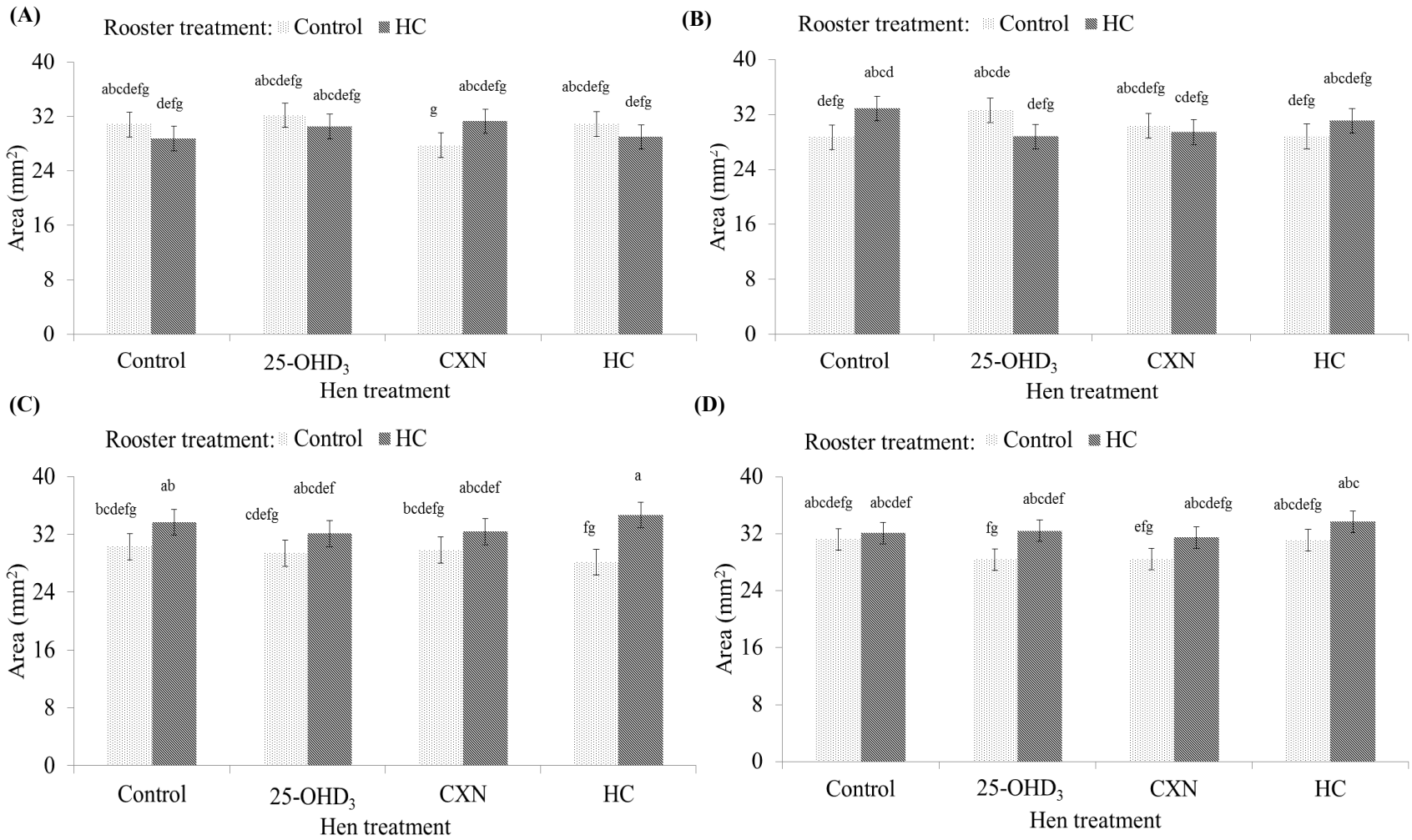

Figure 3.4 Interaction of hen treatments, rooster treatments and breeder ages on cross-sectional trabecular area at 30% of femur bone length from the proximal epiphysis of broilers (P=0.036; n=12). (A) 25 (B) 37 (C) 49, and (D) 59 wk breeder age with different hen treatments and rooster treatments. Means within all panels with no common letters are significantly different (P<0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). Control roosters were fed the same diet as the Control hens. HC roosters were fed the same diet as the HC hens.

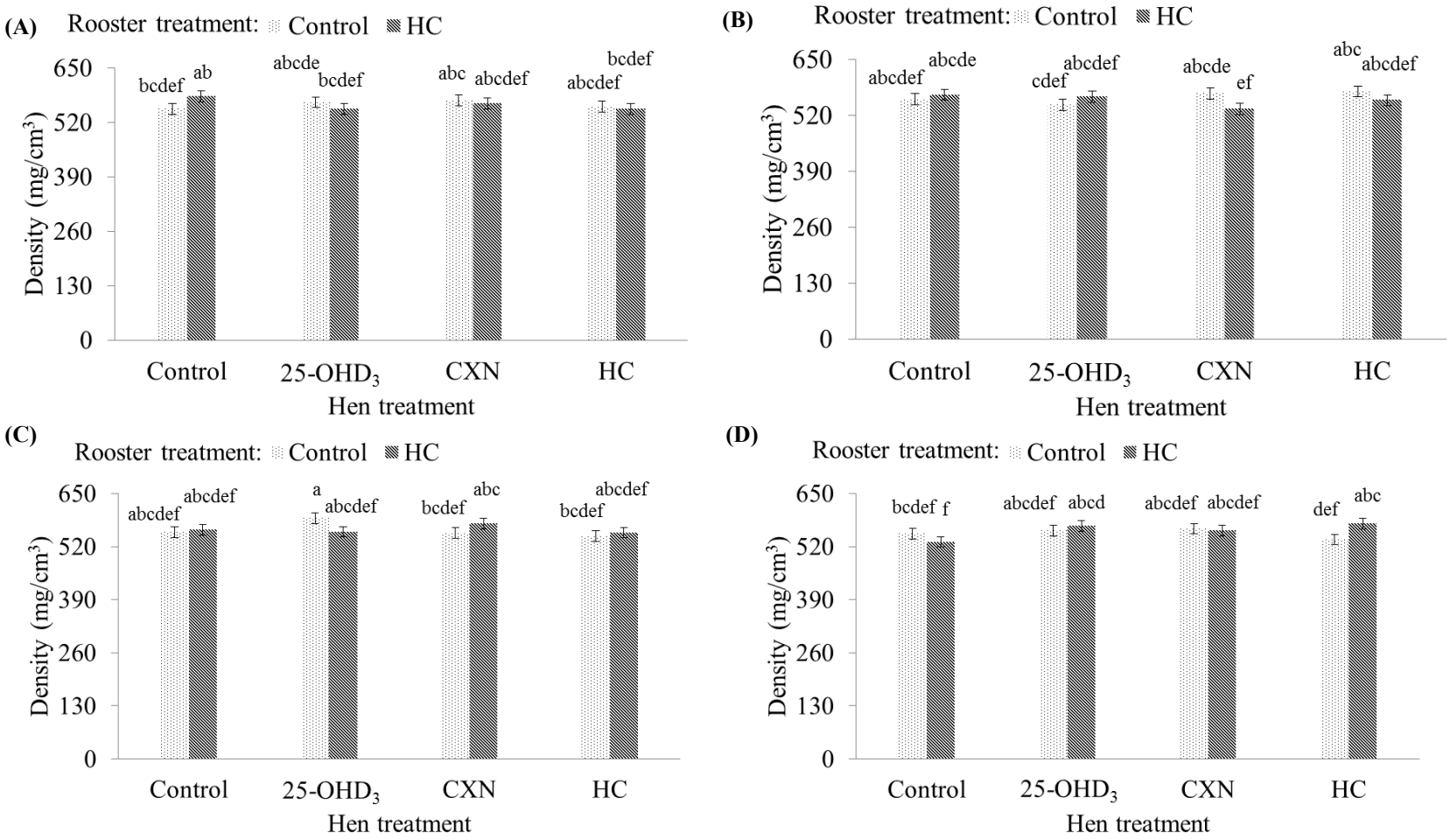

Figure 3.5 Interaction of hen treatments, rooster treatments and breeder ages on total cross-sectional trabecular density at 50% of femur bone length from the proximal epiphysis of broilers (P=0.036; n=12). (A) 25 (B) 37 (C) 49, and (D) 59 wk breeder age with different hen treatments and rooster treatments. Means within all panels with no common letters are significantly different (P<0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products

Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). Control roosters were fed the same diet as the Control hens. HC roosters were fed the same diet as the HC hens.

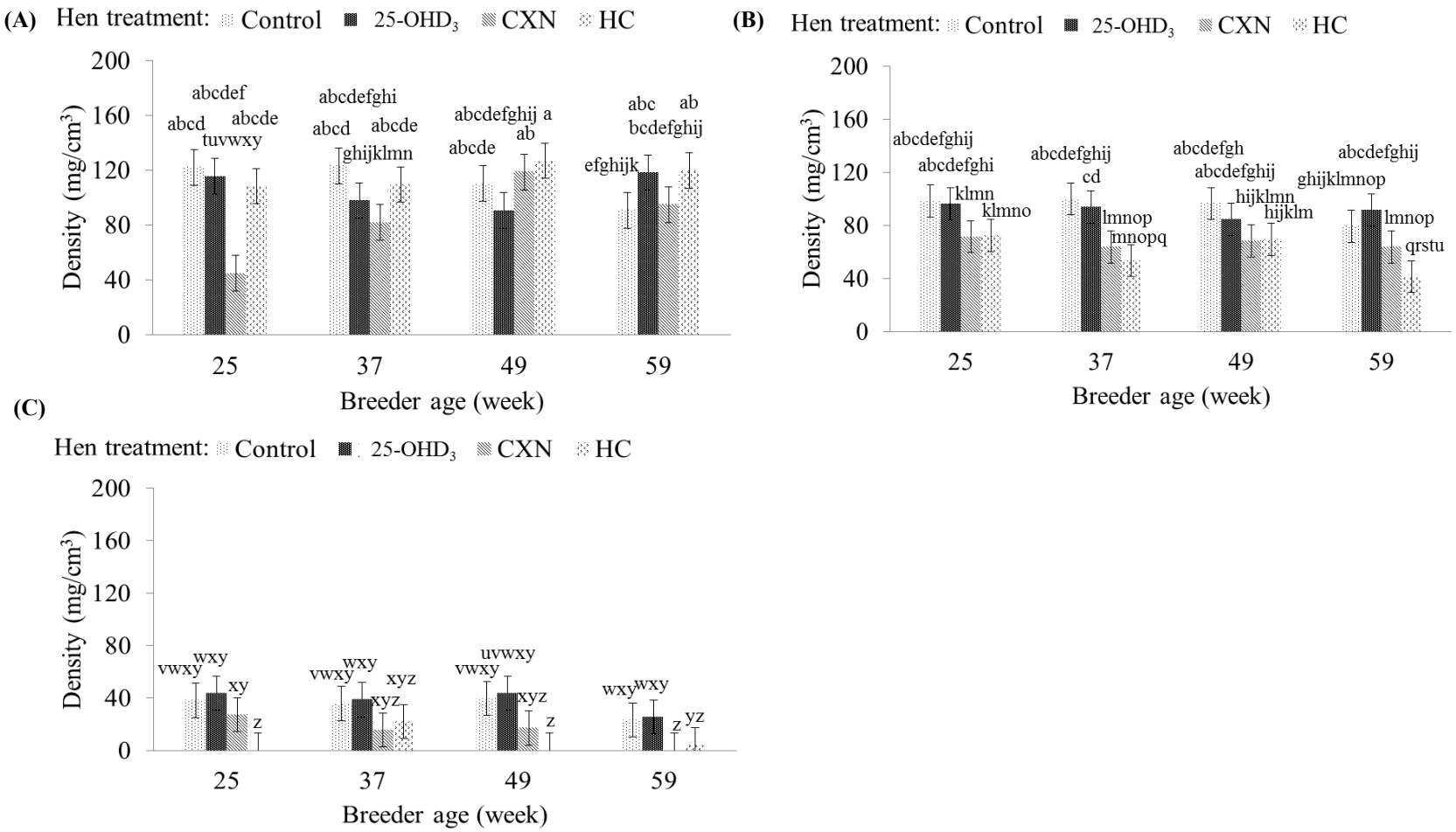

Figure 3.6 Interaction of hen treatments, breeder ages and broiler ages on trabecular density at 50% of femur bone length from the proximal epiphysis of broilers (P=0.005; n=8). (A) 14 (B) 28, and (C) 42 d broiler age with different hen treatments and breeder ages. Means within all panels with no common letters are significantly different (P<0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

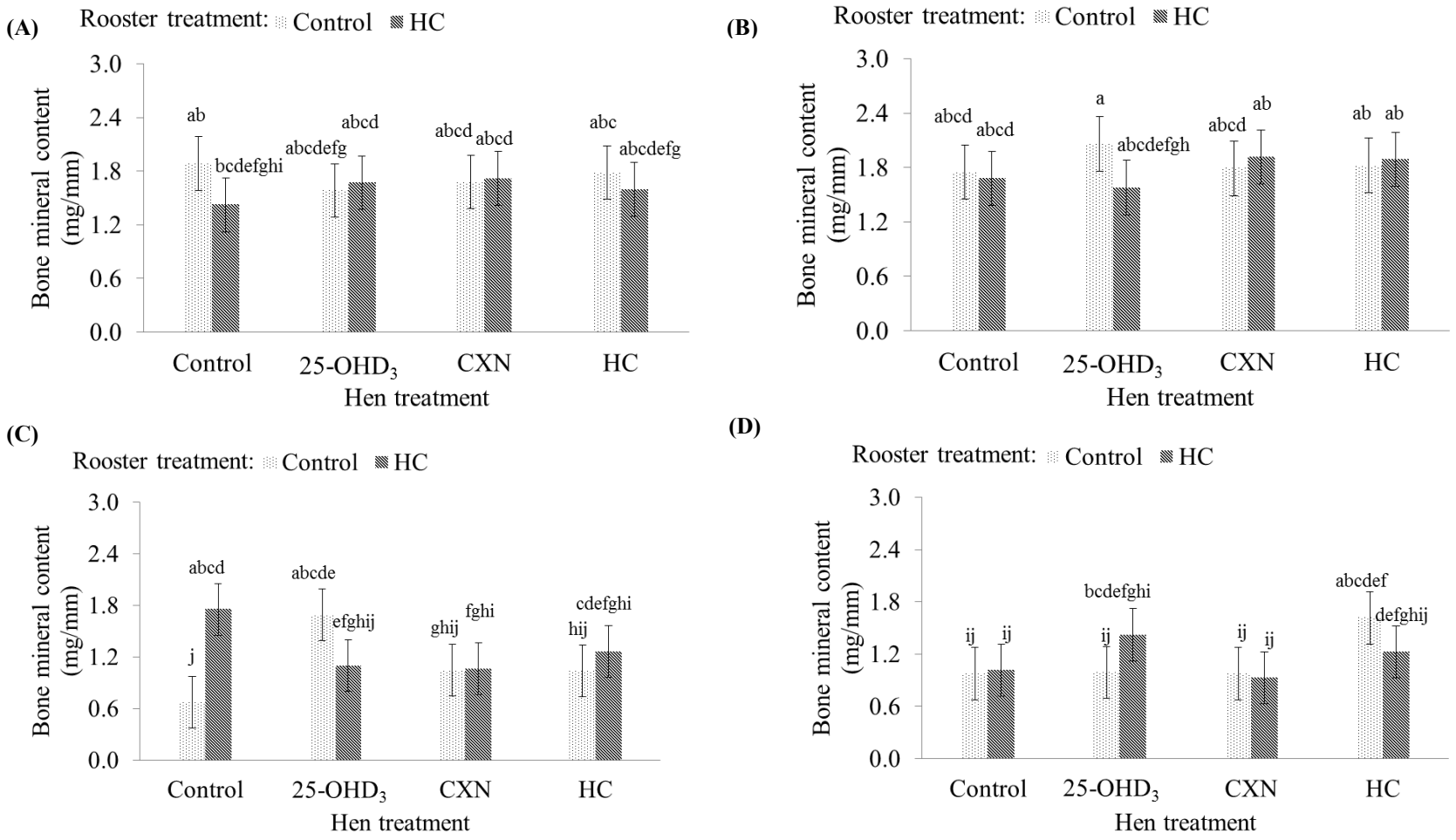

Figure 3.7 Interaction of hen treatments, rooster treatments and breeder ages on trabecular bone mineral content at 50% of femur bone length from the proximal epiphysis of broilers (P=0.007; n=12). (A) 25 (B) 37 (C) 49, and (D) 59 wk breeder age with different hen treatments and rooster treatments. Means within all panels with no common letters are significantly different (P<0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). Control roosters were fed the same diet as the Control hens. HC roosters were fed the same diet as the HC hen.

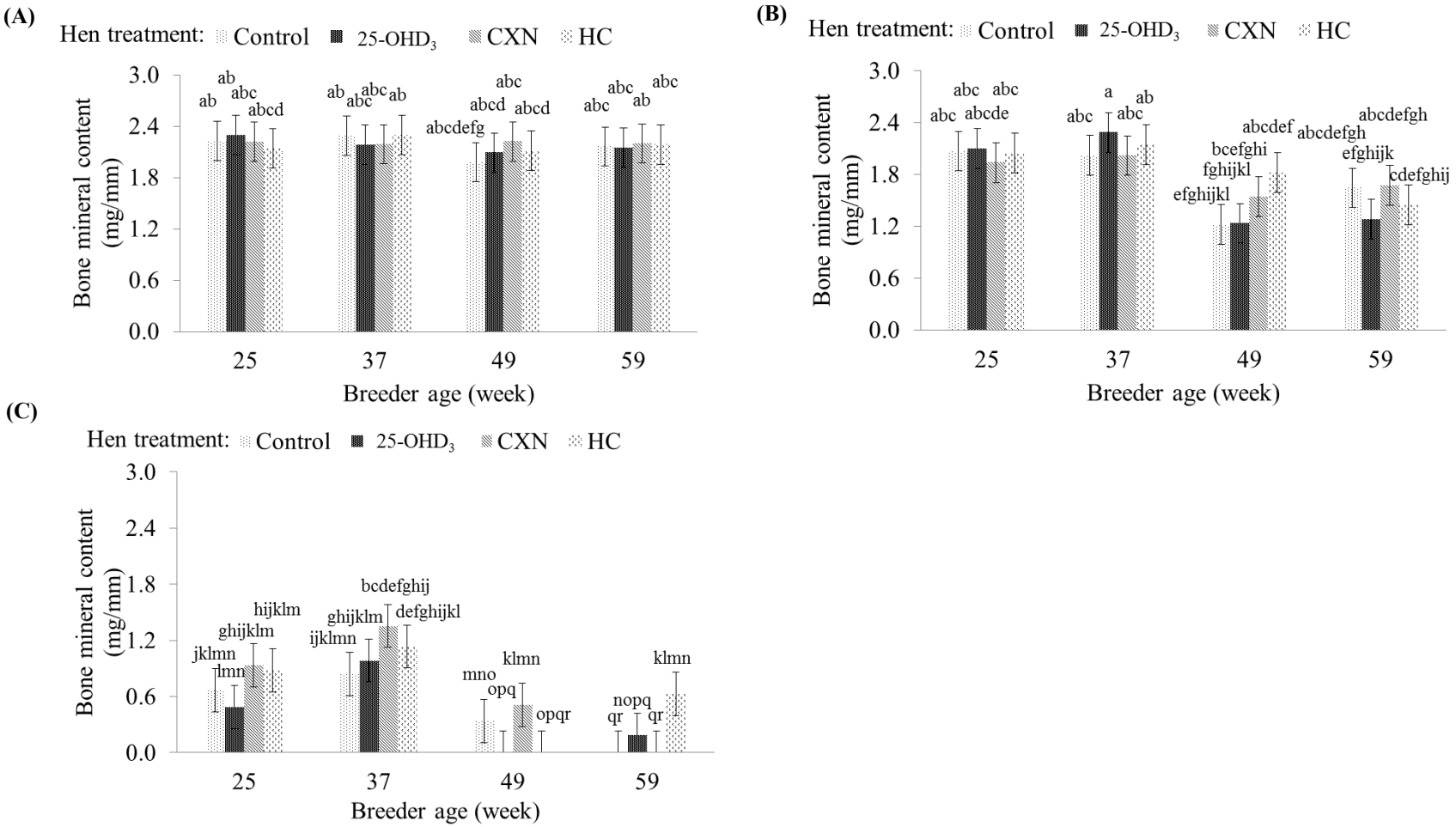

Figure 3.8 Interaction of hen treatments, breeder ages and broiler ages on trabecular bone mineral content at 50% of femur bone length from the proximal epiphysis of broilers (P=0.005; n=8). (A) 14 (B) 28, and (C) 42 d broiler age with different hen treatments and breeder ages. Means within all panels with no common letters are significantly different (P<0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the basal diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the basal diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet with 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

4. THE EFFECT OF MATERNAL DIETARY CANTHAXANTHIN AND 25-HYDROXYVITAMIN D_3 ON BROILER INNATE IMMUNITY

ABSTRACT

Maternal dietary canthaxanthin (**CXN**) and 25-hydroxycholecalciferol (**25-OHD_3**) have each been reported to each increase early innate immunity in broilers. The objective was to determine look at the effects of parental dietary CXN and 25-OHD_3 on early broiler innate immunity and antioxidant capacity. Individually-caged Ross 308 hens (n=288) were fed diets as either 1) Control (Vitamin D_3 at 2,760 IU/kg), 2) CXN (Control with 6 mg/kg CXN) 3) 25-OHD_3 (Control with 25-OHD_3 at 2,760 IU/kg replacing vitamin D_3) or 4) HC (CXN diet with 25-OHD_3 at 2,760 IU/kg replacing vitamin D_3). Individually-caged Ross 544 males (n=60) were fed either the Control or HC diet. Hens were inseminated weekly, and hatching eggs collected at 25, 37, 49 and 59 wk of age and stored for up to 1 wk before incubation. Chick whole blood phagocytic index (cells engulfing at least 1 *Escherichia coli*; ***E. coli***) and phagocytic capacity (relative number of *E. coli* engulfed/cell) were measured at 1 and 4 d of age. The 0 and 14 d old chick plasma antioxidant capacity was determined (n=8/paternal treatment) at each of 37, 49 and 59 wk of breeder age. Data were analyzed using the Proc Mixed procedure; significance was set at $P<0.05$. Overall, maternal dietary 25-OHD_3 increased total phagocytic index, suggesting it increases innate immunity early in life. No maternal CXN effect was observed on phagocytic index or capacity. No maternal dietary effect was seen on chick plasma antioxidant capacity, possibly because chicks were housed at the recommended temperature and a low stocking density. Chicks from hens inseminated by HC roosters had increased total and lymphocyte/thrombocyte phagocytic index compared to chicks from hens inseminated by Control roosters. Hence, paternal CXN and 25-OHD_3 may also increase broiler early innate

immunity possibly due to an epigenetic mechanism. Generally, chick age did not affect phagocytic index and capacity. Chick phagocytic index and capacity was lower at 25 wk of breeder age compared to other older breeder ages.

Key words: canthaxanthin, 25-hydroxyvitamin D_3, broiler breeder, innate immunity, broiler

4.1 INTRODUCTION

Twenty five-hydroxyvitamin D_3 **(25-OHD$_3$)** is a fat-soluble vitamin D_3 metabolite, which is transferred to the egg when fed to hens (Coto et al., 2010). Vitamin D is important for the immune system in the chicken, since vitamin D deficiency decreases macrophage phagocytosis of sheep red blood cells in broiler chickens (Aslam et al., 1998). Dietary 25-OHD_3 has greater intestinal absorption than vitamin D_3 (Bar et al., 1980). Chicken macrophages treated with 200 nM 25-OHD_3 and then stimulated with LPS *in vitro* had greater 1-alpha-hydroxylase mRNA relative to LPS-stimulated macrophages not treated with 25-OHD_3 (Morris and Selvaraj, 2014). Hence, conversion of 25-OHD_3 to 1,25-dihydroxyvitamin D_3 (**1,25(OH)$_2$D$_3$**) after stimulation of LPS may be greater due to increased 1-alpha-hydroxylase mRNA in the immune cells (Morris and Selvaraj, 2014). Human monocytes treated with 1,25$(OH)_2D_3$ (1,25$(OH)_2D_3$ at 24 nM) increased *Escherichia coli* ***(E. coli)*** phagocytic index compared to monocytes without 1,25$(OH)_2D_3$ (Xu et al., 1993). Similary, if 25-OHD_3 is converted more into 1,25$(OH)_2D_3$ in chicken macrophages, then 1,25$(OH)_2D_3$ may increase phagocytosis. Dietary 25-OHD_3 supplementation in broiler chickens had higher *Salmonella typhimurium* phagocytosis compared to vitamin D_3-fed broiler chickens (Chou et al., 2009). Breeder hens fed 25-OHD_3 transferred 25-OHD_3 more efficiently to the egg than breeders fed dietary vitamin D_3 (Coto et al., 2010). Hence, transfer of maternal dietary 25-OHD_3 to the chick may increase broiler phagocytic index and capacity early in life.

Canthaxanthin **(CXN)** is an antioxidant carotenoid (Kull and Pfander, 1995) that is efficiently deposited in the egg when fed to hens (Zhang et al., 2011; Johnson-Dahl et al., 2017; Chapter 2). Therefore, CXN-fed breeders can increase broiler chick plasma antioxidant capacity early in the chick's life (Surai et al., 2003). Broiler chicks hatched from breeder hens fed 6 mg/kg of CXN had increased *E. coli* killing compared to chicks hatched from hens not fed CXN (Johnson-Dahl et al., 2017). Therefore, phagocytosis may also increase from chicks hatched from CXN-fed broiler breeder hens. Little research has been done on the effects of parental dietary CXN on broiler chick innate immunity and hence, further studies are needed in this area. The intestine of the newly hatch chick recruits mature innate cells, such as heterophils first and then as the intestine gradually develops, it recruits mature lymphocytes (Bar-Shira and Friedman, 2006); lymphocyte count and lymphocyte proliferation in response to phytohemagglutinin (**PHA**) was lower in nestling tree swallows than adult tree swallows (Palacios et al., 2009). Therefore chicks rely more heavily on innate immunity for the first few d of life as adaptive immunity develops. Thus, this study focused on the early innate immunity in broiler chicks.

The objective of this research was to investigate the effect of parental dietary CXN and 25-OHD_3 on early broiler chick innate immunity and plasma antioxidant capacity. We hypothesized that when CXN and 25-OHD_3 were fed to broiler breeder hens, these nutrients would be transferred to the egg and therefore to the broiler chick. Thus, there would be increased innate immunity in chicks from CXN and 25-OHD_3 hens compared to hens not fed CXN and 25-OHD_3, as well as increased antioxidant capacity in the broiler chicks hatched from CXN hens compared to hens with no dietary CXN. Since roosters do not transfer nutrients to the egg, we also hypothesized that CXN and 25-OHD_3 fed roosters have no effect on the phagocytic index and capacity, nor on the antioxidant capacity in the broiler chick.

4.2 MATERIALS AND METHODS

4.2.1 Experimental diets and animals

Experimental protocols were approved by the Animal Care and Use Committee: Livestock of University of Alberta. The birds were reared and cared for according to the Canadian Council of Animal Care (2009) guidelines. Individually-caged Ross 308 breeder hens (n=288) and Ross 544 roosters (n=60) were fed the experimental diets from 22 to 60 wk of age. The breeder hens were randomly allocated to individual cages (45 x 48 x 42 cm) and were fed one of 4 experimental diets (72 hens/treatment): Control (vitamin D_3 at 2,760 IU/kg), 25-OHD_3 (vitamin D_3 replaced with 25-OHD_3 at 2,760 IU/kg), CXN (CXN added at 6 mg/kg to the Control diet), HC (vitamin D_3 replaced with 25-OHD_3 at 2,760 IU/kg and CXN added at 6 mg/kg; Chapter 2). Roosters (n=30/treatment) were also randomly allocated to individual cages (42 x 34 x 57 cm) and were fed either the Control or the HC diet. All diets were formulated to contain vitamin D_3 activity at 2,760 IU/kg feed as either vitamin D_3 or 25-OHD_3. All the other nutrients met or exceeded the primary breeder nutrition guide specifications (Aviagen, 2007c). Individual BW were measured weekly and the average of each hen BW/treatment and rooster BW/treatment was used to calculate daily feed allocation for each treatment based on target BW from the primary breeder management guide (Aviagen, 2007c). Within each hen treatment, hens were divided into two groups and assigned to be inseminated by males from one of the rooster treatments. Each wk, hens were artificially inseminated with 0.5 mL of pooled semen from 30 roosters from either the Control treatment or the HC treatment. Egg production for each bird was recorded daily and the fertile eggs were identified by hen and stored in a cooler (16 to 18°C at 70 to 80% relative humidity) for up to one wk. Fertile eggs collected at 25, 37, 49 and 59 wk of age were hatched at University of Alberta. Eggs collected at other ages were used to measure hatchability in a

different experiment (Chapter 2). All broilers had free access to the same starter diet (0 to 14 d; 3,067 ME kcal/kg; 23% crude protein; vitamin D, 4,000 IU/kg; vitamin A, 10,000 IU/kg; vitamin E 35 IU/kg). The diets met or exceeded the broiler nutritional recommendations (Aviagen, 2007b) and the temperature, lighting program and light intensity followed the broiler management guide (Aviagen, 2007a).

4.2.2 Phagocytosis assay

From the hatches at 25, 37, 49 and 57 wk of age, there were 364, 435, 425 and 312 chicks hatched, respectively. On the d of hatch, 80 male chicks (n=10/ parental treatment) were randomly selected and placed in one of the four levels of Petersime battery brooder cages (Petersime Incubator, Gettysburg, OH). At each of 1 and 4 d of age, 5 chicks per parental treatment were randomly selected, weighed, and blood was collected via decapitation in to a heparinized tube. Whole blood was collected to measure phagocytic index and capacity as described by Saunders-Blades and Korver (2015). Briefly, after whole blood was obtained from the chicks, it was diluted with CO_2-independent media (Gibco, Invitrogen, Burlington, ON, Canada) in an Eppendorf tube. Fluorescent *E. coli* particles (100 *E. coli* particles per white blood cell) were added to the diluted blood and mixed. The mixture was then incubated for 15 minutes at 41°C. After incubation, it was washed twice with media and then lysis buffer was added. The white blood cells were fixed with 1% formaldehyde and were acquired through 520 to 530 nm bandpass filter (green fluorescence) for phagocytic index and capacity using BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). At least 10,000 cells were analyzed within the gated region (overall, lymphocyte and thrombocyte, granulocyte and monophage, and macrophage) and data were analyzed using BD FACSDiva software (BD Biosciences, San Jose, CA). Phagocytic index was the proportion of phagocytic cells that engulfed at least 1 fluorescent

E. coli particle (K-12 strain; Molecular Probes (E-2864), Invitrogen, Burlington, ON, Canada) and the phagocytic capacity was the relative number of *E. coli* fluorescent particles that were engulfed by one phagocytic cell. Phagocytic index was measured in percentage while phagocytic capacity was measured in mean fluorescence intensity (MFI).

4.2.3 Antioxidant capacity

A commercial antioxidant kit (709001, Cayman Chemicals, Ann Arbor, MI) was used to measure the plasma antioxidant capacity of 4 male and 4 female broilers per paternal treatment at 0 and 14 d of age as described by Johnson-Dahl et al. (2017). The blood was collected via decapitation at 0 d of age and by brachial venipuncture at 14 d of age for each of the 37, 49 and 59 wk of breeder age. Chick blood was not collected from the hatch at 25 wk of age.

4.2.4 Statistical analysis

Data were tested for normality using Proc Univariate of SAS version 9.2 (SAS Institute, 2001) and then analyzed using Proc Mixed as a 4-way ANOVA for phagocytic index and capacity (4 maternal treatments x 2 paternal treatments x 2 chick ages x 4 breeder ages), and for plasma antioxidant capacity (4 maternal treatments x 2 paternal treatments x 2 chick ages x 3 breeder ages). The experimental unit was the chick (n=10/parental treatment for phagocytosis assay; n=8/parental treatment for antioxidant capacity). Means were separated using least square means and differences were considered significant when $P < 0.05$.

4.3 RESULTS

4.3.1 Phagocytic index

The cell population in the gated region averaged over all chick ages constitutes lymphocyte/thrombocyte (91.1 % ±1.58), monocyte/granulocyte (6.8% ± 0.04) and macrophage (2.0% ± 0.01) populations. Total phagocytic index, the proportion of phagocytic cells that

engulfed at least 1 *E. coli* bioparticle *ex vivo*, was lower in 4-d-old chicks hatched from HC hens compared to other treatments at 25 wk of age (P=0.001; Figure 4.1). No other maternal treatment effects at 25 wk of breeder age in 1-d and 4-d-old chicks were found. At 37 wk of age, 1-d-old chicks from HC hens had higher total phagocytic index compared to those from 25-OHD_3 or CXN hens; 4-d-old chicks from 25-OHD_3 hens had increased total phagocytic index compared to chicks from the other hen treatments. There were no other hen treatment effects at 37 wk of age were observed. At 49 wk of age, there was increased total phagocytic index in 1-d-old chicks from HC hens compared to Control hens but no other maternal treatment effects were seen in 1- or 4-d-old chicks. At 59 wk of age, 1-d-old chicks from HC hens had reduced total phagocytic index compared to those from Control and 25-OHD_3 hens. Four-d-old chicks from Control hens had higher total phagocytic index compared to CXN hens. No other differences were found between maternal treatments at 59 wk of age in 1- or 4-d-old chicks.The chicks from hens inseminated by HC roosters had increased total phagocytic index compared to chicks from hens inseminated by Control roosters (P=0.016; Table 4.1).

At 25 wk of age, 1-d-old chicks hatched from HC hens had higher total phagocytic index than 4 d old chicks from the same hens (Figure 4.1). No other differences due to chick age were observed within any of the other hen treatments at 25 wk of age. Total phagocytic index decreased with increasing chick age for all hen treatments except 25-OHD_3 at 37 wk of age; no differences were seen between the two chick ages for 25-OHD_3 hens. Reduced total phagocytic index was seen with increasing age for chicks hatched from 25-OHD_3 and HC hens at 49 wk of age; no differences between the chick ages were seen for other treatments. No chick age effects were observed within each maternal treatment on total phagocytic index at 59 wk of age. The

chicks from hens inseminated by HC roosters had increased total phagocytic index compared to chicks from hens inseminated by Control roosters (P=0.016; Table 4.1).

Lymphocyte/thrombocyte phagocytic index in 1-d-old chicks from CXN hens was reduced compared to those from HC hens at 25 wk of hen age (P<0.001; Figure 4.2) but no other maternal treatment effects were seen at 1 d of age; 4-d-old chicks from HC hens had decreased lymphocyte/thrombocyte phagocytic index compared to other hen treatments. At 37 wk of age, 1-d-old chicks from HC hens had increased lymphocyte/thrombocyte phagocytic index compared to CXN and Control hens; no other differences between maternal treatments were observed at 1 d of age. Lymphocyte/thrombocyte phagocytic index in 4-d-old chicks from 25-OHD_3 hens was increased compared to other maternal treatments. At 49 wk of age, 1-d-old chicks from HC hens had higher lymphocyte/thrombocyte phagocytic index compared to Control hens, but no other maternal treatment effects were seen at 1 d of age and 4 d of age. Similar to total phagocytic index at 59 wk of age, decreased lymphocyte/thrombocyte phagocytic index in 1-d-old chicks from HC hens compared to Control and 25-OHD_3 hens was observed; at 4 d of age, there was increased lymphocyte/ thrombocyte phagocytic index from Control hens compared to CXN hens. No other interactions between maternal treatments and chick age were found. Chicks from hens inseminated by HC roosters had higher lymphocyte/thrombocyte phagocytic index compared to chicks from hen inseminated by Control roosters (Table 4.1).

Lymphocyte/thrombocyte phagocytic index increased with increasing chick age for the Control and CXN maternal treatment at 25 wk of age, but the opposite was seen for the HC treatment, and no difference between the different chick ages was seen for the 25-OHD_3 hens (Figure 4.2). A similar pattern as for total phagocytic index was observed at 37 wk of age, with decreased lymphocyte/thrombocyte phagocytic index with increasing chick age for all maternal

treatments except 25-OHD_3 hens, in which case there was no chick age effect. Decreased lymphocyte/thrombocyte phagocytic index with increasing chick age for 25-OHD_3 and HC hens was found but no differences were seen for other maternal treatments at 49 wk of age. There was decreased lymphocyte/thrombocyte phagocytic index with increasing chick age for 25-OHD_3 and CXN hens at 59 wk of age; no differences were seen between the two chick ages for any other maternal treatment.

There was decreased granulocyte/monocyte phagocytic index in 1-d-old chicks from Control hens compared to HC hens (P=0.005; Table 4.2), but the opposite was seen in 4-d-old chicks. Higher granulocyte/monocyte phagocytic index was seen at 1 d of age compared to 4 d of age for the HC treatment but the opposite was seen for the Control treatment. No other maternal effects, nor any paternal effects or interactions were seen for granulocyte and monocyte phagocytic index.

At 1 d of chick age, granulocyte/monocyte phagocytic index increased with increasing breeder age until 49 wk of breeder age and then remained constant to 59 wk of breeder age ($p<0.001$; Table 4.2). There was increased 4-d-old chick granulocyte/monocyte phagocytic index from 37 to 49 wk of age, which then remained the same to the end of the experiment (Table 4.2).

Chicks from HC hens inseminated by HC roosters had higher macrophage phagocytic index than from CXN hens inseminated by HC roosters and HC hens inseminated by Control roosters at 1 d of age, but chicks from HC hens inseminated by HC roosters had lower macrophage phagocytic index at 4 d of age compared to all other parental treatments (P=0.021; Figure 4.3). Four-d-old chicks from Control hens inseminated by HC roosters had higher phagocytic index compared to chicks from CXN hens inseminated by HC roosters and HC hens inseminated with Control roosters. No other interactions of parental treatment and chick age

were seen. Macrophage phagocytic index increased with increasing chick age for all hen and rooster treatments (Figure 4.3).

Macrophage phagocytic index in broilers at 1 d of age increased up to 49 wk of breeder age and declined at 59 wk of breeder age ($P<0.001$; Table 4.2). There was increasing macrophage phagocytic index in 4-d-old chicks with increasing breeder age. At 49 and 59 wk of age, 1 d old chicks had decreased macrophage phagocytic index compared to 4 d old chicks. No other interaction between chick age and breeder age was observed.

4.3.2 Phagocytic capacity

Total phagocytic capacity in 1-d-old chicks was higher from 25-OHD_3 hens compared to Control hens at 25 wk of age but no other maternal dietary treatment effect was observed at 1 d of age ($P=0.004$; Figure 4.4). Maternal treatment effect at 4 d of age was not observed. At 1 d of age, chicks from Control hens had increased phagocytic capacity compared to chicks from HC hens. Total phagocytic capacity was higher in 4-d-old chicks from CXN and HC hens compared to those from Control hens at 37 wk of age; no other interactions between maternal treatments were observed at 1 and 4 d of age. At 49 wk of age, no maternal treatment effects for 1-d-old total phagocytic capacity were found. Four-d-old chick total phagocytic capacity increased from Control and CXN hens compared to HC hens at 49 wk of age. No other maternal treatment effects were seen at 4 d of age. At 59 wk of age, no maternal treatment effects were seen at 1 d of age but 4-d-old chicks from Control hens had higher total phagocytic capacity compared to CXN hens. No other maternal treatment effects were found in 4-d-old chicks. Paternal treatment effects were not observed for total, lymphocyte/thrombocyte and macrophage phagocytic capacity (Table 4.3).

One-d-old chicks from Control hens had lower total phagocytic capacity compared to chicks from 25-OHD_3 hens at 25 wk of breeder age. No other differences between the different chick ages within each hen treatment for total phagocytic capacity at 25 wk of age were found (Figure 4.4). At 37 wk of age, there was decreased total phagocytic capacity with increasing chick age for Control, 25-OHD_3 and CXN hen treatments; there were no significant differences between the chick ages in chicks from HC hens. Total phagocytic capacity increased with increasing chick age at 49 wk of age for Control and CXN hens; no differences due to chick age for 25-OHD_3 and HC hens were found. At 59 wk of age, total phagocytic capacity decreased with increasing chick age for all hen treatments.

Maternal treatment had no effect on lymphocyte/thrombocyte phagocytic capacity (Table 4.3). For both 1 and 4 d of age, lymphocyte/thrombocyte phagocytic capacity increased until 37 wk of age, and then decreased after 49 wk of age (P=0.025; Table 4.4). Higher lymphocyte/thrombocyte phagocytic capacity was observed in 1-d-old chicks compared to 4-d-old chicks at 37 wk of age. No other significant differences between chick ages were seen within the different breeder ages.

No maternal treatment or chick age effects on granulocyte/monocyte phagocytic capacity were seen at 25 and 37 wk (Figure 4.5). At 49 wk of age, granulocyte/monocyte phagocytic capacity in 1-d-old chicks from Control and 25-OHD_3 hens increased compared to CXN and HC hens but no other maternal effects were seen in 1- or 4-d-old chicks. Granulocyte/monocyte phagocytic capacity increased with increasing chick age for CXN and HC hens, but no differences were observed for 25-OHD_3 and Control hens. At 59 wk of breeder age, 1-d-old chicks hatched from HC hens had higher granulocyte/monocyte phagocytic capacity compared to 25-OHD_3 and Control hens. One-d-old chicks from CXN hens had greater phagocytic capacity

compared to chicks from 25-OHD_3 hens at 59 wk of age. No other maternal effects were seen in 1- or 4-d-old chicks. No differences in granulocyte/monocyte phagocytic capacity were observed with increasing chick age within each hen treatment at 59 wk of age.

At 1 d of chick age, granulocyte/monocyte phagocytic index increased with increasing breeder age until 49 wk, and then decreased up to 59 wk of breeder age ($p<0.001$; Table 4.4). Granulocyte/monocyte phagocytic index in 4-d-old chick increased from 37 wk of age up to 49 wk of age and then decreased until the end of the experiment.

Macrophage phagocytic capacity increased in chicks from 25-OHD_3 and Control hens compared to HC hens at 49 wk of age. Chicks from 25-OHD_3 hens had higher macrophage phagocytic capacity compared chicks from CXN hens at 49 wk of age. No other differences were seen due to any other hen treatments within breeder ages ($P=0.008$; Table 4.4). Macrophage phagocytic capacity in chicks from 25-OHD_3 remained the same until 37 wk of age, increased at 49 wk of age, and then decreased at 59 wk of age. Chick macrophage phagocytic capacity from other hen treatments increased with increasing breeder age up to 49 wk of age and then remained the same until 59 wk of age (Table 4.4).

There was increased macrophage phagocytic capacity in 1-d-old chicks from 37 to 49 wk, but a decline after 59 wk of hen age ($P<0.001$; Table 4.4). Macrophage phagocytic capacity increased with increasing breeder age in 4-d-old chicks. At 37, 49 and 59 wk of age, 4-d-old chicks had higher macrophage phagocytic capacity compared to 1-d-old chicks but no differences between the chick ages were found at 37 and 25 wk of age.

4.3.3 Antioxidant capacity of broiler plasma

The data is missing for antioxidant capacity at 25 wk of breeder age because broiler plasma was not collected at 25 wk breeder age. There was no maternal ($P= 0.308$; data not

shown) nor paternal treatment effects on broiler antioxidant capacity (Figure 4.6). For each breeder age, chick antioxidant capacity at 14 d of age was higher than at 0 d of age (P=0.022; Figure 4.6). At 37 wk of breeder age, 14-d-old chicks had higher plasma antioxidant capacity than at 49 or 59 wk of age. The 0-d-old chick plasma antioxidant capacity was lower at 49 wk of age compared to other breeder ages.

4.4 DISCUSSION

Phagocytic index and capacity were measured at 1 and 4 d of age to investigate the effects of maternal dietary 25-OHD_3 and CXN on early broiler innate immunity. Previously in our lab, it was shown that maternal dietary 25-OHD_3 and CXN each increased broiler *E. coli* killing at 1 and 4 d of age (Saunders-Blades and Korver. 2015; Johnson-Dahl et al., 2017). Therefore, phagocytic index and capacity were measured at 1 and 4 d of age.

Overall, similar patterns as for total phagocytic index were seen for lymphocyte/thrombocyte phagocytic index because lymphocytes and thrombocytes constitute the majority of the white blood cells in chickens (Scanes, 2015) as well as the cell population analyzed in our experiment (over 90%). Lymphocytes are not professional phagocytes (Rabinovitch, 1995) therefore, lymphocyte phagocytic index was minimal; most phagocytic activity (approximately 35%) was due to thrombocytes which have toll-like receptors (**TLR)**. The TLR are type of pattern recognition receptor important for phagocytosis because they detect specific molecules on the pathogens such as lipopolysaccharides (**LPS;** Janeway Jr. and Medzhitov, 2002). Furthermore, thrombocytes increase phagocytic ability when stimulated with *E. coli* LPS (St. Paul et al., 2012). Since granulocyte/monocyte, and macrophage populations were less than 10% of the total population, the contribution to total phagocytic index were small for these cell types.

Generally, chick total, lymphocyte/thrombocyte, and macrophage phagocytic index from 25-OHD_3 breeder hens were higher compared to those from CXN and HC hens. Total phagocytic capacity in chicks from 25-OHD_3 hens was higher compared to Control hens at young breeder ages (25 wk of age). Breeder hens fed 25-OHD_3 transferred 25-OHD_3 more efficiently to the egg than breeders fed dietary vitamin D_3 (Coto et al., 2010). Chicken macrophages treated with 200 nM 25-OHD_3 and then stimulated with LPS *in vitro*, increased amount of 1-alpha-hydroxylase mRNA compared to chicken macrophages not treated and stimulated with LPS (Morris and Selvaraj, 2014). If chicks from 25-OHD_3 hens had a greater amount of macrophage 1-alpha-hydroxylase mRNA after stimulation of LPS than chicks from hens not fed with 25-OHD_3, the former may have had increased synthesis of 1,25$(OH)_2D_3$. The 1,25$(OH)_2D_3$ can induce immunoglobulin opsonized *E. coli* phagocytic index in human monocytes through an immunoglobulin-dependant mechanism (Xu et al., 1993). Hence, we expected chicks from 25-OHD_3 hens to have increased phagocytic index and capacity compared to chicks from hens not fed with 25-OHD_3. Maternal treatments did not affect egg 25-OHD_3 contents (Chapter 2) and broiler feed did not contain dietary 25-OHD_3, therefore egg 25-OHD_3 contents and broiler feed likely did not influence phagocytic capacity in the broiler chick. DNA methylation is an epigenetic mechanism, which occur by addition of methyl group to DNA molecule (Fetahu et al., 2014). The methylation of gene promotor regions generally represses gene expression (Fetahu et al., 2014). Dietary 25-OHD_3-fed breeders increased plasma 25-OHD_3 content (Coto et al., 2010); increasing human plasma 25-OHD_3 has been shown to increase human vitamin D receptor gene methylation (Beckett et al., 2016). If 25-OHD_3-fed breeders had increased plasma 25-OHD_3 content, vitamin D receptor gene methylation may be higher compared to breeders not fed 25-OHD_3. Vitamin D receptor deficiency reduced formyl-Nle-Leu-Phe-Nle-Tyr-Lys fluorescein

derivative chemotaxis in mouse macrophages (Mathieu et al., 2001). Chemotaxis is important for the movement of the phagocytes for instance, neutrophils and macrophages, toward bacterial attractants such as LPS and hence reduced chemotaxis may decrease phagocytosis (Aderem and Underhill, 1999). If breeders fed 25-OHD_3 had reduced immune cell chemotaxis due to increased vitamin D receptor methylation, then this could possibly be transferred to the chick. Hence chicks from 25-OHD_3-fed breeders might have reduced phagocytic index and capacity compared to chicks from breeders fed without 25-OHD_3. Phagocytosis index and capacity increased in chicks from 25-OHD_3 hens, which was the the opposite of what we expected in this study. Phagocytic index and capacity increased in chicks from 25-OHD_3 hens. Phagocytic capacity in chicks from 25-OHD_3-fed breeder hens increased compared to vitamin D_3-fed breeder hens at 61 to 63 wk of age (Saunders-Blades and Korver, 2015). Though the mechanism is unclear, this may indicate that maternal 25-OHD_3 increase chick phagocytic index and capacity.

Oxygen consumption and heat production in the embryo increases rapidly before hatch and therefore, newly hatched chicks can be prone to oxidative stress (Hamidu et al., 2007). Oxidative stress occurs when there is more generation of reactive oxygen species (**ROS**) than elimination of ROS (Akbarian et al., 2016). The membrane of immune cells, such as neutrophils, contains high amount of polyunsaturated fatty acids (Guerra and Otton, 2011). Hence when there is excess ROS due to oxidative stress, lipid peroxidation occurs in the immune cell membrane and damages the cell (Hughes, 1999). Human mononuclear cells exposed to ROS decreased expression of human leukocyte antigen-antigen D related, which is a major histocompatibility complex class II surface receptor (Gruner et al., 1986). Canthaxanthin, which is an antioxidant, may protect the immune cells from lipid peroxidation and hence immune cells may perform phagocytosis more efficiently. When ROS in human neutrophils were induced by high glucose

and free fatty acids *in vitro*, zymosan phagocytic capacity decreased compared to neutrophils treated without glucose and free fatty acids; addition of astaxanthin, which is a xanthophyll and an antioxidant similar to CXN, to neutrophils treated with glucose and free fatty acids prevented ROS production and recovered neutrophil zymosan phagocytic capacity *in vitro* (Guerra and Otton, 2011).Therefore, we were expecting chicks from CXN hens to have increased phagocytic index and phagocytic capacity compared to chicks from hens not fed with CXN. Maternal dietary treatment did not affect antioxidant capacity and hence, a maternal CXN effect on phagocytic index and capacity was not observed in our study. Eggs incubated under high temperature (39.6 °C for 6 h each day from 10 to 18 d incubation) had chicks hatched with lower BW compared to chicks from eggs incubated under standard temperature (37.8 °C from 1 to 18 d incubation; Yalçin et al., 2005). This suggests that oxidative stress was induced by heat stress in the chick embryos during incubation and hence resulted in lower hatch weight compared to chick embryos that were not under oxidative stress. No maternal dietary effect was seen in chick hatch weight in our study; furthermore, chick hatch weight was heavier in our study compared to the Aviagen guideline (Chapter 3; Aviagen, 2007a). Therefore, chicks in our study were not likely under oxidative stress and this may explain why we did not observe a maternal CXN effect on phagocytic index and capacity.

The chicks from hens inseminated by HC roosters had increased total and lymphocyte/thrombocyte phagocytic index compared to chicks from hens inseminated by Control roosters. Dietary 25-OHD_3 can alter phagocytosis; higher phagocytic index against *Salmonella typhimurium* was reported in chickens fed 25-OHD_3 compared to those fed diets without 25-OHD_3 (Chou et al., 2009). Roosters do not transfer nutrition to the egg but roosters transfer genetic information to the chicken embryo through the sperm. The sperm cell membrane

is mainly composed of polyunsaturated fatty acids (Surai et al., 1998) and hence it is susceptible to lipid peroxidation. Lipid peroxidation can produce reactive electrophiles such as 4-oxo-2-nonenal **(ONE)**, which can further react with lysine contained in histones to form irreversible adducts (Galligan et al., 2014). This causes disruption of gene transcription and chromosome replication in cells because histones are important for chromatin structure and function (Galligan et al., 2014). Human sperm cells produce interleukin-1α (Huleihel et al., 2000). Interleukin-1α is a cytokine which induces *E. coli* phagocytosis (Kabbur and et al., 1995). Therefore if the interleukin-1α gene is disrupted in the sperm due to irreversible adducts, phagocytic index may decrease in broiler chicks. Since CXN is an antioxidant, it may have prevented the lipid peroxidation in the sperm and reduced ONE production ultimately maintaining chromatin structure and gene expression of cytokines (Galligan et al., 2014) such as Interleukin-1α. It is unknown whether the mechanisms that disrupt gene expression in the sperm due to ONE would lead to decreased interleukin-1α production and ultimately decrease phagocytic index in broiler chicks.Therefore, the reason for the cause of higher phagocytic index in chicks hatched from hens mated to HC roosters than hens mated to Control roosters is unknown.

Overall, total, lymphocyte/thrombocyte and granulocyte/monocyte phagocytic indices in 1-d-old chicks from HC hens were higher than at 4 d of age. Breeders fed CXN efficiently deposited CXN in the egg than breeders not fed CXN (Chapter 2). Chicks hatched from carotenoid-containing eggs had high carotenoid content in the plasma and liver at 0 d of age, which declined by 7 d of age (Surai et al., 2003). This is because body CXN would have been used by the chick over time; CXN would have been diluted in the chick because broiler feed did not contain CXN. Cytochrome P450 activity in the chick liver, which is important for conversion of vitamin D_3 to 25-OHD_3, peaked at hatch and then declined rapidly after 1 d of age for a wk

(Hu, 2013). The yolk sac is absorbed during the first few days of the chick's life (Noy et al. 1996). Therefore, chicks may rely on 25-OHD_3 contents in the yolk sac up to 7 d post-hatch instead of vitamin D_3 in the broiler feed. Plasma 25-OHD_3 levels were lower at 4 d of age compared to 1 d of age (Saunder-Blades and Korver, 2014). This could possibly be because the yolk sac, which provides 25-OHD_3 to the chick, was mostly absorbed by the chick at hatch (Şahan et al., 2014) and the 25-OHD_3 was gradually getting used by the chick. The 25-OHD_3 was converted to 1,25$(OH)_2D_3$ in the monocytes and possibly the thrombocytes through 1-alpha-hydroxylase (Liu et al., 1996). The 1,25$(OH)_2D_3$ could then bind to vitamin D receptor and form a heterodimer with a nuclear receptor-associated with CXN, retinoid X receptor, at the vitamin D_3 response element. This increased heterodimer formation with retinoid X receptor, may have increased differentiation of myeloid cells to monocytes and thrombocytes (Liu et al., 1996); ultimately increasing phagocytic index in our study. However, lymphocyte/thrombocyte and granulocyte/monocyte population was not different between 1 and 4 d old chicks from HC hens (data not shown). Therefore, it is unlikely due to increased differentiation of myeloid cells to monocytes and thrombocytes and the cause of increased phagocytic index in chicks at 1 d of age compared to 4 d of age from HC hens is unknown.

Chick age did not affect total phagocytic capacity. This is surprising because we expected phagocytic capacity to be lower in 1-d-old chicks compared to 4-d-old chicks; heterophil phagocytosis increased with increasing chick age because heterophils are immature in chicks at 1 d of age compared to 7 d of age (Wells et al., 1998). Furthermore, 4 d old *E. coli* phagocytic capacity was higher than 1 d old phagocytic capacity in our previous study (Chapter 5). Therefore, the reason for the lack of chick age effect on phagocytic capacity is unclear.

Generally, total, lymphocyte/thrombocyte, granulocyte/monocyte phagocytic index increased with increasing breeder age for all chick ages. Total yolk lipid transferred into the chicken embryo is less from young breeders at 25 wk of age compared to middle aged breeders at 41 wk of age (Noble et al., 1986). Since lipid serves as a primary energy source during embryogenesis (Noble and Cocchi, 1990), the reduced lipid mobilization in the embryo from young breeders may result in embryos with insufficient energy and nutrients as shown by reduced hatchability (Chapter 2) and increased first-wk chick mortality (Yassin et al., 2009) in young breeders compared to middle aged breeder. Furthermore, chicks from young breeders have lower BW and feed intake compared to chicks from middle aged breeders (Chapter 3; Maiorka et al., 2004). Hence, chicks that hatched from young breeders may be energy deficient which may have reduced post-hatch granulocyte and monocyte phagocytic index, as phagocytosis is an energy-demanding process (Chandak et al., 2010). Chicks hatched from older breeders (52 wk of age) have reduced yolk absorption compared to middle-aged breeders (36 wk of age; Şahan et al., 2014). Similar to chick embryos from young breeders, reduced yolk absorption in chicks from old breeders (Şahan et al., 2014) may result in chicks which are energy-deficient compared to middle-aged breeders. This may have resulted in decreased phagocytic capacity in chicks from old breeder age as seen in our study; chicks from young breeder age (25 wk of age) and old breeder age (59 wk of age) had decreased total, lymphocyte and thrombocyte and granulocyte and monocyte phagocytic capacity compared to the middle aged breeders (49 wk of age). The cause for the increased phagocytic index in chicks from old breeder age (59 wk of age) is unknown.

There was no parental treatment effect on broiler plasma antioxidant capacity. Oxygen consumption, heat production and heart ROS production in the chick embryo increases rapidly

before hatch (Hamidu et al., 2007; Zhao et al., 2017), hence newly-hatched chicks may be prone to oxidative stress. Chick plasma antioxidant capacity at hatch was higher from breeder hens fed 6 mg/kg CXN compared to breeder hens not fed CXN (Surai, 2012; Johnson-Dahl et al., 2017). The reason for lack of maternal treatment effects on plasma antioxidant capacity at 0 d of age in the current study is unclear. Paternal treatment effect on antioxidant capacity was not seen at 14 d of age because the chicks were housed at recommended temperatures as listed in the Aviagen guideline (Aviagen, 2007a) and had low stocking density (Sun et al., 2013). This in agreement with Zhang et al. (2011), who did not see differences between the total antioxidant capacity at 1 and 7 d of age in chicks hatched from hens fed CXN compared to hens not fed CXN when chicks were raised under optimal temperature and stocking density. Canthaxanthin is an antioxidant and a free radical scavenger (Mortensen et al., 1997). If chicks were under oxidative stress, we would expect CXN to be used or have an effect on antioxidant capacity; Plasma antioxidant capacity in 5 wk old broilers decreased when broilers were under heat stress (32 °C for 6 h) compared to broilers under normal thermal condition (21 °C; Lin et al., 2006). Therefore, the chicks may have had low levels of oxidative stress in our study and the parental treatment effects were not seen on antioxidant capacity at 14 d of age.

Chick plasma antioxidant capacity was lower at 0 d of age compared to 14 d of age. Chicks hatched from carotenoid-containing eggs have higher carotenoid plasma contents at 0 d of age, which declined by 7 d of age (Surai et al., 2003). This is because chicks will use carotenoids over time and body carotenoid stores will be diluted as the chick grows (Surai et al., 2003). Therefore, we were expecting a similar effect on chick plasma antioxidant capacity; 0 d chick plasma antioxidant to be higher than 14 d. Dietary vitamin A (12,500 IU/kg) and vitamin E (50 IU/kg) fed to each broiler breeder hen (0.132 kg/d/hen) on average was 6.6 and 1,650 IU/d,

respectively (Chapter 2). The breeder hens laid on average 0.533 eggs/d and average egg weight was approximately 63 g and therefore daily egg mass output was 33.58 g of egg/hen/d (Chapter 2). Assuming a transfer efficiency of 70% vitamin A and 25% vitamin E from hen feed to the egg (Cherian, 2006), 1,155 IU/d and 1.64 IU/d would be transferred, respectively. Therefore, each egg would have contained 34 IU/g of egg and 0.05 IU/g of egg of vitamin A and vitamin E, respectively. Broilers on average ate 0.0028 kg/d/ bird of feed containing 10,000 IU/kg vitamin A and 35 IU/kg vitamin E or approximately 28 IU/d/bird and 0.098 IU/d/ bird, respectively (Chapter 3). On average, broiler 10 d BW was 280 g (Chapter 3) and hence the daily gain was 28 g/d. If we assumed that vitamins A and vitamin E were 100 % and 84 %, repectively, absorbed from the feed, 28 IU/d vitamins A and 0.082 IU/d vitamin E would be transferred and deposited into the broiler body, respectively (Sklan et al., 1982; Surai et al., 2001). Therefore vitamin A and vitamin E contents in the broiler would be 1.0 IU/g BW and 0.0029 IU/g BW, respectively. Egg vitamin A and vitamin E contents per g of egg (34 IU/g of egg vitamin A and 0.05 IU/g of egg of vitamin E) were higher than per g of broiler chicken BW (1.0 IU/g BW of vitamin A and 0.0029 IU/g BW of vitamin E). Therefore vitamin A and vitamin E in the broiler feed likely would not be responsible for the greater antioxidant capacity at 14 d than at 0 d in the chick. Eighty one percent of the yolk sac is already absorbed by the chick at hatch (Şahan et al., 2014). Hence, the low antioxidant capacity at 0 d of age was also unlikely due to the delayed absorption of the yolk sac by the chick. Uric acid and glutathione are antioxidants; plasma levels of uric acid and glutathione in chicks at 0 d of age were lower than at 14 d of age (Pflanz and Goodman, 1970; Lin et al., 2004). Although, plasma uric acid and glutathione levels may have increased antioxidant capacity at 14 d of age, it is unclear if these antioxidants alone would make

antioxidant capacity at 14 d of age higher than 0 d of age. Therefore, the cause of increased antioxidant capacity at 14 d of age compared to 0 d of age is unknown.

Overall, maternal dietary 25-OHD_3 increased total phagocytic index and capacity. Since egg 25-OHD_3 content was not different between maternal treatments (Chapter 2) and broilers were not fed 25-OHD_3, we were not expecting a maternal 25-OHD_3 effect on phagocytic index and capacity and the cause of this is unknown. Generally, no maternal dietary CXN effect was seen on chick phagocytic index and capacity. This could be because chicks were raised under a controlled environment and hence low oxidative stress. Therefore, no maternal dietary effect was seen in chick plasma antioxidant capacity. The chicks from hens inseminated by HC roosters had increased total and lymphocyte/thrombocyte phagocytic index compared to chicks from hens inseminated by Control roosters. This indicates that paternal dietary CXN and 25-OHD_3 increase phagocytic index in broilers early in life, possibly due to an epigenetic mechanism.

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4.6 TABLES

Table 4.1 Main effects of hen diet, rooster diet and broiler chick age on whole blood phagocytic index to *Escherichia coli*[1].

Hen treatment	Male treatment	Chick age (d)	Breeder age (wk)	n	Phagocytic index			
					----------%----------			
					Total	Lymphocyte/ thrombocyte	Granulocyte/ monocyte	Macrophage
Control[2]				80	37.3[ab]	36.7[ab]	47.2	58.5[a]
25-OHD_3[3]				80	38.0[a]	37.7[a]	48.7	58.1[a]
CXN[4]				80	36.5[b]	36.0[b]	47.0	57.0[ab]
HC[5]				80	36.7[b]	36.2[b]	46.8	55.6[b]
SEM					0.39	0.38	0.60	0.75
	Control[6]			160	36.7[b]	36.2[b]	46.9	57.4
	HC[7]			160	37.6[a]	37.1[a]	48.0	57.1
SEM					0.28	0.28	0.42	0.53
		1		160	38.5[a]	38.0[a]	47.5	49.2[b]
		4		160	35.8[b]	35.4[b]	47.3	65.4[a]
SEM					0.63	0.62	0.96	1.20
			25	80	34.7[c]	34.9[c]	40.9[c]	43.2[d]
			37	80	35.8[c]	34.9[c]	42.8[b]	49.1[c]
			49	80	37.8[b]	37.1[b]	53.8[a]	74.1[a]
			59	80	40.3[a]	39.7[a]	52.2[a]	62.7[b]
SEM					0.40	0.44	0.62	0.77
					----------Probability----------			
Hen					0.007	0.008	0.104	0.035
Rooster					0.016	0.026	0.066	0.687
Chick age					0.027	0.030	0.951	<0.001
Breeder age					<0.001	<0.001	<0.001	<0.001

[a-d]Means with no common letters within the same column are significantly different ($P<0.05$).

[1]Proportion of phagocytic cells that engulfed at least 1 *Escherichia coli* bioparticle *ex vivo*.

[2]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.

[3]25-OHD_3 hens were fed the Control diet with 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.

[4]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[5]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[6]Control roosters were fed the same diet as the Control hens.
[7]HC roosters were fed the same diet as the HC hens.

Table 4.2 Interactions of hen, broiler chick age and breeder age on whole blood phagocytic index to *Escherichia coli* [1].

Hen treatment	Chick age (d)	Breeder age (wk)	n	Phagocytic index			
				----------%----------			
				Total	Lymphocyte/ thrombocyte	Granulocyte/ monocyte	Macrophage
Control[2]	1		40	37.9^{bcd}	37.2^{bc}	46.3^{cd}	49.5^{c}
Control	4		40	36.7^{bcd}	36.3^{abc}	48.1^{ab}	67.4^{a}
25-OHD$_3$[3]	1		40	39.3^{ab}	38.8^{a}	48.1^{abc}	49.3^{c}
25-OHD$_3$	4		40	37.2^{abc}	36.7^{ab}	49.3^{ab}	66.9^{a}
CXN[4]	1		40	37.4^{cd}	36.9^{bc}	46.9^{abc}	48.2^{c}
CXN	4		40	35.5^{de}	35.0^{c}	47.1^{abc}	65.7^{a}
HC[5]	1		40	39.4^{a}	39.0^{a}	48.6^{ab}	49.8^{c}
HC	4		40	34.0^{e}	33.4^{d}	44.9^{cd}	61.5^{b}
SEM				0.78	0.76	1.22	1.50
	1	25	40	34.4^{de}	34.2^{de}	40.6^{c}	42.6^{f}
	1	37	40	39.0^{bc}	38.1^{bc}	45.5^{b}	47.6^{de}
	1	49	40	39.0^{bc}	38.5^{bc}	52.2^{a}	68.1^{c}
	1	59	40	41.5^{a}	41.1^{a}	51.5^{a}	38.6^{g}
	4	25	40	35.0^{d}	35.6^{d}	41.1^{c}	43.8^{ef}
	4	37	40	32.6^{e}	31.8^{e}	40.1^{c}	50.7^{d}
	4	49	40	36.7^{cd}	35.8^{cd}	55.3^{a}	80.1^{b}
	4	59	40	39.1^{ab}	38.3^{ab}	52.9^{a}	86.8^{a}
SEM				0.73	0.87	1.23	1.35
				----------Probability----------			
Hen x Rooster				0.892	0.822	0.773	0.349
Hen x Chick Age				0.001	<0.001	0.005	0.008
Rooster x Chick Age				0.193	0.223	0.124	0.298
Hen x Breeder age				0.001	<0.001	0.254	0.585
Rooster x Breeder age				0.177	0.133	0.178	0.309
Chick age x Breeder age				<0.001	<0.001	<0.001	<0.001
Chick age x Hen x Rooster x Breeder age				0.755	0.850	0.342	0.161

[a-g]Means with no common letters within the same column are significantly different (P<0.05).
[1]Proportion of phagocytic cells that engulfed at least 1 *Escherichia coli* bioparticle *ex vivo*.
[2]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.

[3]25-OHD_3 hens were fed the Control diet plus 69 μg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.
[4]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[5]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

Table 4.3 Main effects of hen, rooster, broiler chick age and breeder age on whole blood phagocytic capacity to *Escherichia coli* [1].

Hen treatment	Male treatment	Chick age (d)	Breeder age (wk)	n	Mean fluorescence intensity			
					Total	Lymphocyte/ thrombocyte	Granulocyte/ monocyte	Macrophage
Control[2]				80	14,852	13,682	28,311	74,159
25-OHD_3[3]				80	15,088	13,092	27,904	71,648
CXN[4]				80	14,678	12,698	27,399	71,766
HC[5]				80	14,778	12,846	26,795	68,502
SEM					188	379	1005	2655
	Control[6]			160	14,719	13,092	27,794	73,541
	HC[7]			160	14,979	13,067	27,411	69,496
SEM					133	269	713	1883
		1		160	15,404	14,283[a]	25,317	44,969[b]
		4		160	14,293	11,876[b]	29,888	98,068[a]
SEM					302	611	1619	4243
			25	80	13,555[b]	12,183[b]	16,057[d]	24,681[c]
			37	80	16,068[a]	14,610[a]	20,406[c]	42,638[b]
			49	80	16,406[a]	14,957[a]	42,865[a]	112,753[a]
			59	80	13,366[b]	10,568[c]	31,081[b]	106,003[a]
SEM					219	438	1037	2737
					----------Probability----------			
Hen					0.464	0.275	0.736	0.515
Rooster					0.168	0.947	0.704	0.130
Chick Age					0.054	0.039	0.137	<0.001
Breeder age					<0.001	<0.001	<0.001	<0.001

[a-d]Means with no common letters within the same column are significantly different (P<0.05).
[1]Proportion of *Escherichia coli* fluorescent bioparticles that were engulfed per phagocytic cell *ex vivo*.
[2]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.
[3]25-OHD_3 hens were fed the Control diet plus 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.
[4]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

[5]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[6]Control roosters were fed the same diet as the Control hens.
[7]HC roosters were fed the same diet as the HC hens.

Table 4.4 Interactions of hen diet, rooster diet and broiler chick age on whole blood phagocytic capacity to *Escherichia coli* [1].

Hen treatment	Chick age (d)	Breeder age (wk)	n	Mean fluorescence intensity			
				Total	Lymphocyte/ thrombocyte	Granulocyte/ monocyte	Macrophage
Control[2]		25	20	12,898^{ef}	11,885	14,003^{g}	21,895^{e}
Control		37	20	15,815^{a}	14,466	19,972^{ef}	47,026^{d}
Control		49	20	16,658^{a}	17,042	49,275^{a}	120,583^{ab}
Control		59	20	14,037^{bc}	11,334	29,994^{c}	107,132^{bc}
25-OHD_3[3]		25	20	13,973^{bcd}	12,555	16,748^{efg}	23,869^{e}
25-OHD_3		37	20	16,309^{a}	14,836	19,656^{ef}	36,576^{de}
25-OHD_3		49	20	16,276^{a}	13,645	47,198^{a}	127,217^{a}
25-OHD_3		59	20	13,793^{bcde}	11,330	28,015^{cd}	98,930^{c}
CXN[4]		25	20	13,120^{cdef}	11,510	15,872^{fg}	25,670^{e}
CXN		37	20	16,145^{a}	14,577	22,447^{de}	44,369^{d}
CXN		49	20	16,780^{a}	14,944	38,113^{b}	106,010^{bc}
CXN		59	20	12,668^{f}	9,761	33,162^{bc}	111,014^{bc}
HC[5]		25	20	14,231^{b}	12,780	17,606^{efg}	27,289^{e}
HC		37	20	16,003^{a}	14,559	19,547^{efg}	42,580^{d}
HC		49	20	15,911^{a}	14,198	36,874^{b}	97,202^{c}
HC		59	20	12,964^{def}	9,847	33,153^{bc}	106,935^{bc}
SEM				382	775	2018	5328
	1	25	40	13,354^{def}	12,644^{bc}	14,088^{e}	19,202^{f}
	1	37	40	17,560^{a}	16,633^{a}	18,798^{d}	15,002^{f}
	1	49	40	15,836^{b}	15,82 ^{a}	37,961^{b}	91,609^{c}
	1	59	40	14,868^{c}	12,024^{bcd}	30,421^{c}	54,063^{e}
	4	25	40	13,757^{e}	11,721^{c}	18,027^{de}	30,160^{f}
	4	37	40	14,577^{cd}	12,58^{bc}	22,013^{d}	70,274^{d}
	4	49	40	16,977^{ab}	14,085^{ab}	47,769^{a}	133,898^{b}
	4	59	40	11,863^{f}	9,112^{d}	31,741^{bc}	157,942^{a}
SEM				406	711	1799	4750
				----------Probability----------			
Hen x Rooster				0.324	0.236	0.767	0.108
Hen x Chick age				0.279	0.678	0.878	0.813
Rooster x Chick age				0.272	0.466	0.274	0.164
Hen x Breeder age				0.024	0.146	<0.001	0.008
Rooster x Breeder age				0.232	0.887	0.104	0.702
Chick age x Breeder age				<0.001	0.025	0.021	<0.001
Chick age x Hen x Rooster x Breeder age				0.450	0.624	0.102	0.287

[a-g]Means with no common letters within the same column are significantly different (P<0.05).

[1]Proportion of *Escherichia coli* fluorescent bioparticles that were engulfed per phagocytic cell *ex vivo*.
[2]Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3.
[3]25-OHD_3 hens were fed the Control diet plus 69 μg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3.
[4]CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).
[5]HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

4.7 FIGURES

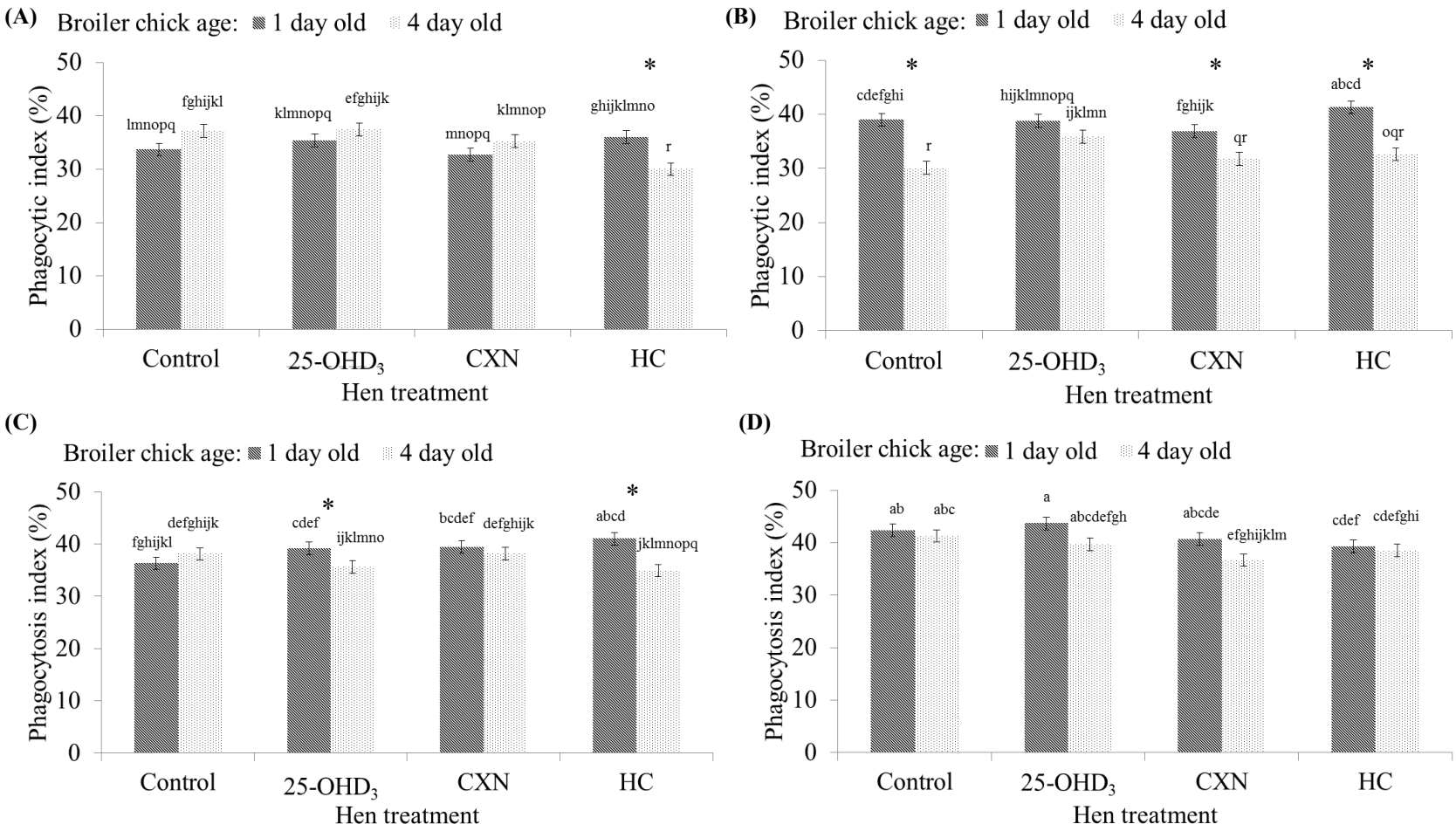

Figure 4.1 Interaction between hen treatment, broiler chick age and breeder age on total phagocytic index ($P<0.001$; n=10) at (A) 25, (B) 37, (C) 49, and (D) 59 wk of age. Phagocytic index is the proportion of phagocytic cells that engulfed at least 1 *Escherichia coli* bioparticle *ex vivo*. Means with no common letters are significantly different ($P<0.05$) for all hen treatment, broiler chick age and

breeder age interaction. Chick ages that are significantly different within maternal treatment in each breeder age ($P<0.05$) are indicated with an asterisk. Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the Control diet plus 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

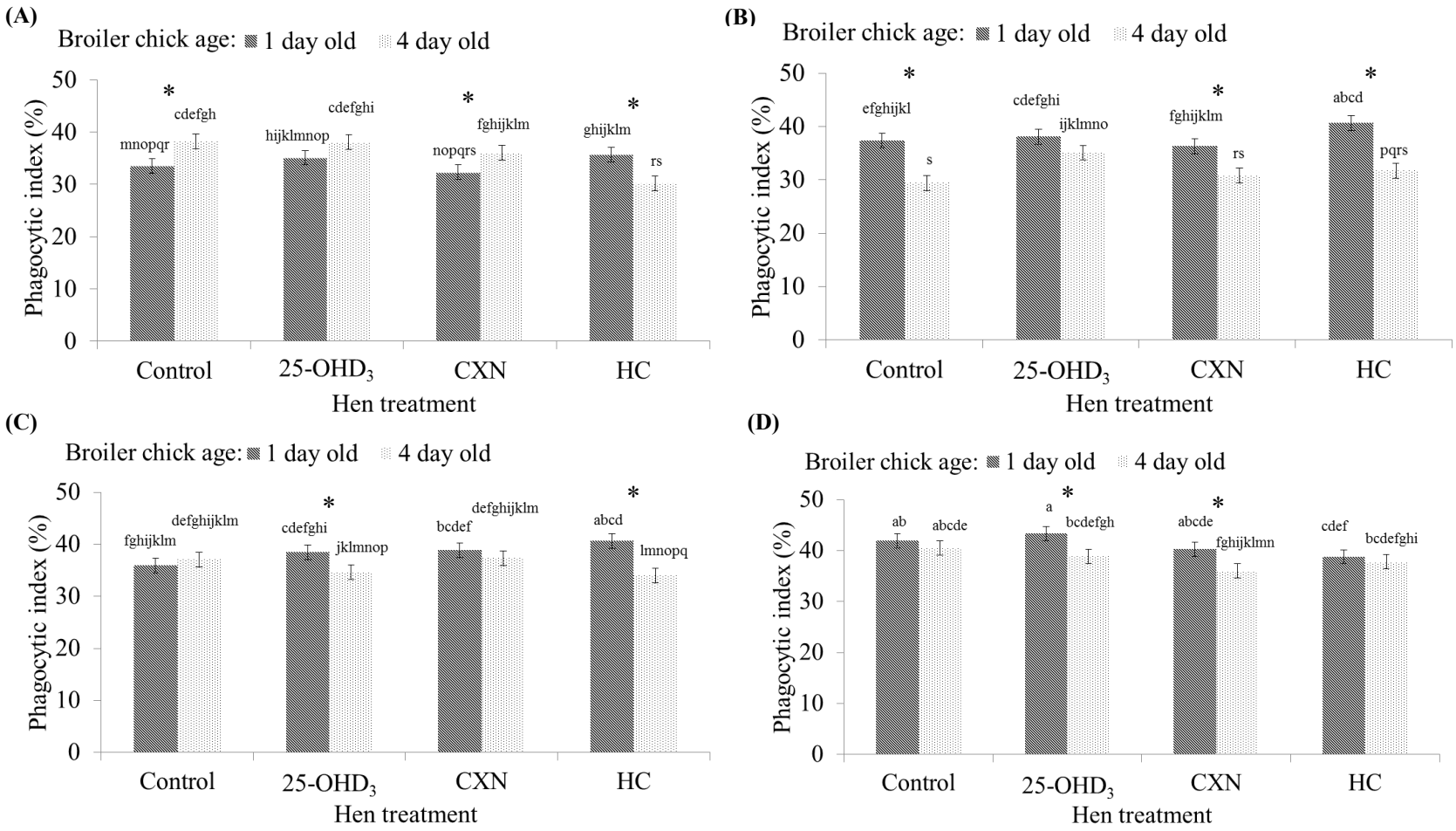

Figure 4.2 Interaction between hen treatment, broiler chick age and breeder age on lymphocyte and thrombocyte phagocytic index ($P<0.001$; n=10) at (A) 25, (B) 37, (C) 49, and (D) 59 wk of age. Means with no common letters are significantly different ($P<0.05$) for all hen treatment, broiler chick age and breeder age interaction. Chick ages that are significantly different within maternal treatment in each breeder age ($P<0.05$) are indicated with an asterisk. Proportion of phagocytic cells that engulfed at least 1 *Escherichia coli* bioparticle *ex vivo*. Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the Control diet plus 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional

Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

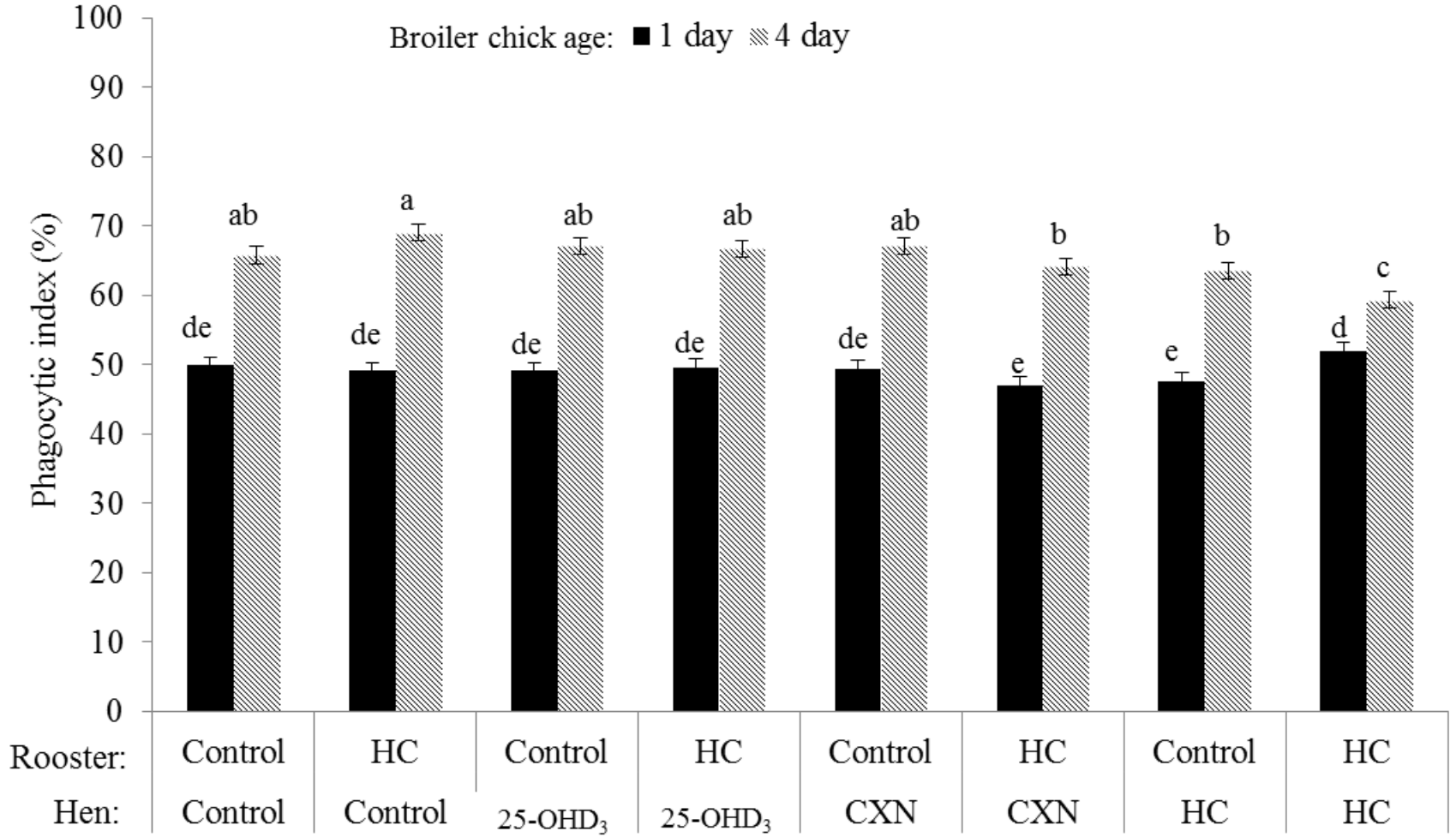

Figure 4.3 Interaction between hen treatment, male treatment and chick age on macrophage phagocytic index (P=0.025; n=20) at 1 d and 4 d of age. Means with no common letters are significantly different (P<0.05). Proportion of phagocytic cells that engulfed at least 1 *Escherichia coli* bioparticle *ex vivo*. Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the Control diet plus 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM

Nutritional Products Ltd., Parsippany, NJ). Control roosters were fed the same diet as the Control hens. HC roosters were fed the same diet as the HC hens.

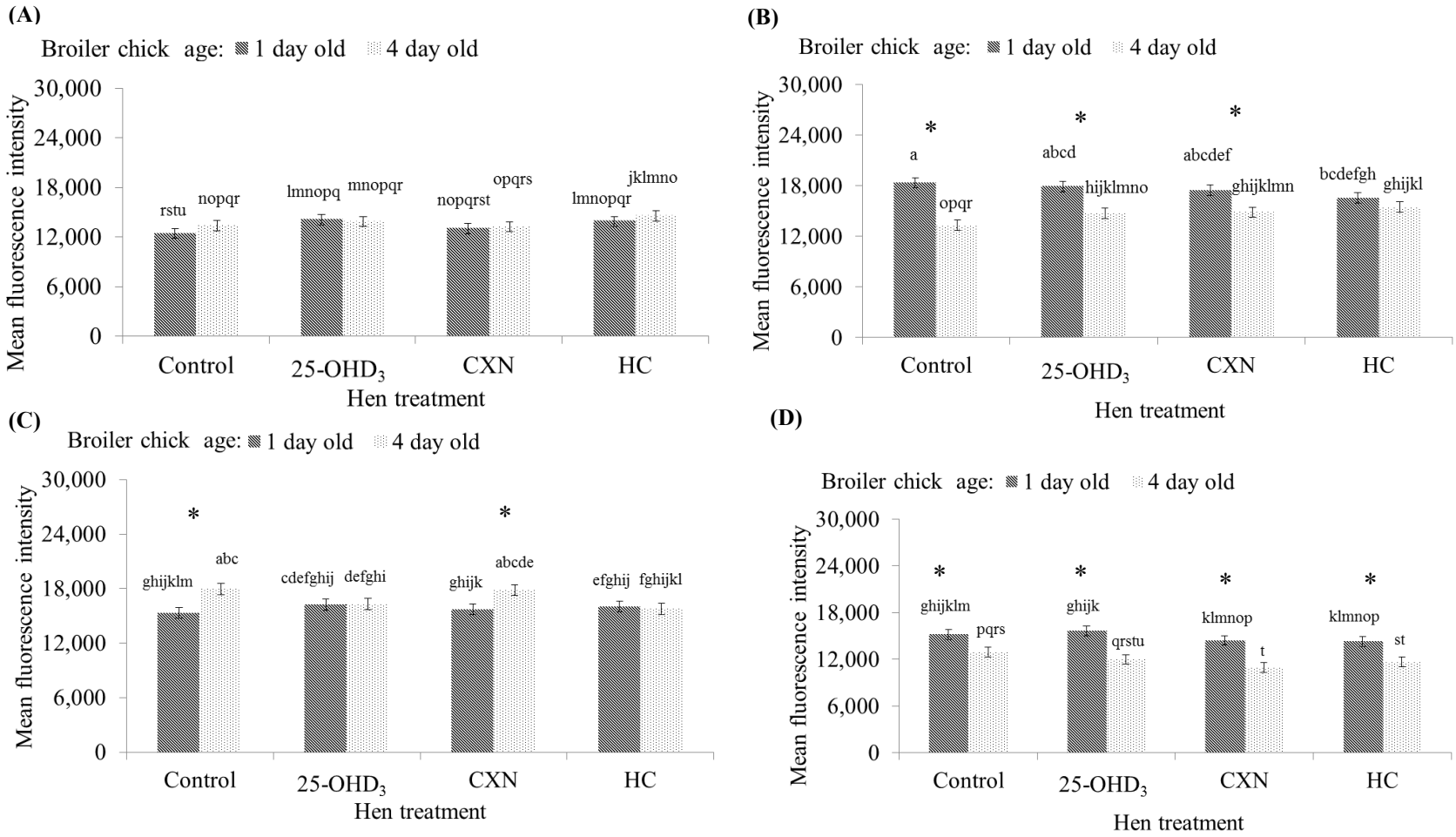

Figure 4.4 Interaction between hen treatment, broiler chick age and breeder age on total phagocytic capacity (P=0.004; n=10) at (A) 25, (B) 37, (C) 49, and (D) 59 wk of age. Means with no common letters are significantly different (P<0.05) for all hen treatment, broiler chick age and breeder age interaction. Chick ages that are significantly different within maternal treatment in each breeder age (P<0.05) are indicated with an asterisk. Proportion of *Escherichia coli* fluorescence bioparticles that were engulfed per phagocytic cell *ex vivo*. Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the Control diet plus 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

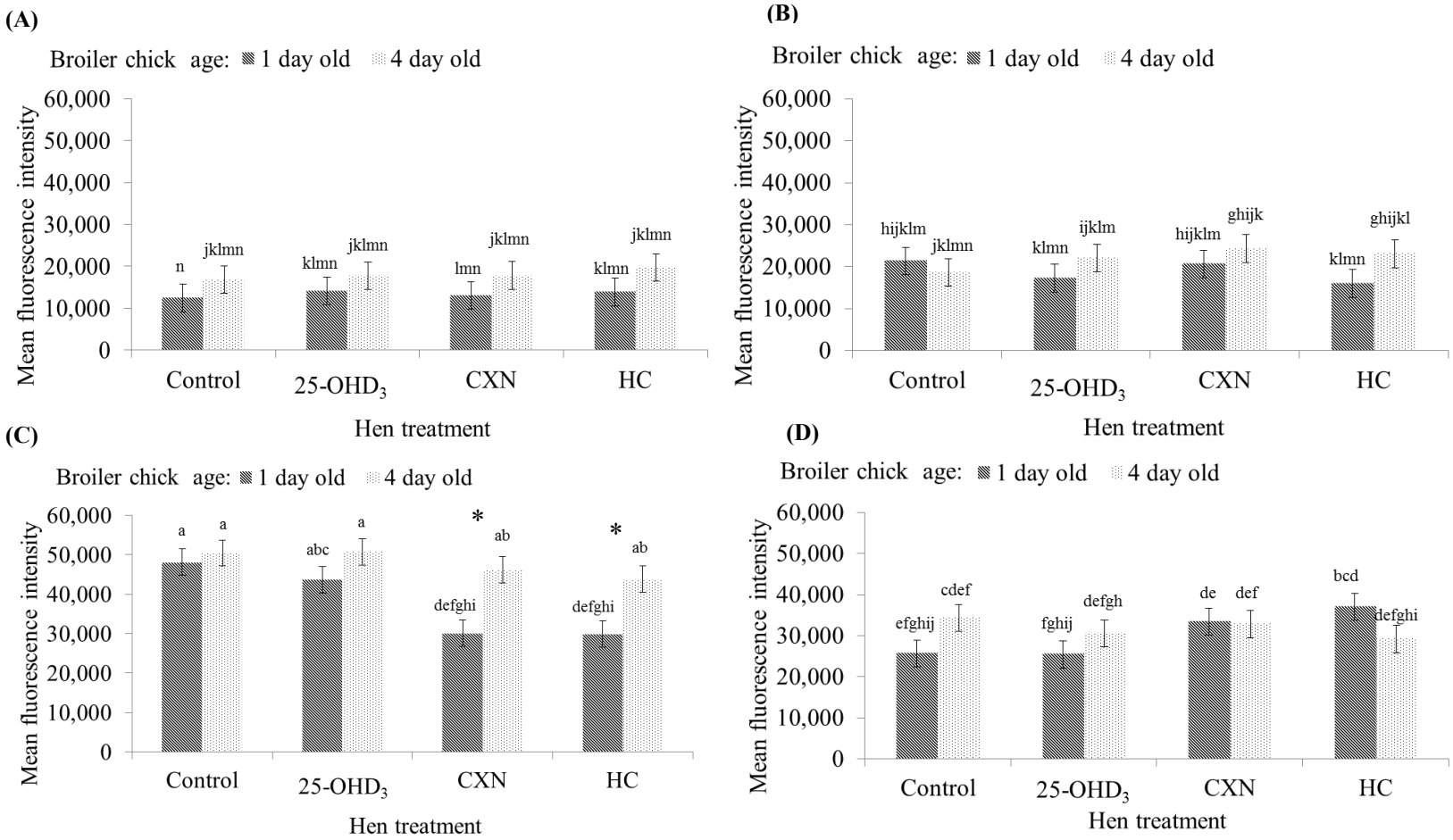

Figure 4.5 Interaction between hen treatment, broiler chick age and breeder age on granulocyte/monocyte phagocytic capacity (P=0.020; n=10) at (A) 25, (B) 37, (C) 49, and (D) 59 wk of age. Means with no common letters are significantly different (P<0.05) for all hen treatment, broiler chick age and breeder age interaction. Chick ages that are significantly different within maternal treatment in each breeder age (P<0.05) are indicated with an asterisk. Proportion of *Escherichia coli* fluorescence bioparticles that were engulfed per phagocytic cell *ex vivo*. Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the Control diet plus 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd.,

Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ).

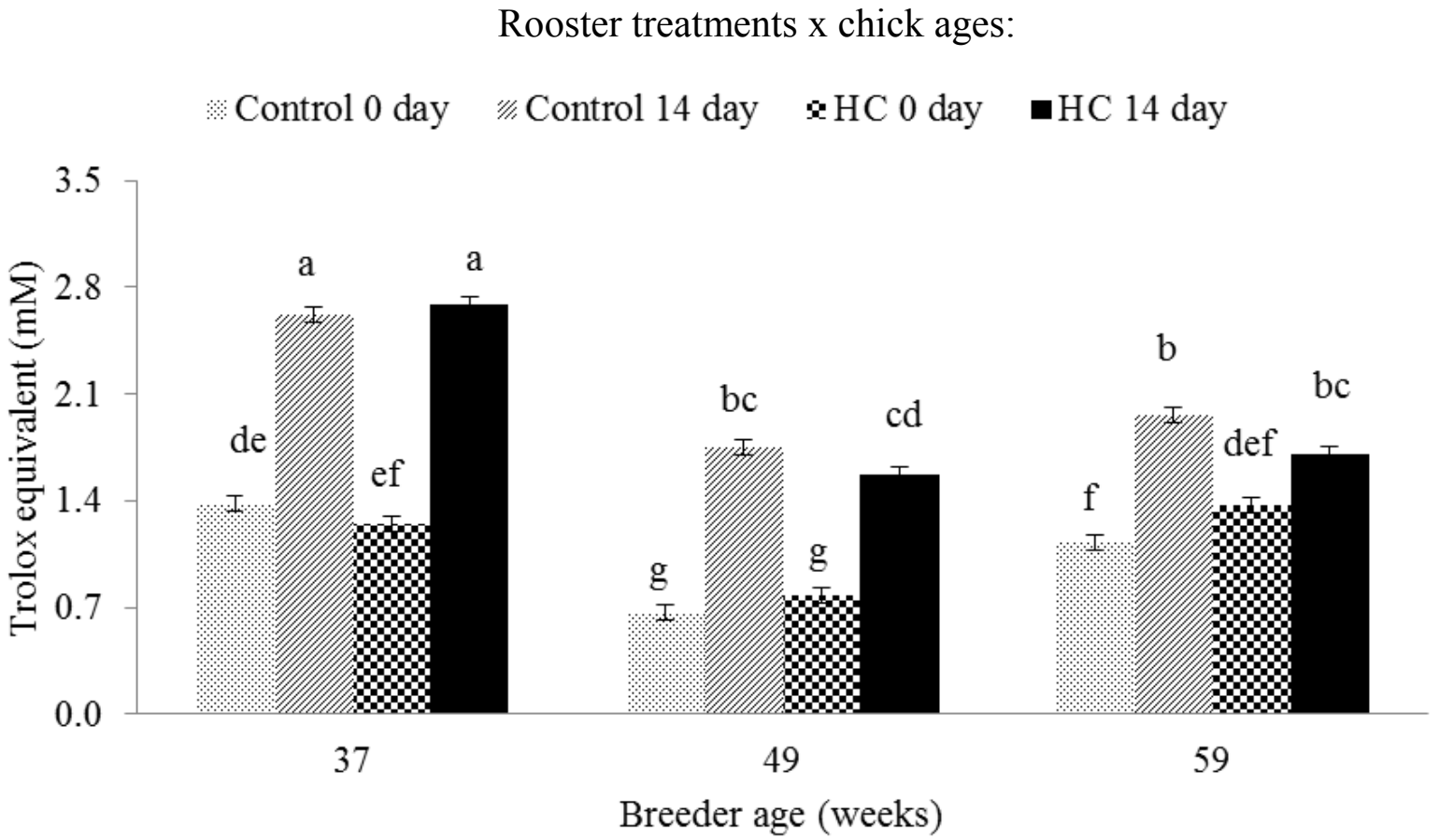

Figure 4.6 Interaction of rooster diet, breeder age and broiler chick age on plasma antioxidant capacity in 0 and 14 d old broiler chick (P=0.023; n=32). Means with no common letters are significantly different (P<0.05). Control hens were fed a nutritionally complete diet containing 2,760 IU of supplemental vitamin D_3. 25-OHD_3 hens were fed the Control diet plus 69 µg 25-OHD_3 per kg of feed (HyD, DSM Nutritional Products Ltd., Parsippany, NJ) in place of vitamin D_3. CXN hens were fed the Control diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). HC hens were fed the 25-OHD_3 diet plus 6 mg CXN per kg of feed (Carophyll-Red, DSM Nutritional Products Ltd., Parsippany, NJ). Antioxidant capacity data is missing for 25 wk breeder age.

5. DEVELOPMENT OF A HETEROPHIL EXTRACELLULAR TRAP ASSAY AS A MEASURE OF *EX VIVO* INNATE IMMUNITY AGAINST *ESCHERICHIA COLI*

ABSTRACT

Young chicks rely heavily on innate immunity early in life as adaptive immunity is immature. However, relatively little is known about immune system development in the young chick. Chicken heterophils release extracelluar DNA to trap and ultimately kill invading pathogens, a mechanism called heterophil extracellular trap (**HET**). A HET assay was developed in young chicks to assess the involvement of the HET in early chick innate immune function. First, an *ex vivo* assay to measure HET was developed using blood from Single-Comb White Leghorn hens. When the method has been validated, the assay was used to assess the effect of chick age and sex on *ex vivo Escherichia coli* (***E. coli***) bactericidal activity, phagocytic index and capacity, and HET release in response to *E. coli* at 1, 4, 6 and 8 d of age in Ross 308 chicks. Phagocytic index was the proportion of phagocytic cells that engulfed at least 1 fluorescent *E. coli* particle and phagocytic capacity was the average relative number of *E. coli* fluorescent particles engulfed by one phagocytic cell. Bacteria killing was greater at 1, 4 and 6 (64.23%, 72.89 %, 82.08%, respectively) as compared to 8 d of age (31.61%). Overall total, leukocyte, thrombocyte, lymphocyte, granulocyte and monocyte phagocytic index and capacity increased with increasing age. The HET release in response to *E. coli* was greater at 1 d (51.01%) than at any other age (23.96 to 29.76 %). The HET release in response to *E. coli* was higher in male than in female chicks, and bacteria killing tended to be greater in male compared to female chicks (P=0.058). Overall, no sex effect was seen for total phagocytic index or capacity. Therefore, different innate immunity mechanisms are more effective at killing *E. coli* at different ages. Based on the second experiment, the HET assay developed is useful to measure white blood cell extracellular trap

production in chicks. The HET response may play an important role in early chick immune protection.

Key words: innate immunity, heterophil extracellular trap, age, broiler chicken, sex

5.1 INTRODUCTION

The immune system is important to protect the host against pathogens and can be divided into two components: innate immunity and adaptive immunity. The innate immune system has many defense mechanisms to protect the host against pathogens; for instance, phagocytosis, oxidative burst and heterophil extracellular trap (**HET**; Chuammitri et al., 2009; Saunders-Blades and Korver, 2015).

Heterophils are the most abundant white blood cells present in the chicken and are similar in function to neutrophils in mammals (Shini et al., 2008). Although chicks at 0 d of age have greater numbers of heterophils than at 8 d of age, younger chicks are more susceptible to diseases compared to older ones (Zulkifli and Siegel, 1994). *Escherichia coli* (***E. coli***) killing and phagocytosis in 1-d old broiler chicks was lower than 4 d old chicks (Saunders-Blades and Korver, 2015; Johnson-Dahl et al., 2017). This suggests that newly-hatched chicks have immature innate immune functions.

Innate immunity is affected by sex of the chicken, as four wk old layer pullets had higher inducible nitric oxide synthase (**iNOS**) gene expression in the liver after LPS challenge than layer cockerels (Kleinert et al., 2004; Wils-Plotz and Klasing, 2017). Increase in iNOS gene expression also increases nitric oxide production (Kleinert et al., 2004) and hence female chickens have greater nitric oxide production compared to male chickens after LPS stimulation.

Neutrophil extracellular trap, a release of extracellular DNA to trap and kill pathogens, was found in many species including humans (Branzk et al., 2014), bovines (Jerjomiceva et al., 2014) and mice (Etulain et al., 2015). After the discovery of neutrophil extracellular trap (**NET**) in mammals (Brinkmann et al., 2004), its functional equivalent in chickens, HET, was reported (Chuammitri et al., 2009). The HET assay reported by Chuammitri et al. (2009) was developed for adult chickens, but not for young chicks which have a much lower blood volume. Additionally, in that study the isolated heterophils were not challenged with any bacteria. To understand the interaction between the host cell and pathogen, bacteria challenge is essential.

The objective of this study was to establish a HET method in young chicks that can be used for future poultry innate immunity study. To achieve this objective, HET assay was first developed in laying hens because it was an easy supply of blood and we were able to obtain blood regularly. Then, based on the adult birds, it was developed for use in young chicks. This second experiment was done to dertermine if the HET assay is capable of detecting differences due to factors such as sex and age that we expected to have an effect on innate immunity. Therefore, in the second experiment, the effect of broiler chick age and sex on innate immunity including HET against *E. coli ex vivo* was investigated. We hypothesized that *E. coli* killing, phagocytosis and HET release in response to *E. coli* would increase with increasing chick age and male chicks would have lower *E. coli* killing, phagocytosis and HET release in response to *E.coli* killing compared to female chicks.

5.2 MATERIALS AND METHODS

Experimental protocols were approved by the Animal Care and Use Committee: Livestock of University of Alberta. All birds were reared and cared for according to the Canadian Council of Animal Care (2009) guidelines.

5.2.1 Experiment 1

5.2.1.1 Isolation of white blood cells

Commercial Single-Comb White Leghorns hens (n=6) at approximately 42 wk of age and housed at University of Alberta were used on 3 different days (2 hens/d). A 1 mL blood sample was obtained through brachial venipuncture from each bird each day. The blood was collected in a 5 mL vacutainer tube (BD Biosciences, Mississauga, ON, Canada) that contained 75 United States Pharmacopeia units of lithium heparin. White blood cells were isolated based on a modification of the NET method described by Behrendt et al. (2010). Heparinized blood was mixed with an equal volume of phosphate-buffered saline at pH 7.4 (**PBS**; Thermo Fischer Scientific Inc., Mississauga, ON, Canada) containing 1% bovine serum albumin (**BSA**). One mL of blood mixture/hen was layered on each of two different density gradients: Biocoll Separating Solution (1 mL of Biocoll Separating Solution; 1.100 g/mL; Biochrom AG, Berlin, Germany) or Histopaque 1.119 (1 mL of Histopaque 1.119; 1.119 g/mL; Sigma-Aldrich, Oakville, ON, Canada). Each tube was then centrifuged at 500 x g for 30 minutes at 4°C and the 1.100 density band (Bicoll) and 1.119 density band (Histopaque 1.119) were collected. Each tube was then washed with PBS with 1% BSA, and centrifuged at 370 x g for 10 minutes at 4 °C. The cell pellet was resuspended in 2 mL ammonium chloride-potassium chloride (**ACK**) lysis buffer

(Lonza, Basel, Switzerland) for 3 minutes and then washed with PBS with 1% BSA. This was done twice. After the lysis procedure, the viability and the purity of hetrophils were determined.

5.2.1.1.1 Cell viability and heterophil purity

After the cells were washed twice in PBS with 1% BSA, 100 µL of the re-suspended cells were transferred to a 500 µL siliconized polypropylene tube (Invitrogen, Carlsbad, CA and BD Biosciences, Mississauga, ON, Canada). Cells were stained *ex vivo* using propidium iodide (Invitrogen, Carlsbad, CA and BD Biosciences, Mississauga, ON, Canada). Propidium iodide was added at a final concentration of 4 µg/mL. Cells were incubated for 15 min in a dark room at room temperature (20 °C) and then a BD FACSCanto II flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA) was used to measure cell viability. The purity of the heterophil preparation was determined by centrifuging the cells at 55 x g for 6 minutes using a cytocentrifuge (Cytospin 4 cytocentrifuge, Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then staining with HEMA 3 stain set (Thermo Fischer Scientific Inc., Mississauga, ON, Canada). Microscopic examination (Leica DM 1000 microscope, Leica Microsystems, Cancord, Ontario, Canada; 1000 x total magnification) of the smear was used to count at least 200 white blood cells. The percentage of heterophils was calculated by dividing the number of heterophils by the total number of white blood cells.

5.2.1.2 HET assay

After cell viability and heterophil purity were measured, the HET assay was developed based on Behrendt et al. (2010) with modifications. The white blood cell suspension from the heterophil isolation was added into a 96-well plate (150 µL/well; approximately 2.1 x 10^5 cells/well; white blood cell suspension from each hen was added to a separate 96-well plate)

coated with poly-L-lysine (Sigma-Aldrich, Oakville, ON, Canada). The wells were each filled with PBS with 1% BSA to a final volume of 200 µL. Each well contained 50 µL of either 0.1% Triton X-100 (Sigma-Aldrich, Oakville, ON, Canada), phorbol 12-myristate 13-acetate (**PMA**; 10 ng/mL dissolved in dimethyl sulfoxide; Sigma-Aldrich, Oakville, ON, Canada), PBS with 1% BSA or *E. coli* (4.3×10^7 cfu/mL; ATCC #51813, Epower, Microbiologics Inc., Saint Cloud, MN). Triton X-100 was used to completely lyse the cells and all the externalized DNA was used as a positive control. The PMA was also used as a positive control to stimulate the cells and determine how many cells released HET. As a negative control, PBS with 1% BSA (no stimulation) was added to the cells. The plate was incubated for 3 hours at 41 °C and 5% CO_2 and then samples were stained using SYTOX orange nucleic acid stain (1 µM/well; Thermo Fischer Scientific Inc., Mississauga, ON, Canada) for 10 minutes at room temperature (20 °C). A UV-Vis SpectraMax Microplate Reader with SoftMax pro data acquisition & analysis software (Molecular Devices, Sunnyvale, CA) was used to measure the fluorescence of SYTOX orange stain using an excitation wavelength of 530 nm and emission at 590 nm. The HET was expressed as % externalized DNA relative to total DNA (DNA released by Triton X-100).

5.2.2. Experiment 2

5.2.2.1 Experimental animals

A total of 177, one-d-old Ross 308 broiler chicks (91 males and 86 females) obtained from a commercial hatchery (Lilydale Hatchery, Edmonton, AB, Canada) were placed in Petersime battery cages (Petersime Incubator, Gettysburg, OH). The chicks were allowed free access to a diet that met or exceeded the Aviagen-recommended nutrient levels (Aviagen, 2014b) and the temperature, lighting program and light intensity followed the broiler management guide

(Aviagen, 2014a). At each of 1, 4, 6 and 8 d of age, 40 chicks (20 male and 20 female) were randomly selected and weighed. Blood was collected from each chick via decapitation at 1, 4 and 6 d of age and via brachial venipuncture at 8 d of age. Approximately 1 mL of whole blood was collected from each chick into 5 mL vacutainer tubes containing 75 United States Pharmacopeia units of lithium heparin (BD Biosciences, Mississauga, Canada); 40 µL were removed for each phagocytosis assay and bacteria killing assay and the remainder was used for the HET assay.

5.2.2.1.1 Bacteria killing assay

Bacteria killing was assessed for 20 chicks/sex at each of 1, 4, 6 and 8 d of age (Saunders-Blades and Korver, 2015). Briefly, *E.coli* stock solution was made by diluting 1 *E. coli* pellet (MicroBiologics Inc., Saint Cloud, MN) in 40 mL PBS (Thermo Fischer Scientific Inc., Mississauga, ON, Canada). The *E.coli* working solution was prepared by adding 2 mL *E.coli* stock solution to 8 mL of PBS. Forty µL of heparinized whole blood from the chicks were diluted with CO_2-independent media (1:10 dilution; Thermo Fischer Scientific Inc., Mississauga, ON, Canada) with 4 mM L-Glutamine (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) in an Eppendorf tube. In each tube, 40 µL of *E.coli* working solution were added (approximately 250 *E. coli*/ 50 µL of diluted blood) and incubated for 90 minutes at 41°C. Fifty µL of each blood and bacteria mixture was plated on a tryptic soy agar plate. The plates were incubated overnight at 37 °C and the number of colonies was counted. Percent *E.coli* killing was calculated by subtracting the total number of *E. coli* colonies in the 50 µL of working solution (control) by the number of *E. coli* colonies from the sample, and dividing it by the total number of *E. coli* colonies in 50 µL working solution (control) and then multiplying by 100. We assumed that the decrease in *E. coli* colonies was due to white blood cell killing of the *E. coli.*

5.2.2.1.2 Phagocytosis assay

Phagocytic index and capacity were measured using whole blood from 20 chicks/sex at each of 1, 4, 6 and 8 d of age (Saunders-Blades and Korver, 2015). Whole blood (40 µL) was diluted with CO_2-independent media (1:20 dilution; Thermo Fischer Scientific Inc., Mississauga, ON, Canada) in an Eppendorf tube and then fluorescent *E. coli* particles (100 *E. coli* particles per white blood cell) were added to the diluted blood. After incubation of the mixture for 15 minutes at 41°C, it was washed twice with CO_2-independent media and then ACK lysis buffer (Lonza, Basel, Switzerland) was added. Cell fluorescence was measured using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). At least 10,000 cells were analyzed within the gated region (total, thrombocyte and lymphocyte, granulocyte and monocyte, and macrophage) and data was analyzed using FACSDiva software (BD Biosciences, San Jose, CA). Phagocytic index was the proportion of phagocytic cells that engulfed at least 1 fluorescent *E. coli* particle (Molecular Probes, Burlington, ON, Canada). The phagocytic capacity was the relative number of *E. coli* fluorescent particles engulfed by one phagocytic cell, and was measured as mean fluorescence intensity.

5.2.2.1.3 HET assay

The HET assay was performed using 20 chicks/sex at each 1, 4, 6 and 8 d of age. One mL of whole blood/chick was mixed with an equal amount of PBS at pH 7.4 (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) containing 1% BSA. It was then layered on Biocoll Separating Solution (1 mL of Biocoll Separating Solution; 1.100 g/mL density; Biochrom AG, Berlin, Germany) and centrifuged at 500 x g for 30 minutes at 4 °C. The 1.100 g/mL density band was collected and then PBS with 1% BSA was added to the collected 1.100 g/mL density

band. This mixture was then centrifuged at 370 x g for 10 minutes at 4 °C. The cell pellet was resuspended in 2 mL ACK lysis buffer (Lonza, Basel, Switzerland) and washed with PBS with 1% BSA. This lysis step was done twice. The rest of the HET assay was performed as described in Experiment 1.

5.2.3 Statistical analysis

Paired t-tests were used to compare the differences in white blood cell viability between the 2 different density gradients using SAS version 9.2 (SAS Institute, 2001). The experimental unit was the hen in experiment 1 (n=6). Data were tested for normality using Proc Univariate of SAS version 9.2 (SAS Institute, 2001) and then analyzed using Proc Mixed as a 2-way ANOVA for bacteria killing, phagocytic index, phagocytic capacity and HET release in response to *E. coli* (4 chick ages x 2 sexes). Experimental unit was the chick in experiment 2 (n=20/sex/age). Differences were considered significant when $P < 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 Experiment 1

The previously-published HET methods (Chaummitri et al., 2009; Redmond et al., 2011) used 15 mL of blood, a volume which cannot be obtained from young chicks. When those methods were attempted using 1 mL of layer blood, the heterophil-containing 1.119 g/mL density band contained a high proportion of RBC. It is important to remove RBC as much as possible and obtain a pure WBC population because high % of RBC interferes with the HET assay by increasing unwanted background fluorescence (data not shown). Neither Chaummitri et al. (2009) nor Redmond et al. (2011) indicated the heterophil purity and hence we do not know if they isolated a pure population of heterophils. In our study, total white blood cells were collected

and the total white blood cell extracellular trap production was measured. Our experiment was conducted before extracellular trap of other white blood cells such as macrophage and basophil had been reported in mice and humans (Liu et al., 2014; Yousefi et al., 2015) and therefore, we assumed that extracellular DNA was produced only from heterophils and no other immune cells. Eighty percent of human neutrophils stimulated with LPS produced NET (Yousefi et al., 2009). Only 10% of the total macrophage population produced extracellular trap when stimulated with *E. coli* in mice (Liu et al., 2014). Although human basophil produced extracellular trap when stimulated with *E. coli*, the percentage of extracellular trap produced in a basophil population was not measured (Yousefi et al., 2015). After stimulation with LPS, 83% of the total eosinophil population produced extracellular trap in humans (Yousefi et al., 2008). Although macrophages, basophils and eosinophils can produce extracellular trap and kill *E. coli*, heterophils are one of the predominant leukocytes in chicken blood; heterophils, macrophages, eosinophils and basophils account for 37.8%, 3.8%, 3.4% and 2.1%, respectively of total white blood cells in the Ross 308 broiler breeder hen (Abdi-Hachesoo et al., 2011). Lymphocytes and thrombocytes constitute the majority of leukocytes in the breeder hen (53%; Abdi-Hachesoo et al., 2011), but no study has reported the production of extracellular trap by lymphocyte and thrombocyte. Therefore, the white blood cell that produce majority of the extracellular trap is the heterophil. Since extracellular trap production from the whole white blood cell population was measured, the mechanism and which cells produced extracellular trap and killed *E. coli* cannot be determined in our study. However, we were able to determine the functional extracellular trap produced from white blood cells that were stimulated with *E. coli* in our study. *Mycobacterium tuberculosis*-induced NET has been reported to contain heat shock protein 72 in humans, which play an important role in the interaction between netutrophils and macrophages during an

infection (Braian et al., 2013). Heat shock protein 72 stimulates the release of TNF-α in macrophages (Braian et al., 2013) and this TNF-α can further induce NET formation (Keshari et al., 2012). Immune cells work together and cells communicate with each other by releasing heat shock proteins, which have cytokine-like functions (Tsan and Gao, 2004), and by secreting cytokines such as TNF-α (Belardelli, 1995). The immune cells that received the cytokine signal perform activities such as HET (Belardelli, 1995). It is unknown if HET contain heat shock protein 72 but if it did, then HET assay using whole white blood cell population may include immune cell-cytokine interaction. Therefore, the HET assay developed in our study is a useful tool to assess innate immunity. Similar to the Behrendt et al. (2010) NET assay in cattle, incubation of white blood cells with Triton X-100 was used as a positive control in our study. Triton X-100 lyses all cells, and hence the entire DNA from the cell population in a sample can be measured (Behrendt et al., 2010). Therefore incubating the white blood cell population with Triton X-100 will provides an indication of the potential total DNA release and thus provide a standard of basis for comparison. This positive control is important to make sure that the assay is working properly and this control was not included in the Chaummitri et al. (2009) HET assay. The HET release in response to *E. coli* was also not included in the assay from Chaummitri et al. (2009) but it was included in our study.

The Biocoll density gradient that was used to isolate white blood cells resulted in a greater proportion of viable cells (63.6%) than the use of the Histopaque 1.119 density gradient (36.4%; data not shown). The use of Biocoll density gradient also resulted in a cell population with approximately 35% heterophils, whereas the use of Histopaque 1.119 resulted in a cell population with only 20% heterophils (data not shown). The viability of cells from each density

gradient was lower than other studies that used Histopaque density gradient in chickens (over 95% cell viability; Kapczynski, 2008; Redmond et al., 2011). This could be because lysis of RBC was performed 3 minutes twice for a total of 6 minutes in our study; cell viability decreased when lysis of RBC was performed for more than 5 minutes (Duda et al., 2007). In other chicken white blood cell isolation assays (Kapczynski, 2008; Redmond et al., 2011), RBC lysis was not done or was done for only 3 minutes. In our study, RBC lysis was done twice for 3 minutes because performing RBC lysis once did not remove enough RBC to perform the HET assay and we wanted to remove RBC as much as possible. Both Biocoll and Histopaque density gradient composition is similar; both contain the same compounds such as polysucrose and sodium diatrizoate as indicated in the product manuals (Sigma-Aldrich, Oakville, ON, Canada; Biochrom AG, Berlin, Germany). Furthermore, cell viability was 99% when cells were prepared using Histopaque 1.119 in adult chickens (Finkelstein et al., 2003). When cells were prepared using Biocoll separating solution in male pigs, the cells had at least 95% cell viability (Engert et al., 2017). Therefore, we expected the same cell viability between Biocoll and Histopaque and the cause of different cell viability between the two density gradient in our study is unknown. Centrifugal force was 500 x g and the duration was 30 minutes in our study. This is because lower centrifugal force (250 x g) with various duration (40, 50 and 60 minutes) as well as higher centrifugal force (500 x g) with shorter duration time (10 and 20 minutes) resulted in RBC contamination in the 1.10 g/mL (Biocoll) and 1.119 g/mL (Histopaque) band (data not shown). Chicken heterophils found at densities of 1.092 and 1.108 g/mL had the highest purity (96.9 and 99.8%, respectively) compared to densities below1.056 (Mills and Wilcox, 1993). Therefore, we expected heterophil purity at the 1.10 g/mL (Biocoll) density band to be greater than 1.119 g/mL (Histopaque) band. Centrifugal force was lower (500 x g) and duration was longer (30 minutes)

in our study than reported by Mills and Wilcox (1993; 800 x g for 15 minutes) as well as the blood and density gradient volume was smaller in our study (1 mL blood and 1 mL density gradient) compared to Mills and Wilcox (1993; 4 mL of blood and 6 mL Percoll density gradient). In human monocytes, a longer centrifugal time (20 minute) compared to shorter centrifugal time (5 minute) increased apparent density by 0.01 g/mL (280 x g; Loos et al., 1976). As the centrifugal force increases, the sedimentation rate also increases (Majekodunmi, 2015). Furthermore, the higher blood and density gradient volume will result in less cell separation (Sharpe, 1988), Mississauga, ON, Canada). However we do not know how much centrifugal force, and blood and density gradient volume would affect the apparent heterophil density. Although we had longer centrifugal duration and lower blood and density gradient volume than Mills and Wilcox (1993), if the apparent density increase was less than 0.011 g/mL it may explain why the, heterophil purity at 1.10 g/mL (Biocoll) density band had higher heterophil purity compared to1.119 g/mL (Histopaque) band in our study. Although the HET assay developed in our study does not explain the mechanism, it is a useful tool to measure part of the innate immunity.

5.3.2 Experiment 2

In experiment 2, Biocoll separation solution (1.10 g/mL density) was used to isolate young chick white blood cells for the HET assay because we found that Biocoll was more suitable density gradient than Histopaque 1.119 density gradient; cell preparation using Biocoll had higher cell viability and heterophil purity compared to the Histopaque 1.119 density gradient (experiment 1). Innate immune response such as phagocytosis is different depending on the chick age; *Salmonella enteritidis* heterophil phagocytosis was lower at 1 d of age compared to 7 d of

age (Wells et al., 1998). Therefore knowing that there are differences in innate immunity response between different chick ages, HET production was measured through the different chick ages to ensure that the HET assay is capable of detecting differences due to factors such as sex and age. Currently, no studies have measured HET release in response to *E.coli* in young broiler chicks, and hence this would be the first study to report it. Bacteria killing was lower at 8 d of age compared to the other chick ages ($P<0.0001$; Table 5.1); male chicks tended ($P=0.058$) to have having higher bacteria killing than female chicks. No interaction between chick age and sex was observed. Total phagocytic index, which includes the phagocytic activity of lymphocytes and thrombocytes, granulocytes and monocytes, and macrophages, increased from 1 to 4 d of age, decreased at 6 d of age and then increased at 8 d of age ($P<0.001$). No sex effects nor interactions were found. Lymphocyte and thrombocyte phagocytic index followed the same pattern as chicks aged as for total phagocytic index ($P<0.001$). Since lymphocytes and thrombocytes constitute the majority of the white blood cells (over 90%; data not shown; Sturkie, 2015), the similarity in age response for total and lymphocyte and thrombocytes phagocytic index is to be expected. Most of the phagocytic activity included in total, and lymphocyte and thrombocytes phagocytic index is due to chicken thrombocytes, which express cell surface toll-like receptors (**TLR**); TLR are important for phagocytosis of *E. coli* (St. Paul et al., 2012). Since lymphocytes are not professional phagocytes (Rabinovitch, 1995), their phagocytic activity is minimal. Granulocytes, monocytes and macrophages represented less than 10% of the total population (data not shown), and thus the impact of these cells on the effects of chick age and sex on total phagocytic index were small. At 8 d of age, female chicks had higher granulocyte and monocyte phagocytic index than at other ages; male chicks at 6 and 8 d of age had greater granulocyte and monocyte phagocytic index than at 1 and 4 d of age ($P=0.0007$).

Male granulocyte and monocyte phagocytic index was higher than that of females at 6 d of age, but there were no sex differences at any other ages. No chick age nor sex effects were found for macrophage phagocytic index.

Total phagocytic capacity in female chicks increased from 1 to 4 d of age, decreased at 6 d of age and then increased at 8 d of age (P=0.029; Table 5.2). Male chicks had increased total phagocytic capacity from 1 to 4 d of age, which decreased to 6 d of age and remained the same at 8 d of age. At 6 d of age, total phagocytic capacity was higher in males than females, but no other sex differences were seen in any other ages. Similar to total phagocytic capacity, the highest lymphocyte and thrombocyte phagocytic capacity was found at 4 d of age; 4 d old chicks had greater lymphocyte and thrombocyte phagocytic capacity than 1- and 8-d-old chicks (P<0.0001). No sex effect was found for lymphocyte and thrombocyte phagocytic capacity. Female chicks at 8 d of age had increased granulocyte and monocyte phagocytic capacity compared to all other ages (P=0.008); male chicks had higher granulocyte and monocyte phagocytic capacity at 6 d of age than at 1 and 4 d of age. Granulocyte and monocyte phagocytic capacity was greater in males than females at 6 d of age, but no other sex differences were seen in any other ages. At 4 d of age, macrophage phagocytic capacity was lower than at 1 and 6 d of age (P<0.0001). A sex effect was not observed for macrophage phagocytic capacity.

Total, lymphocyte and thrombocyte phagocytic index and total phagocytic capacity was the lowest at 6 d of age compared to all the other ages. This could be because we had difficultly collecting whole blood through decapitation at 6 d of age when the chicks were bigger; we observed some blood samples with clotting. Hence that may have affected the blood quality and immune function assay. However, we did not observe any changes in HET release in response to

E. coli and bacteria killing at 6 d of age. Therefore, it is unlikely that the decreased phagocytic index and capacity at 6 d of age was due to the blood collection method, and the cause for the decreased phagocytic index and capacity at 6 d of age is unclear.

There were no chick age or sex effects on HET release in response to PMA (Table 5.3), but overall HET release in response to PMA was lower than HET release in response to *E.coli*. Myeloperoxidase activity is required for PMA-induced NET formation in human neutrophils (Parker et al., 2012). On the other hand, human neutrophils stimulated with *E.coli* produce NET by using NADPH oxidase-dependant mechanism and hence do not require myeloperoxidase activity for NET formation (Parker et al., 2012). Chickens lack myeloperoxidase (Harmon, 1998) and therefore, PMA may induce small or no HET production in chickens. This may explain the cause of lack of chick age and sex effects on HET release in response to PMA, and a lower HET release in response to PMA compared to *E. coli*. At 1 d of age, HET release in response to *E. coli* was greater than at other ages (P=0.0001) and 8 d old chicks had the lowest HET release in response to *E. coli*. Leukocytes from human neonates had lower NET production compared to adults when stimulated with LPS for 2 hours (Lipp et al., 2016). This decreased NET production in neonates is due to delayed neonate NET production; human neonate neutrophils stimulated with LPS for 3 hours had no difference in NET production compared to adults (Marcos et al., 2009). If 3 hour *E. coli* stimulation in younger chicks was a long enough time to produce the same amount of extracellular trap as in older chicks, we expected the chick HET production against *E. coli* to be the same for all ages. The higher HET release in response to *E. coli* at 1 d of age compared to all the other ages is unknown.

Bacteria killing was higher at 1, 4 and 6 d of age compared to 8 d of age, possibly because HET release in response to *E.coli* and other innate immunity defense mechanism that were not measured in this study such as degranulation, were functional immediately after hatch. The HET response to *E. coli* was higher at 1 d of age compared to 4, 6 and 8 d of age in our study. When heterophils were stimulated with zymosan, no differences in degranulation from heterophils were seen between 1 and 7 d of age (Kogut et al., 2002). Although HET release in response to *E. coli* and degranulation from heterophils are functional at a young age, phagocytic activity is low when chicks are young; overall phagocytic index and capacity was lower at 1 d of age compared to 8 d of age in our study. Furthermore, chick heterophil phagocytic index and phagocytic capacity was lower at 1 d of age than at 7 d of age (Wells et al., 1998). The difference in innate immune function response between and young older chicks could be due to differences in cytokine production. Human infant monocytes produced less tumor necrosis factor-α (**TNF-α**), and more interleukin-8 (**IL-8**) and interleukin-6 (**IL-6**) than adult monocytes when monocyte TLR4 was stimulated or monocytes were stimulated with LPS (Schultz et al., 2002; Corbett et al., 2010). Tumor necrosis factor-α induced *Staphylococcus aureus* neutrophil phagocytosis but did not induce neutrophil *Staphylococcus aureus* killing in humans (Pechkovsky et al., 1996). Serum-opsonized microsphere phagocytosis was lower when stimulated with IL-8 compared to TNF- α in human granulocytes; IL-8 had similar *Staphylococcus aureus* killing as TNF- α (Knowles et al., 1997). Therefore, IL-8 may induce small or no bacteria phagocytosis and *Staphylococcus aureus* killing. Interleukin-6 stimulated neutrophil *Staphylococcus aureus* killing but did not stimulate *Staphylococcus aureus* neutrophil phagocytosis in humans (Pechkovsky et al., 1996). The enhanced neutrophil bacteria killing from IL-6 could be because IL-6 can directly stimulate neutrophil degranulation (Borish et al., 1989). If young chicks under 7 d of age

produced more IL-8 and IL-6 and less TNF-α, it may explain why we saw lower *E. coli* phagocytic index and capacity and higher *E. coli* killing up to 7 d of age in our study. It may also explain why degranulation from heterophils did not change between 1 and 7 d of age (Kogut et al., 2002). Furthermore, IL-8 and TNF-α are cytokines that enhace NET production in human and bovine neutrophils (Mitchell et al., 2003; Keshari et al., 2012). Although the reason for increased HET release in response to *E.coli* at 1 d of age is unknown, the HET release in response to *E.coli* did not change between 4 and 8 d of age possibly because IL-8 and TNF-α stimulated HET production until 7 d and after 8 d of age, respectively. We suggest that different innate immunity defense mechanisms may be more effective at killing *E. coli* at different ages. The reason for decreased *E. coli* killing and increased phagocytic index and capacity at 8 d of age in our study may be due to decreased IL-6 and IL-8, and increased TNF-α.

Overall, total, lymphocyte and thrombocyte, and granulocyte and monocyte phagocytic indices, and phagocytic capacity increased from 1 to 8 d of age. There are currently no studies reporting the effects of age on thrombocyte and monocyte phagocytosis in chickens. However, low monocyte *E. coli* phagocytic index in human neonates compared to adults had been found (Strunk et al., 2004). Furthermore, thrombocyte *Staphylococcus aureus* phagocytosis decreased in young carp (3 months) compared to older carp (17 months; Stosik et al., 2001). Furthermore, chick heterophil phagocytic index and phagocytic capacity decreased with decreasing age (Wells et al., 1998). Our results from the HET release in response to *E.coli*, and heterophil degranulation from a different study (Kogut et al., 2002) suggests that heterophils can function immediately after post-hatch. Therefore, the decreased phagocytic index and capacity at a young age could be due to a low quantity of specific cytokine production as described above.

The HET release in response to *E. coli* was higher for male chicks than for female chicks (P=0.020). Roosters had higher plasma testosterone concentration than capons, and had increased delayed-type hypersensitivity response, a measure of cellular innate immunity (Mashaly, 1984). However, plasma testosterone concentration at hatch is the same between female and male chicks (Motelica-Heino, 1995). Therefore, the increased HET response *to E. coli* in male chicks was not likely due to a difference in plasma testosterone concentration. The heterogametic sex in birds is the female (ZW) and the homogametic sex is the male (ZZ); type 1 interferon genes, which include interferon-α and -β, are located on the Z chromosome (Ankra-Badu and Aggrey, 2005). Therefore, male chicks, having two Z sex chromosomes have increased interferon-β production compared to female chicks when triggered with LPS (Sheikh et al., 2014). Interferon-β increases iNOS gene expression to increase nitric oxide production and antimicrobial activity in mice macrophages (Utaisincharoen et al., 2004). If interferon-β can stimulate certain cytokines and expression of genes related to HET production such as IL-8 (Alfaro et al., 2016), HET release in response to *E.coli* may be greater in male chicks. Furthermore, the trend of greater *E.coli* killing in male chicks compared to female chicks could be due to the higher HET release in response to *E.coli*, and possible greater nitric oxide production from increased interferon-β production in male chicks.

Overall, there were no sex effects on total phagocytic index, nor on thrombocyte, lymphocyte, and macrophage phagocytic index and capacity. Thrombocyte phagocytosis is induced by LPS through the TLR4 pathway (St. Paul et al., 2012), but no cytokine receptors such as IL-8 receptor (CXCR-1 and CXCR-2) have been reported in thrombocytes. If LPS-stimulated interferon-β production increased in male chicks and ultimately increased cytokine gene

expression such as IL-8 as described above, thrombocytes may not be activated due to the lack of IL-8 receptors; neutrophils have IL-8 receptors and hence IL-8 can activate neutrophils and increase phagocytosis (Knowles et al., 1997; Zeilhofer and Schorr, 2000). The majority of phagocytic index and capacity was from thrombocytes in our study. Therefore, the overall lack of sex effects on total, thrombocyte and lymphocyte phagocytic index could be due to an absence of cytokine receptors in the thrombocyte. The granulocyte and monocyte phagocytic index and capacity was greater in males compared to females at 6 d of age possibly because granulocyte and monocyte have receptors such as IL-8 as found in human neutrophils (Zeilhofer and Schorr, 2000). The reason why we only found this sex effect at 6 d of age is unknown. We found that there was no sex effect on macrophage phagocytic index and capacity even though macrophages have IL-8 receptors (Bishayi et al., 2015). If the lack of sex effect on thrombocyte phagocytic index and capacity is due to absence of cytokine receptor, we would have expected to see a sex effect on macrophage phagocytic index and capacity. Therefore, the cause is unknown for the lack of sex effect on macrophage phagocytic index and capacity.

The HET assay was modified from the previous HET assay for use in adult chickens (Chaummitri et al., 2009) to measure extracellular trap production in response to *E. coli* in young chicks. This new assay requires less blood than the previously-published assay (Chaummitri et al., 2009) and hence can be used in young chicks. The HET assay developed in our study using white blood cell population includes the effects of interactions between immune cell and cytokine. Therefore, this assay could be used to assess involvement of the HET in early chick innate immune function. Generally, bacteria killing was higher under 6 d of age compared to 8 d of age. Contrary to bacteria killing, total phagocytic index generally increased with increasing

age. The HET release in response to *E. coli* was the highest at 1 d of age compared to all the other ages. This indicates that different mechanisms are more effective at killing the bacteria at different chick ages. Based on experiment 2, HET assay can detect differences from factors such as age and sex that are known to affect innate immunity. Therefore, HET assay is a useful tool to measure part of the innate immunity and could be applied to young chicks. This is the first time reporting HET release in response to *E. coli* in young chicks. The HET plays an important role in early immune protection of the chick and HET production is higher in male chicks compared to female chicks.

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5.5 TABLES

Table 5.1 Effects of broiler chick age and sex on phagocytic index and bacteria killing of whole blood to *Escherichia coli*.

Chick age (d)	Sex	n	Bacteria killing	Phagocytic index[1]			
				Total[2]	Lymphocyte/ thrombocyte	Granulocyte/ monocyte	Macrophage
			----------%----------				
1		40	64.23[a]	16.57[c]	16.40[c]	27.51[c]	30.97
4		40	72.89[a]	20.47[b]	19.63[b]	24.97[c]	29.55
6		40	82.08[a]	13.63[d]	13.34[d]	38.23[b]	28.09
8		40	31.61[b]	23.90[a]	23.52[a]	55.49[a]	27.53
SEM			9.06	0.79	0.45	4.01	2.82
	Female	80	59.53	18.51	18.08	35.62	29.56
	Male	80	65.87	18.77	18.37	37.48	28.51
SEM			2.42	0.29	0.29	1.62	1.03
1	Female	20	59.36	16.34	16.14	26.88[c]	33.38
1	Male	20	69.11	16.80	16.67	28.14[c]	28.55
4	Female	20	70.41	21.05	20.20	27.02[c]	30.46
4	Male	20	75.37	19.89	19.06	22.92[c]	28.64
6	Female	20	81.90	13.25	13.03	30.55[c]	28.53
6	Male	20	82.25	14.00	13.66	45.91[b]	27.66
8	Female	20	26.44	23.41	22.94	58.03[a]	25.90
8	Male	20	36.77	24.39	24.09	52.95[ab]	29.17
SEM			7.59	0.88	0.87	4.30	3.11
			----------Probability----------				
Chick Age			<0.001	<0.001	<0.001	<0.001	0.871
Sex			0.058	0.507	0.462	0.347	0.447
Chick age x Sex			0.673	0.197	0.186	<0.001	0.228

[a-d]Means with no common letters within the same column are significantly different (P<0.05).
[1]Proportion of phagocytic cells that engulfed at least 1 *Escherichia coli* bioparticle *ex vivo*.
[2]Overall phagocytic index including lymphocyte and thrombocyte, granulocyte and monocyte, and macrophage phagocytic index.

Table 5.2 Effects of broiler chick age and sex on phagocytic capacity of whole blood to *Escherichia coli*.

Chick age (d)	Sex	n	Mean fluorescence intensity[2]			
			Total[3]	Lymphocyte/ thrombocyte	Granulocyte/ monocyte	Macrophage
1		40	8,582[d]	6,679[b]	28,256[b]	75,296[a]
4		40	15,741[a]	17,220[a]	21,212[b]	36,335[b]
6		40	10,772[c]	7,134[ab]	59,011[a]	68,140[a]
8		40	12,217[b]	7,000[b]	71,013[a]	57,543[ab]
SEM			588	3,614	7,624	6,072
	Female	80	11,801	10,332	44,424	58,613
	Male	80	11,855	8,684	45,322	60,044
SEM			320	1,325	2,795	4,950
1	Female	20	8,633[c]	6,653	28,612[cd]	71,552
1	Male	20	8,531[c]	6,705	27,900[cd]	79,039
4	Female	20	16,083[a]	21,005	24,617[d]	37,652
4	Male	20	15,400[a]	13,434	17,807[d]	35,018
6	Female	20	10,072[c]	6,958	47,674[bc]	64,131
6	Male	20	11,472[b]	7,308	70,347[a]	72,148
8	Female	20	12,417[b]	6,712	76,794[a]	61,116
8	Male	20	12,016[b]	7,289	65,232[ab]	53,969
SEM			659	3,916	8,394	7,967
			----------Probability----------			
Chick age			<0.001	<0.001	<0.001	<0.001
Sex			0.853	0.356	0.811	0.765
Chick age x Sex			0.029	0.274	0.008	0.568

[a-d]Means with no common letters within the same column are significantly different (P<0.05).
[2]Relative number of *E. coli* fluorescent particles engulfed by one phagocytic cell *ex vivo*.
[3]Total phagocytic capacity, including lymphocyte and thrombocyte, granulocyte and monocyte, and macrophage phagocytic capacity,

Table 5.3 Effects of broiler chick age and sex on heterophil extracellular trap release in response to phorbol 12-myristate 13-acetate and *Escherichia coli ex vivo*.

Chick age (d)	Sex	n	PMA[1,2]	*Escherichia coli*[2]
			----------%----------	
1		40	11.75	51.01[a]
4		40	9.06	26.77[b]
6		40	12.08	29.76[b]
8		40	26.57	23.96[b]
SEM			13.62	6.37
	Female	80	10.83	29.20[b]
	Male	80	18.90	36.55[a]
SEM			3.80	2.23
			----------Probability----------	
Chick age			0.434	<0.001
Sex			0.114	0.012
Chick age x Sex			0.539	0.411

[a,b]Means with no common letters within the same column are significantly different (P<0.05).
[1]Phorbol 12-myristate 13-acetate concentration dissolved in dimethyl sulfoxide at 10 ng/mL.
[2]The heterophil extracellular trap release in response to phorbol 12-myristate 13-acetate and *Escherichia coli* was expressed as % externalized DNA relative to total DNA.

6. DEVELOPMENT OF A HETEROPHIL ISOLATION METHOD IN LAYING HENS

ABSTRACT

A heterophil isolation method was developed in this study for future use in a heterophil extracellular trap (**HET**) assay. In Experiment 1, the optimal centrifugation time (10, 15, 20 or 25 minutes at 500 x g) was determined for cell separation from the whole blood from Single-Comb White Leghorn hens using Histopaque 1.077/1.119 density gradient. Percentage of white blood cell (**WBC**), red blood cell (**RBC**) and heterophils were measured. In Experiment 2, the effect of different density gradient treatments (Histopaque 1.077/1.119 with lysis buffer, Histopaque 1.077/1.119 with no lysis buffer, Histopaque 1.083/1.119 with no lysis buffer and Histopaque 1.077/1.1083 with no lysis buffer) on total cell populations from hen whole blood were observed. In Experiment 3, we investigated the effect of 2 different density gradients using Dextran T500, an RBC sedimentation buffer, on total cell populations from hen blood. The 2 different density gradients used to prepare cells were Histopaque 1.077/1.119, and Mono-poly resolving medium. The WBC viability, percentage of WBC, RBC and heterophils were measured in Experiments 2 and 3. Centrifuging the cells for 15 minutes at 500 x g on Histopaque 1.077/1.119 g/mL resulted in higher heterophils (58.8%) than for 20 (20.0% heterophils) or 25 minutes (12.1% heterophils) centrifugation times and resulted in lower RBC (96.3%) and higher WBC (3.7%) than the 10 minute (1.1% WBC) centrifugation time. Using Histopaque 1.077/1.119 with lysis buffer reduced the level of WBC viability (31.8%), heterophil (44.3%) and increased WBC (94.2%) but no differences in level of WBC viability, RBC and WBC were found with any other density gradient treatments. A pure isolation of heterophils was not possible using the different density gradients and procedures tested. The heterophil isolation

method closest to obtaining a pure heterophil population was Histopaque 1.077/1.119 with Dextran T500; 77.6% WBC viability, 59.5% WBC and 40.5 % RBC and 50.5% heterophils. Further refinements, including reducing RBC to lower unwanted background fluorescence, and increasing heterophil purity, are needed to confidently measure HET production.
Key words: white blood cell, heterophil, laying hen, method, immunology

6.1 INTRODUCTION

The innate immune system uses many defense mechanisms to protect the host against pathogens. Heterophils are one of the most abundant granulocyte cells present in the chicken, with functions similar to neutrophils in mammals (Harmon, 1998). Neutrophil extracellular trap (**NET**) is part of the innate defence mechanism; neutrophils release extracellular DNA that bind to various bacteria and kill the bacteria (Brinkmann et al., 2004). Chicken heterophils also release extracellular DNA (heterophil extracellular trap, **HET**; Chuammitri et al., 2009).

In a previous experiment, HET was measured using total white blood cells (**WBC**; Chapter 5). Although we were able to measure extracelluar trap production from WBC and observe the effect of chick age and sex on extracellular trap production in our previous experiment (Chapter 5), we were not able to determine which cells were involved in the extracellular trap production; immune cells other than heterophils, such as macrophages, can also produce extracellular traps (Liu et al., 2014). Therefore, a heterophil isolation method was developed in this study for use in future HET assay and to understand heterophil function. Many NET studies using isolated neutrophils in humans (Pilsczek et al., 2010; Branzk et al. 2014) and bovines (Saffarzadeh et al., 2014; Gondaira et al., 2016) have been done, but there has only been one study that has attempted to isolate heterophils and measure HET in chickens (Chuammitri et al., 2009). In that study, heterophil viability and ratio of heterophils to non-heterophil cells after

heterophil preparation were not reported. Hence, we do not know if pure population of viable heterophils were isolated. Obtaining a pure population of heterophils and having high heterophil viability is important for proper measurement of HET production and understanding heterophil function. In this study, various isolation procedures were tested to determine the optimum conditions for optimizing heterophil purity for future use in HET assays. The objective of this study was to develop a heterophil isolation method in laying hens to allow for an improved HET method in future studies.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Experimental protocols were approved by the Animal Care and Use Committee: Livestock of the University of Alberta, and all birds were reared and cared for according to the Canadian Council of Animal Care (2009) guidelines. Commercial Single Comb White Leghorns hens at approximately 30 wk of age, housed at University of Alberta, were used as the source of heterophils.

6.2.1.1 Experiment 1

Approximately 6 mL of blood was obtained through brachial venipuncture from each of 2 commercial Single-Comb White Leghorns hens. The blood was collected in 2 tubes from each hen; each 4 mL sterile vacutainer tube contained 7.2 mg of Ethylenediaminetetraacetic acid (**EDTA**; BD Biosciences, Mississauga, ON, Canada). The 2 blood tubes from the same hen was combined and put into 15 mL Falcon conical centrifuge tube (Thermo Fischer Scientific Inc., Mississauga, ON, Canada). The blood from each hen was mixed with 1% bovine serum albumin (**BSA**) containing 1 x phosphate-buffered saline (**PBS**) at pH 7.4 (Thermo Fischer Scientific Inc.,

Mississauga, ON, Canada) to make a total volume of 12 mL. Three mL of this mixture was layered onto Histopaque 1.077/1.119 (3 mL of Histopaque 1.077 and 3 mL of Histopaque 1.119; specific gravity of 1.077 over 1.119 g/mL; Sigma-Aldrich, Oakville, ON, Canada). It was then centrifuged at 500 x g for 10, 15, 20 or 30 minutes at 20 °C. The upper band between the plasma and the 1.077 band contained mononuclear cells such as lymphocytes, the middle band between the 1.077/1.119 interface and the 1.119 band contained polynuclear cells such as heterophils, and the lower band under the 1.119 band contained RBC (Figure 6.1; Chuammitri et al., 2009). The interface between the 1.077 and 1.119 bands, and the 1.119 density band were collected and washed with 1 x PBS with 1% BSA, and centrifuged at 370 x g for 10 minutes at 20 °C. The WBC viability, red blood cell (**RBC**), WBC and heterophil percentage was determined for all treatments.

6.2.1.1.1 White blood cell viability

Cells were stained *ex vivo* using annexin V (**AnnV**) /propidium iodide (**PI**; Invitrogen, Carlsbad, CA and BD Biosciences, Mississauga, ON, Canada) to measure WBC viability as described by Bayona et al. (2017). Cells (100 µL) were washed with 1 mL of AnnV binding buffer (BD Biosciences, Mississauga, ON, Canada) twice and then resuspended in AnnV binding buffer (100 µL). Annexin V (1:20 dilution; BD Biosciences, Mississauga, ON, Canada) was added to the cell solution and then PI (final concentration of 4 µg/mL; Invitrogen, Carlsbad, CA) was added and then the cell solution was incubated for 30 minutes at 41 ºC. At least, 10,000 cells were acquired and analyzed using The BD FACSCanto II flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA).

6.2.1.1.2 Red and white blood cell and heterophil determination

The cells were centrifuged at 55 x g for 6 minutes using a cytocentrifuge (Cytospin 4 cytocentrifuge, Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then deposited onto a glass slide. It was then stained with Hema 3 stain set (Thermo Fischer Scientific Inc., Mississauga, ON, Canada). The glass slides were rinsed with deionized water and air-dried and the cells counted and confirmed using a brightfield microscopy (Leica DM 1000 microscope, Leica microsystems, Cancord, Ontario, Canada; 1000 x total magnification). At least 200 cells were counted and the percentage of RBC and WBC was calculated by dividing the number of WBC or RBC by the total cell population. Percentage of heterophils was calculated by dividing the number of heterophils by the total WBC.

6.2.1.2 Experiment 2

A blood sample (approximately 6 mL) was collected through brachial venipuncture from each of 4 commercial Single-Comb White Leghorns hens on 2 different days (2 hens/day). The blood for each hen was obtained in two 4 mL sterile vacutainer tubes containing 7.2 mg of EDTA (BD Biosciences, Mississauga, ON, Canada). The two blood tubes from the same hen were combined into a 15 mL Falcon conical centrifuge tube (Thermo Fischer Scientific Inc., Mississauga, ON, Canada). Each sample was mixed with 1% BSA containing 1 x PBS at pH 7.4 (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) to make a total volume of 12 mL. Three mL of this mixture was layered onto each of the density gradient treatments: Histopaque 1.077/1.119 (3 mL each of the Histopaque densities; specific gravity of 1.077 over 1.119 g/mL; Sigma-Aldrich, Oakville, ON, Canada) with lysis buffer (Sigma-Aldrich, Oakville, ON, Canada), Histopaque 1.077/1.119 (3 mL each of the Histopaque) with no lysis buffer, Histopaque 1.083/1.119 (3 mL each of the Histopaque; specific gravity of 1.083 over 1.119

g/mL; Sigma-Aldrich, Oakville, ON, Canada) with no lysis buffer and Histopaque 1.077/1.1083 (3 mL each of the Histopaque; specific gravity of 1.077 over 1.1083 g/mL; Sigma-Aldrich, Oakville, ON, Canada) with no lysis buffer. It was then centrifuged at 500 x g for 15 minutes at 20 °C. The cells collected from the interface between the 1.077 and 1.119 bands, and the 1.119 density band from Histopaque 1.077/1.119 with lysis buffer, Histopaque 1.077/1.119 with no lysis buffer, and Histopaque 1.083/1.119 with no lysis buffer were collected. The interface between the 1.077 and 1.1083 band, and 1.1083 density band was collected from Histopaque 1.077/1.1083 with no lysis buffer. Cells from each of the combined interface and bands were then washed with 1 x PBS with 1% BSA, and centrifuged at 370 x g for 10 minutes at 20 °C. The WBC viability, RBC, WBC and heterophil percentage were then determined after this for all treatments except Histopaque 1.077/1.119 with lysis buffer. The cell pellet from this treatment was resuspended in 2 mL ammonium chloride-potassium chloride lysis buffer (Lonza, Basel, Switzerland) for 3 minutes and then washed with 1 x PBS containing 1% BSA. This was repeated again, and then the WBC viability and the percentages of RBC, WBC and heterophils were determined as described in Experiment 1.

6.2.1.3 Experiment 3

Blood was collected from 4 laying hens over 2 different days (2 hens/day) as described in Experiment 2. Each blood sample was mixed with 1 x PBS at pH 7.4 containing 1% BSA (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) to make a total volume of 12 mL/hen as described in Experiment 2. Three mL of this blood mixture was layered on 3 mL of 6% Dextran T500 (Pharmacosmos, Holbaek, Denmark) with 1 x PBS (Thermo Fischer Scientific Inc., Mississauga, ON, Canada). It was then centrifuged at 20 x g for 20 minutes at 20 °C. The

upper layer was removed and washed using 6 m L of 1% BSA with 1x PBS. It was centrifuged at 370 x g for 10 minutes at 20 °C and the solution was decanted. The 1% BSA with 1x PBS was added to make a total volume of 12 mL and then 3 mL was layered on either: Histopaque 1.077/1.119 (3 mL of Histopaque each; specific gravity of 1.077 over 1.119 g/mL; Sigma-Aldrich, Oakville, ON, Canada) or Mono-poly resolving medium (6 mL; combination of Ficoll400 and Hypaque to yield density of 1.114 g/mL; MP Biomedicals, Solon, OH). It was then centrifuged at 500 x g for 15 minutes at 20 °C. The interface between 1.077/1.119, and 1.119 density band from Histopaque 1.077/1.119, and the middle layer below the top white band of the Mono-poly resolving medium were collected. Cells from each of the combined interface and band, or band alone was then washed with 1x PBS with 1% BSA, and centrifuged at 370 x g for 10 minutes at 20 °C. No lysis buffer was added to the cells. The WBC viability, RBC, WBC and heterophil percentages were determined for all treatments as described in Experiment 1.

6.3 RESULTS AND DISCUSSION

6.3.1 Experiment 1

The cells were maintained at room temperature (20 ºC) and centrifuged at room temperature (20 ºC) to maximize heterophil purity in the 1.119 band of the Histopaque 1.077/1.119 g/mL in this study. The RBC aggregation is enhanced when human whole blood and density gradients are at high temperature; some WBC are trapped with RBC aggregation at high temperature and hence WBC migrate with the RBC (Fuss et al., 2009). When human whole blood and density gradients are at low temperature (4 ºC), RBC aggregation is reduced but also cell separation is reduced using density gradients (Ferrante and Thong, 1980). Hence, maintaining or centrifuging cells at above or below room temperature (18 to 20ºC) can reduce

human mononuclear cell and neutrophil purity and yield (Ferrante and Thong, 1980; Fuss et al., 2009). It is unknown whether centrifugation and cell temperature affect chicken heterophils and therefore heterophil isolation, but chicken heterophil isolation has been performed at room temperature (Stabler et al., 1994). Therefore, to maximize heterophil purity in the 1.119 band of the Histopaque 1.077/1.119 g/mL, cells were maintained and centrifuged at room temperature (20ºC) in this study.

Data was not analyzed using one-way ANOVA in this study because the sample size was small. Power analysis is a useful technique to determine the number of samples required to have statistical power (Cohen, 1992). Statistical power for animal studies is mostly kept at 0.8 (Charan and Kantharia, 2013); this indicates that we would detect statistical significant differences between the different treatments 80% of the time. When Proc Power of SAS version 9.2 (SAS Institute, 2001) was used to do power analysis, 5 replication/treatment was required to have a statistical power over 0.8 in our study. We only had 2 replication/centrifugation time in Experiment 1 (statistical power of 0.23) and 4 replications/density gradient treatment in Experiment 2 and 3 (statistical power of 0.69). Hence, statistical significant differences between the different treatments most likely would not be detected if we analyzed our data using one-way ANOVA in this study; this could lead to misinterpretation of our data. Therefore, only the mean and variance was reported in this study.

To maximize the heterophil purity and to reduce the proportion of RBC in the 1.119 band of the Histopaque 1.077/1.119 g/mL, the optimal centrifuge time for cell separation using Histopaque 1.077/1.119 g/mL was determined. Cells were also prepared using low centrifugal force (250 x g) for various duration (40, 50 and 60 minutes) but % RBC was high (over 95%;

data not shown) for all centrifugation times. Furthermore, long contact of cells with Ficoll, which is contained in Histopaque (description from MP Biomedicals, Solon, OH), is toxic to the cells (Maqbool et al., 2011). Therefore, cells were prepared using high centrifugal force (500 x g) for shorter durations (10, 15, 20 and 25 minutes) in our study.

Overall, % WBC was greater, and % RBC and % heterophils were lower in the 1.119 band of the Histopaque 1.077/1.119 g/mL with increasing centrifugation times (Table 6.1). Density gradients separate the cell types such as heterophils based on size and density (Loos et al., 1976), and hence length of centrifugation times affect cell separation; a longer centrifugation time (20 minute) compared to shorter centrifugation time (5 minute) increased human monocyte migration by increasing apparent density by 0.01 g/mL (280 x g; Loos et al., 1976). When length of centrifugation time was short (10 minutes), the cells did not separate and % RBC was high in the 1.119 band of Histopaque 1.077/1.119 g/mL. On the other hand, when length of centrifugation time was long (25 minutes), % RBC and % heterophil decreased compared to other shorter centrifugation time in the 1.119 band of Histopaque 1.077/1.119 g/mL. Highest chicken heterophil (99.8%) and RBC (99.9%) purity is observed at a density of 1.108 g/mL and 1.129 g/mL, respectively (Mills and Wilcox, 1993). Hence, RBC and heterophils appeared to have migrated to the bottom with long centrifugation time (25 minutes). We determined that 15 minutes at 500 x g on Histopaque 1.077/1.119 g/mL (3 mL of Histopaque each) using 3 mL of laying hen blood mixed with PBS containing 1% BSA was the optimal length of centrifugation time. This is because 15 minutes had higher % heterophils (58.5%) than 20 (20.0%) and 25 minutes (12.0%) centrifugation times and had lower % RBC (96.0%) and higher % WBC (4.0%) than 10 minutes (RBC 98.5 % and WBC 1.5%) centrifugation time. In Experiment 2, cells were

prepared using different combinations of double density gradients; chicken lymphocytes have been successfully isolated using Histopaque at density of 1.077 g/mL or 1.083 g/mL (Lavoie and Grasman, 2005), and heterophils have been isolated using double density of Histopaque at density of 1.077 g/mL and 1.119 g/mL (Chuammitri et al., 2009). The whole chicken blood layered on top of the density gradient would slowly migrate towards the bottom of the tube; Histopaque at density of 1.077 g/mL or 1.083 g/mL would retain lymphocytes and the Histopaque at density of 1.119 g/mL would retain heterophils (Figure 6.1).

6.3.2 Experiment 2

White blood cell viability from cells prepared using Histopaque without lysis buffer in our study (approximately 80%; Table 6.2 and Figure 6.2) was lower than another study which isolated leukocytes from chickens without using lysis buffer (over 95%; Mills and Wilcox, 1993). Mills and Wilcox (1993) used trypan blue exclusion to assay WBC viability, in which live cells exclude trypan blue, and dead cells are stained (Tennant, 1964). In our study, the combination of AnnV and PI was used to measure WBC viability to differentiate between live and dead cells (Figure 6.2; Havixbeck et al., 2015). Cells die through 2 different processes: apoptotic and necrotic cell death (Kromer et al., 1998). Apoptosis is a programmed cell death, while necrosis is a cell death which is stimulated by external factors such as trauma or infection (Kromer et al., 1998). Propidium iodide stains DNA but cannot cross the plasma membrane in live cells (Kromer et al., 1998). Annexin V binds to phosphatidylserine (**PS**), which is a phospholipid located in the inner leaflet of the plasma membrane of healthy cells (Crowley et al., 2016). Hence, live cells do not get stained by PI and AnnV. Phosphatidylserine is exposed to the outer leaflet of the cell plasma membrane during apoptosis and hence AnnV binds to PS

(Crowley et al., 2016). However, cell membranes are not ruptured during early apoptosis and therefore PI is excluded (Crowley et al., 2016). Cell membranes are ruptured during necrosis due to impairment of mitochondria, such as over production of superoxide ions, which causes disruption of plasma cell membrane integrity (Kromer et al., 1998). Hence, cells during necrosis are stained with both AnnV and PI; AnnV bind to PS and PI binds to DNA during necrosis of cell (Kromer et al., 1998). The lower WBC viability in our study compared to other study (Mills and Wilcox, 1993) is due to the exclusion of early apoptotic cells from live cells in our study; early apoptotic cells constituted approximately 15% of total WBC viability in our study (data not shown).

The cell preparation using Histopaque 1.077/1.119 g/mL with RBC lysis buffer had lower WBC viability (33.9%) compared to cells prepared using other density gradients without RBC lysis buffer (Table 6.2). Cell preparation using Histopaque 1.077/1.119 g/mL with RBC lysis buffer also resulted in the highest % WBC (95.0%) and lowest % RBC (5.0%) relative to cell preparations using other density gradients without RBC lysis buffer (Table 6.2). Lysis buffer was used to lyse the RBC and remove RBC as much as possible (De Boever et al., 2010). Since chicken RBC are nucleated and are difficult to remove using the lysis buffer only once (De Boever et al., 2010). The RBC lysis step was performed twice for 3 minutes each, for a total of 6 minutes. Lysis of RBC performed for more than 3 to 5 minutes may decrease white blood WBC viability (Duda et al., 2007); hence cell preparation using Histopaque 1.077/1.119 g/mL with lysis buffer had the lowest WBC viability (33.9%) but the highest % WBC population (95.0%). Percent heterophils were lower in cell preparation using Histopaque 1.077/1.119 g/mL with lysis buffer (44.0 %). Ten minute incubation of lysis buffer with mixture of RBC and WBC

population, lysed and damaged granulocytes in human (Van Oss et al., 1981). In our study, the total incubation time was 6 minutes. We do not know if heterophils would be lysed and damaged due to 6 minute incubation time with the lysis buffer. But if it did, it would explain why % heterophils were numerically lower in cells prepared using Histopaque 1.077/1.119 g/mL with lysis buffer. Although % heterophils was higher from cells prepared using density gradients without lysis buffer (over 59.0%), % RBC was also high (over 97.5% RBC) compared to cells prepared using Histopaque 1.077/1.119 g/mL with lysis buffer.

Cell preparation using Histopaque1.077/1.119 g/mL without lysis buffer had the highest % heterophil (62.0%) compared to other density gradient treatments. Mono-poly resolving medium is a solution that can separate mononuclear and polymorphonuclear cells in human blood (Hoffman et al., 1992). Therefore, cells were prepared using one of the two different density gradients in Experiment 3: Histopaque1.077/1.119 g/mL or Mono-poly resolving medium. To decrease % RBC in the total cell population without using the lysis buffer during cell preparation, the RBC sedimentation buffer Dextran T500, was used to remove RBC before using the different density gradients in Experiment 3.

6.3.3 Experiment 3

Overall, no or small differences in level of WBC viability, % WBC, % RBC or % heterophil were observed when cells were prepared with Mono-poly resolving medium and Histopaque 1.077/1.119 g/mL (Table 6.3). Percent heterophils was greater in the 1.119 band of Histopaque 1.077/1.119 g/mL compared to 1.114 g/mL of Mono-ploy resolving medium. The density at 1.092 and 1.108 g/mL had the highest purity of heterophils (96.9 and 99.8% heterophil purity, respectively) compared to other densities under 1.056 g/mL (Mills and Wilcox, 1993).

Therefore, we expected cells obtained from a density closer to 1.092 and 1.108 g/mL to have higher % heterophil; 1.114 g/mL of Mono-poly resolving medium was expected to have higher % heterophil than cells obtained from 1.119 g/mL of Histopaque 1.077/1.119 g/mL. The opposite of what we expected were seen in our study and the cause of this is unknown.

When cells were prepared using Dextran T500, % RBC decreased (40.5% RBC; Table 6.3) compared to when cells were prepared without Dextran T500 (98% RBC; Table 6.2). Although the % RBC was lower when Dextran T500 was used, % RBC was still relatively high (40.5 % RBC). This may be due to the presence of lower density immature RBC mixed with hetrophils in the 1.119 band (Mills and Wilcox et al., 1993). Percent heterophils decreased when Dextran T500 was used (50.5%) compared to when Dextran T500 was not used (62.0%). This is possibly due to some heterophils settling at the bottom of the tube of Dextran T500. Overall Dextran T500 was a useful solution to remove RBC in chicken whole blood.

Cells prepared using Histopaque 1.077/1.119 with Dextran T500 had higher % heterophil (50.5%) than cells prepared using Mono-poly resolving medium with Dextran T500 (41.0%). Furthermore, cells prepared using Histopaque 1.077/1.119 with Dextran T500 had increased % WBC (59.5%) and decreased % RBC (40.5%) compared to cells prepared using Histopaque 1.077/1.119 without Dextran T500 (2.0% WBC and 98% RBC). Therefore, cells prepared using Histopaque 1.077/1.119 with Dextran T500 was the method that was close to isolating a pure heterophil population.

Isolation of a pure heterophil population was not possible using different Histopaque density gradients in our study. Cells prepared using each of the different combinations of double density Histopaque gradients in this study had higher % heterophil purity (over 44. 0%)

compared to the hetrophil concentration from the whole white blood cell isolation method in our previous study (30%; Chapter 5). However, % RBC (at least 59% RBC) was higher in this study than in our previous study (approximately 5 %; data not shown). SYTOX orange nucleic acid stain is a fluorescent dye which stains extracellular DNA and is used to measure HET production (Chapter 5). Relative fluorescence units, which were measured using SYTOX orange nucleic acid stain, increased with increasing % RBC of the total cell population (data not shown). This indicates that high % RBC contributes to unwanted background fluorescence and hence interferes with the HET assay. Removing RBC as much as possible and obtaining a pure WBC population is important to reduce background fluorescence. Furthermore, a neutrophil purity of over 90% was used in a NET assay using PicoGreen, a fluorescent nucleic acid stain, in human and bovine (Aulik et al., 2010; Tanaka, 2014). Therefore, heterophil purity may need to be over 90% to confidently measure HET production using SYTOX orange nucleic acid stain. Cell viability of neutrophils used for human and bovine NET assay was over 95% using trypan blue exclusion (Aulik et al., 2010; Gupta et al., 2010). Therefore, heterophil viability may be an important factor for the HET assay, in order to accurately measure heterophil-specific extracellular trap production. We were not able to identify and explain the cells that were involved in the extracellular trap production in our previous experiment (Chapter 5). This is because immune cells other than heterophils, such as macrophages, can also produce extracellular traps (Liu et al., 2014). Although both heterophils and macrophages produce extracellular traps, the extracellular trap release in response to stimulus is different: 80% of human neutrophils stimulated with LPS produced NET (Yousefi et al., 2009) while only 10% of the total macrophage population produced extracellular trap when stimulated with *E. coli* in mice (Liu et al., 2014). Furthermore, macrophages have been shown to uptake NET (Farrera and

Fadeel, 2013). Therefore, obtaining a pure population of heterophils and then measuring extracelluar trap will be important to allow us to study and understand heterophil mechanism and function that is unique to heterophils. Further refinement in heterophil isolation method is needed to perform HET assay using SYTOX orange nucleic acid stain; % RBC needs to be reduced and % heterophil increased to ultimately isolate pure heterophil population which could be used for future HET assays.

Overall, we determined that 15 minutes at 500 x g on Histopaque 1.077/1.119 g/mL (3 mL of each Histopaque) using 3 mL of laying hen blood mixed with PBS containing 1% BSA was the optimal centrifugation time. The highest heterophil purity (50.5%) was obtained using Histopaque 1.077/1.119 with Dextran T500. This heterophil isolation method can not be used for future HET assay because further reduction of RBC contamination is needed to reduce the unwanted background fluorescence and allow HET measurement. Therefore, further refinements are needed to reduce the RBC contamination, increase heterophil purity and ultimately isolate pure heterophil population for future use in HET assays. Although, the heterophil isolation method developed in this study could not be achieved, the total WBC extracellular trap developed in the previous experiment (Chapter 5) would still provide useful information in the study of broiler chick innate immune function. The total WBC extracellular trap assay includes the effects of cytokine-immune cell interactions, which is important for cell communication, and activities such as HET release. Hence, total WBC extracellular trap is a useful tool to measure innate immunity.

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6.5 TABLES

Table 6.1 Effect of centrifugation times on cell population from laying hen whole blood using Histopaque 1.077/1.119[1].

Centrifugation time[2] (minutes)	n	Red blood cells[3]	White blood cells[3]	Heterophils[4]
		----------%----------		
10	2	98.5	1.5	60.0
15	2	96.0	4.0	58.5
20	2	85.5	14.5	20.0
25	2	70.5	29.5	12.0
Variance		157.4	157.4	695.7

[1]Sigma-Aldrich, Oakville, ON, Canada

[2]500 x g centrifugal force was used.

[3]White blood cells and red blood cells were identified using Hema 3 staining (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then percentage of white blood cells or red blood cells was calculated by dividing the number of white blood cells or red blood cells by the total cell population.

[4]Heterophils were identified using Hema 3 staining (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then percentage of heterophils was calculated by dividing the number of heterophils by the total white blood cell population.

Table 6.2 Effects of density gradients and use of lysis buffer on cell populations from laying hen whole blood.

Density gradient (Histopaque[1])	Lysis buffer[2]	n	Red blood cells[3]	White blood cells[3]	White blood cell viability[4]	Heterophils[5]
			----------%----------			
1.077/1.119 g/mL	Yes	4	5.0	95.0	33.9	44.0
1.077/1.119 g/mL	No	4	98.0	2.5	80.31	62.0
1.083/1.119 g/mL	No	4	98.0	2.0	79.31	60.0
1.077/1.083 g/mL	No	4	97.5	2.0	80.21	59.0
Variance			1730	1730	469	140

[1]Sigma-Aldrich, Oakville, ON, Canada.

[2]Ammonium chloride-potassium chloride lysis buffer (Lonza, Basel, Switzerland).

[3]White blood cells and red blood cells were identified using Hema 3 staining (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then percentage of white blood cells or red blood cells was calculated by dividing the number of white blood cells or red blood cells by the total cell population.

[4]Cells were stained *ex vivo* using annexin V/propidium iodide (Invitrogen, Carlsbad, CA and BD Biosciences, Mississauga, ON, Canada) and then BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) was used to measure white blood cell viability.

[5]Heterophils were identified using Hema 3 staining (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then percentage of heterophils was calculated by dividing the number of heterophils by the total white blood cell population.

Table 6.3 Effects of density gradients after cell preparation using Dextran T500[1] on cell population from laying hen whole blood.

Density gradient	n	Red blood cells[4]	White blood cells[4]	White blood cell viability[5]	Heterophils[6]
		----------%----------			
Mono-poly resolving medium[2]	4	41.0	59.0	80.0	41.0
Histopaque 1.077/1.119 g/mL[3]	4	40.5	59.5	77.6	50.5
Variance		169.4	169.4	63.1	233.1

[1]Pharmacosmos, Holbaek, Denmark

[2]MP Biomedicals, Solon, OH.

[3]Sigma-Aldrich, Oakville, ON, Canada.

[4]White blood cells and red blood cells were identified using Hema 3 staining (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then percentage of white blood cells or red blood cells was calculated by dividing the number of white blood cells or red blood cells by the total cell population.

[5]Cells were stained *ex vivo* using annexin V/propidium iodide (Invitrogen, Carlsbad, CA and BD Biosciences, Mississauga, ON, Canada) and then BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) was used to measure white blood cell viability.

[6]Heterophils were identified using Hema 3 staining (Thermo Fischer Scientific Inc., Mississauga, ON, Canada) and then percentage of heterophils was calculated by dividing the number of heterophils by the total white blood cell population.

6.6 FIGURES

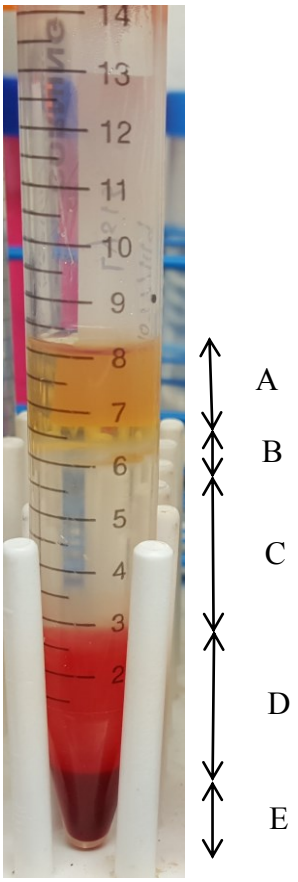

Figure 6.1 Cell separation from hen whole blood mixed with 1 x phosphate-buffered saline at pH 7.4 with 1% bovine serum albumin (3 mL; Thermo Fischer Scientific Inc., Mississauga, ON, Canada) by centrifugation (500 x g for 15 minutes) using Histopaque 1.077/1.119 g/mL (3 mL each; Sigma-Aldrich, Oakville, ON, Canada). Histopaque 1.077/1.119 g/mL fractions indicated by letters are: A) plasma and phosphate-buffered saline with 1% bovine serum albumin, B) mononuclear cells such as lymphocytes and monocytes, C) Histopaque of density 1.077 g/mL, D) mixture of leukocytes with red blood cells in Histopaque of density 1.119 g/mL and E) red blood cells.

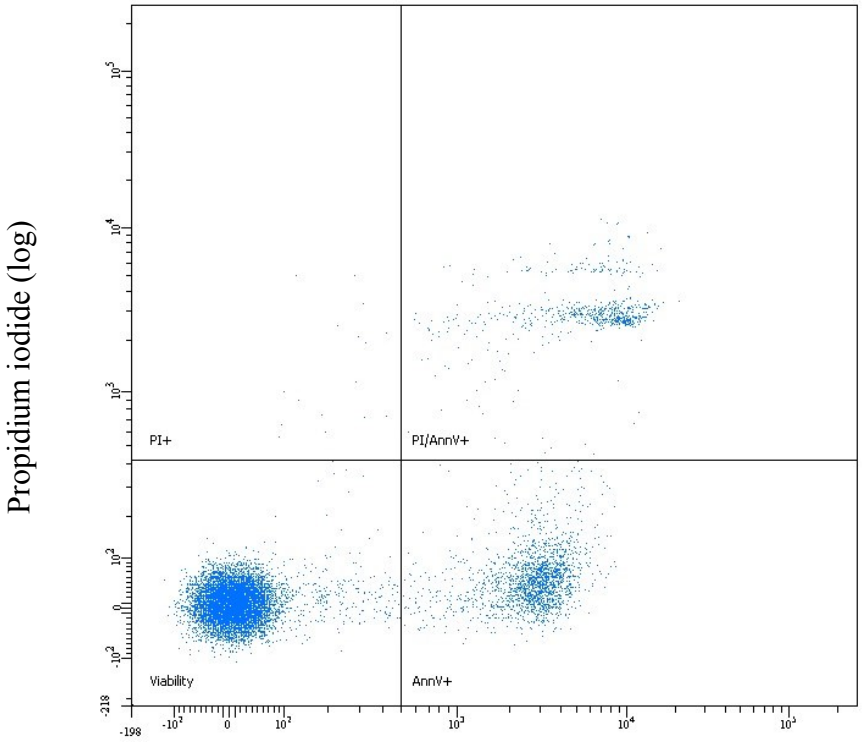

Figure 6.2 Effect of Histopaque 1.077/1.119 g/mL^3 without using lysis buffer on cell viability (78.2% cell viability). Cells stained with Propidium iodide but not Annexin V (PI+), cells stained with Propidium iodide and Annexin V (PI/AnnV+), cells not stained with Propidium iodide and Annexin V (viability) and cells stained with Annexin V but not Propidium iodide (AnnV+) indicates late apoptotic/necrotic, necrotic, viable, and early apoptotic cell population, respectively.

7. RESEARCH SYNTHESIS

7.1 SUMMARY

Twenty five-hydroxyvitamin D_3 (25-OHD_3) is a metabolite of vitamin D_3 and canthaxanthin (CXN) is an antioxidant; both compounds are commercially-available feed supplements used in poultry diets. Vitamin D is associated with calcium and phosphorous metabolism, muscle and bone development, and regulation of the immune system (Wasserman et al., 1982; Norman and Hurqitz, 1993; Aslam et al., 1998; Hutton et al., 2014). Canthaxanthin is a free radical scavenger and therefore, it reduces lipid peroxidation (Mortensen et al., 1997; Rosa et al., 2012). The objective of the first part of the research was to investigate the effect of dietary 25-OHD_3 and CXN on broiler breeder productivity and broiler performance.

Innate immunity involves many defense mechanisms, one of which is heterophil extracellular trap (HET). It may play an important role in protection against pathogens in chickens (Chaummitri et al., 2009). Therefore, the objective of the second set of studies was to develop a HET assay in chickens.

The objectives were addressed with the following hypotheses:

1. Breeder hens fed dietary CXN and 25-OHD_3 would have decreased embryonic mortality, and increased hatchability and antibody titers. When dietary 25-OHD_3 is fed to broiler breeders, it would also increase eggshell thickness. Since roosters do not transfer nutrients to the egg, roosters supplemented with dietary CXN and 25-OHD_3 would not affect egg production, hatchability, embryonic mortality or egg components. These hypotheses were stated and discussed in Chapter 2. However, broiler breeder total and settable egg production, mid, late and total embryonic mortality, hatchability of fertile, shell thickness and IBD antibody titers were not

affected by dietary CXN nor 25-OHD_3. Roosters fed CXN and 25-OHD_3 maintained fertility longer than roosters not fed CXN and 25-OHD_3.

2. Dietary CXN and 25-OHD_3 fed to the breeder hens are transferred to the egg and therefore, chicks hatched from hens fed CXN and 25-OHD_3 would have increased body weight and decreased feed conversion ratio early in life. Breast meat yield and bone mineral density would increase in chicks hatched from 25-OHD_3-fed hens. Roosters supplemented with dietary CXN and 25-OHD_3 would not affect broiler performance. These hypotheses were stated and discussed in Chapter 3. Contrary to the hypotheses, overall there were no or only small effects of parental dietary treatment on broiler performance traits.

3. When CXN and 25-OHD_3 are fed to broiler breeder hens, there would be increased indices of chick innate immunity such as phagocytic index and capacity. Antioxidant capacity would increase in the broiler chicks hatched from CXN-fed hens. Dietary CXN and 25-OHD_3 fed roosters would have no effect on the phagocytic index and capacity, nor on the antioxidant capacity in the broiler chick. This hypothesis was stated and discussed in Chapter 4. As expected, maternal dietary 25-OHD_3 increased total phagocytic index and therefore, suggesting increased innate immunity during the first few d of the chick's life. However, this was not seen in chicks hatched from CXN hens. The chicks from hens inseminated by CXN and 25-OHD_3 fed roosters had increased total phagocytic index compared to chicks from hens inseminated by roosters not fed CXN and 25-OHD_3; the cause of this effect is unknown.

4. The HET assay would be developed using laying hen whole blood. The *Escherichia coli* (*E. coli*) killing, phagocytosis and HET would increase with increasing chick age and male chicks would have decreased *E. coli* killing, phagocytosis and HET compared to female chicks.

This hypothesis was stated and discussed in Chapter 5. Surprisingly, bacteria killing was greater at 1, 4 and 6 compared to 8 d of age but it was the opposite for total phagocytic index. The HET response to *E. coli* was greater at 1 d than any other age. The HET response to *E. coli* was higher in male than female chicks but no sex effect was seen in phagocytic index. Bacteria killing tended to increase in male chicks compared to female chicks. The HET assay in our study is a useful tool to measure part of the innate immunity and could be applied to young chicks.

5. It would be possible to isolate a pure population of heterophils from laying hen whole blood using density gradients. This hypothesis was stated and discussed in Chapter 6. Contrary to the hypothesis, heterophil isolation was not possible using different density gradients. Further refinements are needed to reduce the RBC contamination and isolate pure heterophil population for future use in HET assays.

7.2 ANALYSIS AND IMPLICATIONS

Dietary CXN and 25-OHD_3 fed to broiler breeders at 6 mg/kg and 2,760 IU/kg, respectively, did not affect broiler breeder hen productivity, egg components or antibody titres. This is likely because all breeder treatment diets were well above the NRC (1994) vitamin D activity requirement (300 IU/kg); hatchability was not affected when broiler breeders were fed 25-OHD_3 at 2,760 IU/kg or vitamin D_3 at either 1,200 or 2,400 IU/kg (Coto et al., 2010). Egg shell quality, measured as specific gravity, was not different between layers supplemented 25-OHD_3 or vitamin D_3 at 2,760 IU/kg (Keshavarz, 2003). When breeder hens were fed less than the NRC (1994) vitamin D requirement (125 IU/kg), vitamin D_3 reduced egg production and hatchability of fertile compared to 25-OHD_3 fed at the same level (Atencio et al., 2005). This indicates that 25-OHD_3 and vitamin D_3 contents (2,760 IU/kg) fed to broiler breeders in this study meet the

broiler breeder nutritional requirements, and hence we did not see any 25-OHD_3 effects in breeder productivity. Canthaxanthin did not affect breeder productivity possibly because breeders had adequate dietary antioxidants other than CXN (50 IU/kg vitamin E; 0.3 mg selenium); hatchability and fertility increased when breeders were provided with 6 mg/kg CXN and when other dietary antioxidant levels were low (Rosa et al., 2012; 7.6 IU/kg vitamin E; 0.108 mg/kg selenium). Furthermore, dietary vitamin E contents over 40 IU/kg had no effect on breeder IBD antibody titer (Lin and Change, 2006). This suggests that when other dietary antioxidant contents such as vitamin E and selenium are high (50 IU/kg vitamin E; 0.3 mg/kg selenium) in broiler breeders, dietary CXN does not affect breeder productivity or antibody titres.

Supplementation of the combination of CXN and 25-OHD_3 in roosters increased fertility between 22 and 56 wk of age by 3.6% over 34 wk. The CXN may have scavenged free radicals and therefore protected the sperm from oxidative damage. This may have increased the fertility in the CXN and 25-OHD_3-fed roosters (Surai et al., 1998). Fertility is important in the poultry industry because if the egg is not fertile or there is no embryo in the egg, the egg will not hatch. The difference in fertility between HC (on average 89.50% fertility over 34 wk) and Control (on average 86.38% fertility over 34 wk) roosters over the 34 wk from 22 to 56 wk is 3.6%. The number of broiler breeder hens in Alberta is 519,313/year (Alberta Agriculture and Rural Development, 2011). If a 3.6% increase in hatchability due to increased fertility from HC-fed roosters result in 3.6% less broiler breeder hens required to hatch the same amount of chicks, then it would result in approximately 18,695 fewer breeder hens in Alberta. The ratio of roosters to hens is 1:10 (Aviagen, 2013) and hence, the number of roosters in Alberta would be

approximately 51,931; a 3.6% decrease would reduce that number to 50,061. On average each rooster ate 40.98 kg of feed over a year (Chapter 2) and therefore the total amount of feed that the 51,931 and 50,061 roosters would eat would be 2,128 and 2,051 tonnes of feed, respectively. The cost of rooster feed is approximately $367/tonne (K. L. Nadeau, University of Alberta, Edmonton, Alberta, Canada, personal communication). The 77 tonne reduction in feed required would result in $28,259 more farm cash to the breeder industry. The cost of 25-OHD_3 is $2.69 CAD/tonne of feed and the cost of CXN is $6.99 CAD/tonne of feed (C.A. Lozano, DSM Nutritional Products, Tocancipa, Bogota, Colombia, personal communication) and hence the total cost of dietary 25-OHD_3 and CXN is $9.68 CAD/tonne of feed. The additional cost of feeding dietary 25-OHD_3 and CXN to roosters would be $14,271 CAD over 34 wk (50,061 roosters x 29.45 kg feed/rooster/34 wk x $9.68 CAD/tonne). Therefore, the net benefit from feeding dietary 25-OHD_3 and CXN to rooster would therefore be $13,988 CAD ($28,259 - $14,271). This does not include the feed cost for the extra breeder hens, and housing cost for the extra breeder hens and roosters over a year and hence, the net benefit from feeding dietary 25-OHD_3 and CXN to roosters is likely greater than shown in above calculations. The increased fertility from HC roosters would likely result in increased hatchability and ultimately, will lead to increased profitability.

Parental dietary CXN and 25-OHD_3 did not affect broiler performance traits, broiler carcass and portion yield, bone parameters and antioxidant capacity. Broilers were fed a common diet and 25-OHD_3 and CXN were only included in the egg in the current study. Body weight increased when 25-OHD_3 (Yarger et al., 1995; Fritts and Waldroup, 2003) and marigold flower extract, lutein, (Rajput et al., 2012) was consistently fed to broilers. Lutein is a xanthophyll,

which is a carotenoid that contains oxygen in its structure, and is also an antioxidant similar to CXN (Terao, 1989). Dietary 25-OHD_3 fed to broilers consistently also increased tibia ash (Fritts and Waldroup, 2003).

Maternal dietary 25-OHD_3 and paternal dietary CXN and 25-OHD_3 increased chick total phagocytic index. Egg 25-OHD_3 content was not different between maternal treatments (Chapter 2) and therefore egg 25-OHD_3 contents likely did not influence phagocytic capacity in the broiler chick. The reason for the effect of maternal dietary 25-OHD_3 on broiler innate immune function is not clear, but paternal CXN and 25-OHD_3 may have increased broiler total phagocytic index. Dietary CXN may have prevented the lipid peroxidation, which produce reactive electrophiles such as 4-oxo-2-nonenal **(ONE)**, in the sperm cell membrane (Surai et al., 1998). This ONE can cause disruption of gene transcription and chromosome replication in cells (Galligan et al., 2014). Human sperm cells produce interleukin-1α (Huleihel et al., 2000) and interleukin-1α has been reported to induce *E. coli* phagocytosis (Kabbur et al., 1995). It is unknown whether the mechanisms that disrupt gene expression in the sperm due to ONE would lead to decreased interleukin-1α production and ultimately decrease phagocytic index in broiler chicks. Therefore, the reason for the cause of increased phagocytic index in broiler chicks from hens inseminated by CXN fed roosters compared to chicks from hens inseminated by Control-fed roosters is unknown. Overall, maternal dietary 25-OHD_3 and paternal CXN and 25-OHD_3 may increase innate immunity during the first few d of the chick's life.

The innate immune system has many defense mechanisms such as phagocytosis and HET. A HET method was established in young chicks so that it could be used for future poultry innate immunity studies. Once the HET method was developed using laying hens as a model, the

second experiment was done to show that the HET assay is capable of detecting differences due to factors such as sex and age. Therefore, in the second experiment, the effect of broiler chick age and sex on innate immunity including HET against *E. coli ex vivo* was investigated. The overall innate immunity, *E. coli* bacteria killing, and the HET release in response to *E. coli* were greater when chicks were younger (1 d of age) compared to when chicks were older (8 d of age). However, total phagocytic index against *E. coli* showed an opposite pattern; total phagocytic index increased with increasing age. The resealse of HET in response to *E. coli* was higher in male than female chicks, but no sex effect was seen in bacteria killing or phagocytic index. This indicates that different innate mechanisms are more effective at killing the bacteria depending on chick age and sex. The HET plays an important role in early innate immunity of the chicken.

In Chapter 5, we were able to establish a HET method that can be used for future poultry innate immunity in young chicks. We were able to measure the functional extracellular trap produced from WBC that were stimulated by *E. coli*, and observe the effects of chick age and sex on extracellular trap production. *Mycobacterium tuberculosis*-induced NET contains heat shock protein 72 in humans, which stimulates the release of TNF-α by macrophages (Braian et al., 2013). This TNF-α can further induce NET formation from neutrophils (Keshari et al., 2012). Cytokines, such as TNF-α are important for cell communication and the immune cells, which receive the cytokine signals could perform certain activities such as NET (Belardelli, 1995). It is unclear if HET contain heat shock protein 72, but if they do, then HET assay developed in our study may be influenced by immune cell-cytokine interactions. Hence, HET assay developed in our study is a useful tool to assess innate immunity in chickens. We were not able to determine which cells were involved in the extracellular trap production because macrophages and other

immune cells can also produce extracellular traps (Liu et al., 2014). Therefore, an effort was made to develop a heterophil isolation method to use it in future HET assays. We determined that optimal centrifuge time was 15 minutes at 500 x g on Histopaque 1.077/1.119 g/mL (3 mL of each Histopaque) using 3 mL of laying hen blood mixed with PBS containing 1% BSA. Furthermore, cells prepared using histopaque 1.077/1.119 with Dextran T500 (77.6% WBC viability, 59.5% WBC and 40.5 % RBC and 50.5% heterophils) resulted in greatest enrichment of heterophils. The high proportion of RBC resulted in excessive background interference which made it impossible to reliably measure HET production. Therefore, the procedure from Chapter 6 could not be used to for HET assays. More refinements of the heterophil isolation method are needed to isolate a pure population of heterophils for future use in HET assays. Although, heterophil isolation was not possible in Chapter 6, the HET assay developed in Chapter 5 requires less blood and includes the effect of immune cell-cytokine interactions than the previously-published method (Chaummitri et al., 2009). Hence, the new assay could provide valuble information on chick innate immune function.

Feeding roosters dietary CXN and 25-OHD_3 from 47 and 56 wk of age increased fertility compared to Control roosters. Based on the calculation above, it will lead to increased profitability and revenue. Breeder hens fed dietary CXN and 25-OHD_3 had no effect on breeder productivity. Furthermore, parental dietary CXN and 25-OHD_3 had no or only small effects on broiler performance and innate immunity. Therefore, we would recommend feeding roosters dietary CXN and 25-OHD_3 after 47 wk of age. The HET assay was developed in young chicks and was capable of detecting differences due to factors such as sex and age. However, isolation

of a pure heterophil population was not achieved and further refinements are required to develop a heterophil isolation method that could be used in future HET assays.

7.3 RECOMMENDATIONS FOR FURTURE RESEARCH

Dietary CXN and 25-OHD_3 fed to breeder hens had no effect on breeder productivity (Chapter 2). This could be because all breeder treatment diets were above the NRC (1994) vitamin D activity requirement (300 IU/kg). Egg production and hatchability of fertile has been earlier reported to be lower when breeders were fed vitamin D_3 at 125 IU/kg compared to breeders fed 25-OHD_3 at the same level (Atencio et al., 2005). However, no difference in egg production and hatchability of fertile was observed when breeders were fed higher level of vitamin D_3 and 25-OHD_3 (500 IU/kg; Atencio et al., 2005). Hence, it would be useful to provide different dietary 25-OHD_3 levels, including levels as low as 300 IU/kg which is close to the deficient level, to the breeder hens and look at the effect of breeder productivity in future studies. Breeder hens fed 25-OHD_3 at 2,760 IU/kg had increased hatchability from 61 to 63 wk of age compared to hens fed vitamin D_3 at the same level (Saunders-Blades and Korver, 2015). In the same study, hatchability from 29 to 31 and 46 to 48 wk of age was not different between 25-OHD_3- and vitamin D_3-fed breeder hens (Saunders-Blades and Korver, 2015). Therefore, it would also be useful to extand the study beyond 56 wk of age, to observe the effect of breeder productivity to older ages. The lack of CXN effects on breeder productivity could be because the breeders were housed at a low stocking density (stocking density of 0.216 m^2 per bird) and at a recommended temperature. Breeder productivity increased when breeders were fed with CXN and were group housed at a higher stocking density (Rosa et al., 2012; 0.117 m^2 per bird). Plasma antioxidant capacity has been previously shown to decrease when broilers were subject

to heat stress (32 °C for 6 h) in comparison to broilers under thermoneutral conditions (21 °C; Lin et al., 2006). Hence, the decreased antioxidant capacity and increased oxidative stress in the heat-stressed broiler may cause cell damage and metabolic changes in the broiler and ultimately decrease BW (Altan et al., 2000; Lin et al., 2006). Similarly, heat stressed broiler breeder hens have decreased FI, BW and egg production (McDaniel et al., 1995). Therefore, it would be interesting if dietary CXN was provided to the breeder hens under high stocking density such as group housing and heat stress.

The combination of dietary CXN and 25-OHD_3 fed to roosters increased fertility after 47 wk of age and overall increased total phagocytic index (Chapter 2 and 4). However, whether this effect was from 25-OHD_3, CXN or the combination of both supplements is unclear. Hence, performing a study looking at the effect of dietary CXN and 25-OHD_3 contents individually and the combination of both in roosters on breeder productivity and immune function will be interesting.

Parental dietary CXN and 25-OHD_3 did not affect broiler performance traits, broiler carcass and portion yield, bone parameters and antioxidant capacity (Chapter 3). Broilers were fed a common diet and 25-OHD_3 and CXN were only included in the egg. Therefore, it would be interesting to feed dietary 25-OHD_3 and CXN to the broiler chicken and observe the broiler performance, broiler carcass and portion yield, bone parameters and antioxidant capacity.

The overall innate immunity against *E. coli*, bacteria killing, decreased as chicks aged from 1 to 8 d of age (Chapter 5). However, adaptive immunity was not measured and hence, it would be useful to measure adaptive immunity, such as antibody tire and immunoglobulin levels, in the

chick to investigate if there is a relationship between the innate immunity, adaptive immunity and chick age.

Heterophil isolation was not achieved using different isolation procedures (Chapter 6). Hence, further refinement on the heterophil isolation method should be done in future studies.

7.4 STUDY LIMITATIONS

Calculated dietary 25-OHD3 content was 2,760 IU/kg, but the analyzed values for 25-OHD_3 and HC treatments were 2,017, and 1,892 IU/kg, respectively (Chapter 2). Therefore, analyzing dietary 25-OHD_3 before feeding it to breeder hens and roosters would have made the experiment more consistent and less variable. Gating the cells in flow cytometry is a useful tool because it is used to identify the sub-population such as lymphocyte and thrombocyte, granulocyte and macrophages (Chapter 4 and 5). However, the limitation to gating the cells is that it is subjective. Furthermore, phagocytosis analysis in flow cytometry may include *E. coli* particles that are membrane-bound and not internalized. This is because flow cytometry does not distinguish between membrane-bound *E. coli* particle and internalized *E. coli* particle for phagocytosis analysis. Using imaging flow cytometry will eliminate these limitations (Smirnov et al., 2015) and would have been useful to develop and use it in chickens before we conducted all the immunology experiments.

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