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The effect of maternal antioxidant nutrient supplementation and age on chick post-hatch innate immune function

by

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ABSTRACT

The chick's innate immune system may affect post-hatch liveability and performance, but is immature at hatch. The development of the immune system begins *in ovo* and may be affected by hen age and nutrition. Antioxidants are molecules capable of preventing and correcting oxidative damage and may play an important role in the innate immune response because reactive oxygen species are produced as a bactericidal mechanism. Supplementing hens with antioxidant ingredients may have a priming effect on the chick's innate immune development and increase the response post-hatch. The objective of this thesis was to investigate how hen age and nutrition affected chick post-hatch innate immunity.

Hens were supplemented with one of the antioxidant ingredients, canthaxanthin, synthetic or natural vitamin E or L-carnitine. At three hen ages: 31-33, 45-47, and 57-59 wks, fertile eggs were incubated and hatched. Whole blood was collected for the determination of innate immune function indices which included *Escherichia coli* bactericidal capacity (**EBC**), phagocytosis, oxidative burst and plasma total antioxidant capacity.

The maternal supplementation of each of these three ingredients demonstrated their immuno-modulatory potential in post-hatch chicks. Supplementing with 6 ppm canthaxanthin or natural vitamin E increased chick EBC which was a general measure of innate immune function; while L-carnitine supplementation increased chick oxidative burst. Supplementation of all three ingredients resulted in a three way interaction between hen treatment and age and chick age with respect to chick plasma total antioxidant capacity. The most consistent observation was that chick EBC decreased as hens aged leading to a

study investigating the effects of maternal age and incubator temperature on chick innate immune function. Eggs from 26 to 34, 35 to 44 and 45 to 54 wk old hens were incubated at one of four incubator temperatures, 36.0, 36.5, 37.0, or 37.5°C. It was observed that chicks from 26 to 34 wk old hens had greater innate immune responses and that incubating eggs at 36.5°C resulted in the greatest innate immune response. This research supports published data that chick immune function is affected by incubation conditions and strongly suggests that it is greater in chicks from young hens.

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LIST OF ABBREVIATIONS AND SYMBOLS

α - Alpha

ADG – Average daily gain

AOC – Plasma total antioxidant capacity

ATOC – α-tocopherol

α-TTP – α-tocopherol transport protein

β - Beta

CAT - Catalase

CHP – Chicken heterophil peptides

CpG-ODN - Cytosine guanine di-nucleotide containing oligodeoxynucleotides

CXN - Canthaxanthin

d - Day

δ - Delta

EBC – *ex vivo E. coli* bactericidal capacity

E. coli – *Escherichia coli*

EST – Eggshell temperature

ϒ - Gamma

GIT – Gastrointestinal tract

HDL – High density lipoprotein

HET – Heterophil extracellular traps

HPLC – High performance liquid chromatography

H_2O_2 – Hydrogen peroxide

IL – Interleukin

i.p. – intra-peritoneal

IFN-ϒ – Interferon gamma

LC – L-carnitine

LPS - Lipopolysaccharide

M - Mid

MDA - Malondialdehyde

NDV – Newcastle Disease Virus

NET – Neutrophil extracellular trap

NO – Nitric oxide

NRC – National Research Council

NVE – Natural vitamin E, RRR-α-tocopherol

O - Old

OB – *ex vivo* oxidative burst

OCTN - Organic cation transporters

PA – Phagocytic activation

PC – Phagocytic capacity

PAMP – Pathogen associated molecular patterns

PMN – Polymorphonucleotyes

Post-hatch – Refers to the first seven days of a chick's life

PRR – Pattern recognition receptors

RH – Relative humidity

ROS – Reactive oxygen species

SOD – Superoxide dismutase

SRBC – Sheep red blood cells

SVE – Synthetic vitamin E, all-*rac*-α-tocopherol

THP – Turkey heterophil peptides

TLR – Toll-like receptors

TNF-α – Tumour necrosis factor alpha

VE – Vitamin E

Y - Young

1. LITERATURE REVIEW

1.1 INTRODUCTION

Many factors affect the well-being and growth of chicks including hen nutrition and age, incubation conditions, and the chick's immune function and nutrition. Although much research has focused on the effect of hen age on chick growth and liveability (Tuft and Jensen, 1991; Wolanksi et al., 2006; Hulet et al., 2007; Wolanski et al., 2007; Ulmer-Franco, 2010), little attention has been paid to the immune system which is integral to chick health. The immune system consists of innate and acquired responses. The innate immune response is frequently described as the first line of defense against pathogenic threat (Erf, 2004); and is the part of the immune system the chick relies on during the first week of life while its immune system continues to mature (Crhanova et al., 2011; Bar-Shira et al., 2003). The immaturity of the immune system may contribute to high first week mortality (1.82%) of chicks from young hens (26 weeks of age; Yassin et al., 2009). While the effect of hen age on chick first week mortality suggests there may be a relationship between hen age and the development of the immune system, there are data supporting that the development of the immune response is impaired by high or low incubation temperatures as well (Oznurlu et al., 2010; Ardia et al., 2010). Further adding to the complexity of immune system development is the relationship between hen age and egg size, and egg size and embryonic heat production which affects the temperature of the microenvironment surrounding the incubating egg (Lourens et al., 2006).

There are nutrients which possess immunomodulatory properties (reviewed by Klasing, 1998b); many of these nutrients are also antioxidants. Antioxidant ingredients may play an important role in the innate immune response because of the production of reactive oxygen species as a bactericidal mechanism by innate immune cells (Yamamoto and Johnston, 1984). Given the immaturity of the immune response of post-hatch chicks (Crhanova et al., 2011; Bar-Shira et al., 2003) it would be beneficial if the immune response could be primed prior to hatch. Hen supplementation of the antioxidant and immunomodulatory ingredients canthaxanthin, (Koutsos et al., 2006), vitamin E (Lin et al., 2005; Weiss et al., 2009) or L-carnitine (Peebles et al., 2007; Abd-Allah et al., 2009) resulted in increased levels of deposition into eggs. It is possible that by making these nutrients more available to the chick, the chick's immune response post-hatch could be enhanced. The objective of this review is to explore how immune function could be affected by hen age and antioxidant nutrients.

1.2 HEN AGE AND INCUBATION CONDITIONS

Traditionally, broiler chick performance is measured by carcass yield at market age. As a result, efforts to determine at hatch which chicks would result in the highest performing chicks have been undertaken. Measurements of chicks at hatch thought to indicate which chicks will be the highest performing include body weight, body length, shank length, and residual yolk sac weight (Wolanksi et al., 2006; Wolanski et al., 2007). However, the relationships between many of these measurements at hatch and market age body weight are inconsistent. For example, there are a number of studies reporting that older hens lay larger eggs

which hatch larger chicks which are larger at market age (Ulmer-Franco et al., 2010; Peebles et al., 1999), but there are also studies which state that market age body weight is unaffected by hen age (Tuft and Jensen, 1991; Hulet et al., 2007). Additionally, the correlation values of many of these factors with final body weight are relatively weak. For example, chick hatch length is not highly correlated to body weight at market age (average 0.33 as calculated and reviewed by Willemsen et al., 2008; Wolanski et al., 2006). The lack of strong relationships between the measurements currently favoured in chick quality determination and carcass yield, suggest that better measures of chick quality are needed and should include the impact of immune function.

First week mortality of chicks was greatest (1.82%) among chicks from 26 week old hens, lowest (1.02%) among chicks from 38 to 44 week old hens and then increased slightly (1.20%) among chicks from 60 week old hens (Yassin et al., 2009). The reasons for this observation are not understood. It could be because young (36 wks) and old (63 wks) hen ages are associated with lower usages of yolk sac fatty acids as indicated by greater residual yolk sac weights compared to chicks from middle aged hens (51 wks; Latour et al., 1998). Additionally, eggs from 30 week old hens had less fat per gram of yolk than eggs from 50 week old hens suggesting that chicks from younger hens have fewer post hatch resources than chicks from older hens (Yadgary et al., 2010).

Although chick performance may be affected by hen age, other factors such as incubation conditions could also be important. The effects of incubation conditions on hatchability and chick growth performance post-hatch have been

well studied. Increasing the incubation temperature to 43.5°C from 37.5°C at three days of incubation for 12 hours reduced hatchability of fertile eggs to 50% of controls and as embryos aged their tolerance for heat stress decreased (Ande and Wilson, 1981). Additionally, exposing developing embryos to a high temperature (38.9 °C) during incubation (d7 to hatch) increased post-hatch mortality due to ascites which was attributed to reduced heart development (Molenaar et al., 2011). The reduced hatchability and increased post-hatch mortality observed when incubation temperature was too high indicates that chick development was impaired. Furthermore, the results of the cited literature suggest that incubation temperature could have effects beyond incubation into post-hatch performance, which was greater when the eggshell temperature was 37.8°C compared to 39.5°C (Joseph et al., 2006).

The relationship between egg size and hen age is well established; as hens age they lay larger eggs (Anderson et al., 1978; Gibson et al., 2008; Tůmová and Gous, 2012). This relationship has a significant impact on the incubation requirements of eggs. Larger embryos produced greater amounts of metabolic heat which effectively increased the experienced incubation temperature from day 15 of incubation to hatch (Lourens et al., 2006). To maintain the eggshell temperature of large eggs at 37.8°C, the machine temperature needed to be adjusted downward from day 15 of incubation to hatch (Lourens et al., 2006). The location of eggs in the incubator also affected the incubator temperature experienced by embryos as well as the hatchability, which was further affected by egg size. Elibol and Brake (2008) found that embryos of large eggs placed

furthest away from the incubation fan experienced a nearly 1°C greater incubation temperature than large eggs placed closer to the setter fan or smaller eggs regardless of their position. The hatchability of the large eggs which experienced the increased incubation temperature was about 10% lower than large eggs which were not heat stressed or smaller eggs regardless of position (Elibol and Brake, 2008).

The development of chick immune function is also affected by incubation conditions. Oznurlu et al., (2010) incubated eggs from 40 wk old Ross 308 broiler breeder hens and found that when eggs were incubated at 37.8°C and 65% relative humidity, the actual egg temperature was between 39.0°C and 39.4°C as embryos aged from day 11 to 18 of incubation. When the incubator temperature was raised by one degree to 38.8°C (at 65% RH), the actual egg temperature was between 40.1°C and 40.6°C from day 11 to 18 of incubation. This one degree increase in egg temperature induced immunosuppression of the resulting chicks as indicated by reduced development of the bursa and thymus and reduced peripheral lymphocyte numbers (Oznurlu et al., 2010). Similarly, tree swallow nestlings (*Tachycineta bicolor*) which were exposed to reduced (by 6°C) incubation temperatures had lower abilities to kill *Escherichia coli* (***E. coli***) *in vitro* (Ardia et al., 2010), further demonstrating the effect of incubation temperature on the development of the immune system. Given that optimal incubation conditions are influenced by egg size and by association hen age, and immune system development is influenced by incubation conditions, it could be argued that hen age may influence the development of the chick's immune system. The

relationship between hen age and chick post-hatch innate immune function has not been discussed in the literature, but it is possible that the differences in chick first week mortality associated with hen age observed by Yassin et al., (2009) could be a reflection of differences in the development of the chicks' immune system.

1.3 INNATE IMMUNE FUNCTION

The immune system consists of several layers of defense strategies to protect the host from a variety of pathogens. The innate immune system consists of physical and chemical barriers (e.g. skin and mucosal layers), as well as cellular and soluble components (Erf, 2004). The mechanisms of protection employed by the innate response involve the production of antimicrobial peptides (Evans et al., 1995), heterophil extracellular traps (**HET;** Chuammitri et al., 2009) and reactive oxygen species (**ROS**; Yamamoto and Johnston, 1984). The immune system of the newly hatched chick is functionally immature. In the GIT chicks are not able to mount an acquired response or produce antibodies prior to eight days of age (Bar-Shira et al., 2003); therefore a greater reliance on the innate response is required. In fact, until day four of age chicks rely almost solely on the presence of the antimicrobial peptides, gallinacins 1, 2, 4 and 6, which are part of the innate response, for immune protection in the GIT (Crhanova et al., 2011).

The cells of the innate response, particularly the macrophages, drive the immune response by recognizing the presence of pathogens and activating the inflammatory response which in turn activates the acquired response (Klasing, 1998a). Although the innate response is frequently described as non-specific,

there is a certain amount of specificity in it. The innate immune system identifies pathogens through pathogen-associated molecular patterns (**PAMP**) and pattern recognition receptors (**PRR**) (Köllisch et al., 2005). Pathogen-associated molecular patterns are components of microbes which are essential to their survival and therefore are highly conserved. They include lipopolysaccharide (**LPS**), peptidoglycan, lipoteichoic acid, bacterial lipoproteins, bacterial heat shock proteins, mycobacterial phosphoinositol mannosides, bacterial cytosine guanine di-nucleotide containing oligodeoxynucleotides (**CpG-ODN**), viral single and double stranded RNA and bacterial flagellin (reviewed by Kannaki et al., 2010). Pattern recognition receptors enable immune cells to respond to pathogens by recognizing the molecular patterns (PAMP) which are integral to the survival of pathogens, but which do not exist in host cells. Different PAMP interact with different PRR and to that end, will activate different pathways (Köllisch et al., 2005), a discussion of which is beyond the scope of this review. The most extensively studied PRR are the toll-like receptors (**TLR**) (reviewed by Kannaki et al., 2010).

Toll-like receptors are highly conserved receptors which aid in the initiation of the immune response. Chickens possess ten TLRs: TLR1-LA (Roach et al., 2005), TLR1-LB (Roach et al., 2005), TLR2-A (Higgs et al., 2006), TLR2-B (Fukui et al., 2001), TLR3 (Iqbal et al., 2005), TLR4 (Higgs et al., 2006), TLR5 (Keestra et al., 2007), TLR7 (Philbin et al., 2005), TLR15 (Higgs et al., 2006) and TLR21 (Brownlie et al., 2009). Some TLRs are expressed more prevalently on certain cell types than others, which may reflect the type of immune response

that cells are associated with (Iqbal et al., 2005). For example, chicken heterophils express TLR1, 2, 3, 4, 5 and 7 allowing them to recognize a wide variety of PAMP, conferring on them the ability to protect against a wide variety of pathogens (reviewed by Kannaki et al., 2010). Toll-like receptor-4 is highly expressed on monocytes and macrophages and its activation results in the production of TNF-α, IL-12 and IFN-γ (Beutler and Rietschel, 2003), all of which are pro-inflammatory cytokines which are crucial in propagating the innate response.

Cells of the innate response include dendritic cells, basophils, monocytes/macrophages and heterophils (Carlson, 1972; Harmon and Glisson, 1989; Andreasen et al., 1993). The cells of the innate response act as sentries. Specifically, macrophages are able to detect, phagocytize and kill pathogenic microbes (Dietert and Golemboski, 1998). They also produce pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Serbina et al., 2003), which initiate a cascade of events which may culminate in the full activation of the immune response. Immediately upon activation the endothelium of blood vessels in the vicinity of the pathogen invasion (insult) becomes more permeable and selectively allows leukocytes (mainly heterophils) access to the insulted tissue (Proebstl et al., 2012). At the site of insult, any heterophils which had not already been activated by the cytokines produced by the macrophages become active and set about trying to rid the host of the pathogen. Mechanisms employed by the innate response include degranulation (Farnell et al., 2006), phagocytosis and oxidative

burst (**OB**) (Yamamoto and Johnston, 1984), and heterophil extracellular traps (Chuammitri et al., 2009).

Heterophils contain large amounts of intracellular granules (Kannan et al., 2009), thus they are characterized as granulocytes. The primary granules found in heterophils consist largely of antimicrobial peptides (reviewed by Harmon, 1998). There are 14 described avian cationic peptides which are classified as gallinacins (extensively reviewed by van Dijk et al., 2008). The antimicrobial peptides found within heterophils are chicken heterophil peptides (**CHP**) 1 and 2, and turkey heterophil peptides (**THP**) 1, 2 and 3 (Evans et al., 1995). Chicken heterophil peptides 1 and 2 and THP 1 and 3 are microcidal to a wide array of microbes, including fungi, mycoplasma and gram-negative bacteria (Evans et al., 1995). Degranulation is the process by which heterophils release the contents of their antimicrobial granules into the phagocytic vacuole containing the phagocytized pathogen for the purpose of killing it (Segal et al., 1980). Oxidative burst refers to the production of ROS via the activation of the enzyme NADPH oxidase which coincides with degranulation (Segal et al., 1980). The primary product is superoxide anion which is rapidly dismutated to hydrogen peroxide (**H_2O_2**). Hydrogen peroxide is then halogenated, catalyzed by myeloperoxidase, to form hypochlorous acid (reviewed by Winterburn, 2008; Figure 1.1). However, avian heterophils, which are the counterpart of mammalian neutrophils, lack the enzyme myeloperoxidase, so the conversion of H_2O_2 to hypochlorous acid does not occur. Although OB is not a major mechanism of clearing pathogens in birds (Styrt et al., 1989), ROS are still produced (Farnell et al., 2006). Heterophil extracellular

traps are composed of DNA, histones and granular enzymes (Chuammitri et al., 2009). In chickens, the production of HET can be stimulated using phorbol myristate acetate and H_2O_2 (Chuammitri et al., 2009). Although avian heterophils lack the enzyme myeloperoxidase required for the full activation of the oxidative burst pathway, oxidative products produced by activated heterophils are required for the production of HET (Chuammitri et al., 2009). The products of these lethal mechanisms do not specifically target the pathogen and the host may suffer substantial amounts of collateral damage. For example, the ROS produced during oxidative burst may result in oxidative damage to tissues (Shanmugasundaram and Selveraj, 2011; Boon and Park, 2004). The use of ROS and potential for collateral oxidative damage suggests that antioxidants may play an important role in the innate immune function.

1.4 ANTIOXIDANTS

Reactive oxygen species are normal by-products of metabolism and immune defense (Yamamoto and Johnston, 1984). They include superoxide anion (Pryde and Hirst, 2011), peroxides (Pryde and Hirst, 2011), and nitric oxide (Diesen and Kuo, 2010). Reactive oxygen species also contribute to the formation of free radicals, which are molecules possessing an unpaired electron. The presence of an unpaired electron may alter the structure, and thus function, of a molecule, effectively damaging it (for a complete review on free radical formation and consequences refer to Magder, 2006). In small amounts, ROS may function as signalling molecules (Tozer et al., 2012; Lee et al., 2011) but in high concentrations they are toxic; damaging lipids, DNA and protein (Boon and Park,

2004; Lü et al., 2010). As a result, their levels are carefully controlled by the body via antioxidant molecules (Lü et al., 2010). An antioxidant is a molecule capable of neutralizing ROS and free radicals thereby preventing and correcting oxidative damage (Huang et al., 2005). Antioxidants present within the body include dietary antioxidants such as carotenoids (He et al., 2011), and vitamins E and C (Ryan et al., 2010), amino acids (Li et al., 2012); small plasma molecules such as uric acid (Cohen et al., 2007) and endogenous enzymes – glutathione peroxidase, catalase (**CAT**), and superoxide dismutase (**SOD**) (Hisalker et al., 2012), which occurs in two forms. The cytosolic form of superoxide dismutase contains zinc and copper, while the mitochondrial form contains manganese (Reddi et al., 2009).

Antioxidants may function in several ways to protect the body from oxidative damage. They may directly interact with ROS as in the case of SOD, CAT and glutathione peroxidase. Superoxide dismutase converts superoxide anion to H_2O_2 and oxygen and then H_2O_2 is reduced by CAT and glutathione peroxidase to water and oxygen (Lin et al., 2005). Antioxidants may also act as chain breaking molecules. Fat soluble antioxidant molecules, such as vitamin E and carotenoids, are located in biological membranes and can accept unpaired electrons, preventing membrane oxidation (Howard et al., 2011). Others, such as vitamin C, which is water soluble and is found in the extracellular matrix, not only scavenge (react directly with ROS and free radicals), but also regenerate other antioxidant molecules such as vitamin E (as reviewed by Traber and Stevens, 2011).

The presence of antioxidants during an immune response could be a double-edged sword. Reactive oxygen species are produced as anti-pathogenic effector molecules in the innate immune response as part of OB (Yamamoto and Johnston, 1984). They are also required for the formation and release of neutrophil extracellular traps (**NET**; Kirchner et al., 2012), which are analogous to HET in birds (Styrt, 1989); and also function to regulate chemotaxis of neutrophils to sites of inflammation (Hattori et al., 2010), this presumably is also true of heterophils. Theoretically, excessive amounts of antioxidants could impair these important roles of ROS. However, the enzyme required for the production of ROS, NADPH oxidase, resides within the cellular membrane (Kirchner et al., 2012), and immune cells contain high amounts of polyunsaturated fatty acids and are highly susceptible to oxidative damage (Brambilla et al., 2008). Therefore, in addition to mitigating collateral damage to tissues caused by OB products, maintaining a healthy cellular membrane would be critical for the proper functioning of the immune cell and production and release of ROS. Chain breaking antioxidants such as vitamin E (Howard et al., 2011), which reside within the cellular membrane would be important in this manner.

1.5 ANTIOXIDANT NUTRIENTS

1.5.1 Canthaxanthin

Canthaxanthin (**CXN**) is one of more than 600 known carotenoid molecules (Kull and Pfander, 1995), and is commonly added to the diets of poultry to increase tissue pigmentation (Koutsos et al., 2003a). As a carotenoid it is lipid soluble and possesses many biologically important properties, including

antioxidant and immunomodulatory functions (Bendich and Shapiro, 1986). Carotenoids exist as either xanthophylls (Figure 1.2; eg. canthaxanthin), or carotenes (Figure 1.3; eg. β-carotene) (Rice-Evans et al., 1996). Xanthophylls contain oxygen within their structures, while carotenes are hydrocarbons (Kotake-Nara and Nagao, 2011). The structure of carotenoids affects their antioxidant capacity. Carotenoids with nine or more conjugated double bonds (eg. CXN) are the most effective antioxidant carotenoids (Bendich. 1989); likely because the quantity of double bonds allows the molecules to be highly interactive with other ions (El-Agamey et al., 2004).

Carotenoids scavenge free radicals produced by regular metabolic events within the body (Navara and Hill, 2003), and also by phagocytic cells during an immune response (Boon and Park, 2004). In scavenging and quenching free radicals and ROS within the body, carotenoids may be destroyed, reducing their quantity in the tissues (Koutsos et al., 2003b; Darvin et al., 2008). The antioxidant properties of CXN are apparent in both eggs and chicks of hens supplemented with CXN. Eggs from Cobb 500 hens supplemented with 6 ppm CXN contained fewer oxidative products in stored eggs than eggs from non-supplemented hens (Rosa et al., 2012). Similarly, chicks from 23 week old Chinese Three Yellow broiler breeder hens supplemented with 6 ppm CXN had increased serum antioxidant capacity and lower levels of malondialdehyde (**MDA)** (Zhang et al., 2011); a similar result was also obtained in a field trial of Ross broiler breeder hens (Robert et al., 2007). It is also possible that the antioxidative properties of CXN could have an impact on the innate immune response. *In vitro* application

of CXN to rat macrophages resulted in reduced production of ROS during the OB response of activated macrophages (Zhao et al., 1998), which could have an attenuating effect on collateral damage to host tissues normally incurred during the OB response.

The ability of carotenoids to immuno-modulate is well documented. In 10 month old female cats, lutein induced B-cell proliferation and maturation and increased T-cell populations (Kim et al., 2000). Lutein may also reduce the physical symptoms of inflammation by reducing body weight loss in chicks challenged with LPS compared to chicks not supplemented with lutein (Koutsos et al., 2006); and dietary astaxanthin reduced cecal colonization of *Clostridum perfringens* in experimentally infected broiler chickens (Waldenstedt et al., 2003).

Carotenoids are obtained by animals and birds via their diet, the carotenoid profile of which directly affects the carotenoid profile of the body. For example, the major carotenoids in corn-based diets are lutein and zeaxanthin and chickens consuming a corn-based diet will have large stores of these two carotenoids (Surai and Sparks, 2001). Carotenoids, or CXN, are absorbed along with other fat soluble molecules. They are passively absorbed in the intestine (Hamilton, 1992; Tyczkowski and Hamilton, 1986) in their free alcohol form into enterocytes (Cohn, 1997). The absorption of carotenoids appears to be dependent on the fat content of the diet. Beta-carotene requires a relatively small amount of fat to be present, while lutein requires a substantially greater amount of fat to aid its absorption (Roodenburg et al., 2000). Additionally, there is evidence that the absorption of carotenoids is competitive with other nutrients. For example, the

absorption of vitamin E is inhibited by the presence of carotenoids such as CXN, but not β-carotene in rats (Blakely et al., 1991).

Once absorbed into the enterocyte, carotenoids are packaged into large lipoproteins and transferred to the portal blood as portomicrons (Walzem, 1996), for delivery to the liver. In the liver, carotenoids may undergo some modification and metabolism, but many are left unchanged (Surai et al., 2001). As portomicrons travel through the circulatory system, they are catabolised by lipoprotein lipase which reduces the size of the portomicron. Parts of the remaining portomicron are transferred to high density lipoprotein (**HDL;** Traber, 1996), and the remnants of the portomicron are removed by the liver which is how the liver accumulates carotenoids.

Hen dietary carotenoid content determines the carotenoid content of eggs and subsequently the carotenoid levels in the tissues of post-hatch chicks (Koutsos et al., 2003a). However, published data describing the transport mechanisms of carotenoids into eggs are unavailable and it is likely that most are transported and deposited with fatty acids (yolk formation reviewed by Moran, 1987). Carotenoid deposition in tissues is a saturable process depending on the bioavailability and levels of dietary carotenoids (Koutsos et al., 2003a). When Ross broiler breeder hens were supplemented with 96.5 mg/kg carotenoids (lutein, canthaxanthin, citranaxanthin, and β-apo-89-carotenoic acid) it took 19 to 23 days of supplementation for egg carotenoid levels to plateau (Surai and Speake, 1998). Carotenoids appear to be poorly absorbed by the chick from the yolk sac (Haq and Bailey, 1996), but exposure to carotenoids *in ovo* increased the ability of chicks to

incorporate dietary carotenoids into their tissues post-hatch (Koutsos et al., 2003a). Most yolk-derived carotenoids are deposited into the embryonic liver (Speake et al., 1998), whereas carotenoids provided post-hatch are deposited into many tissues, including the liver (Koutsos et al., 2003a). When CXN is supplemented to broiler breeder hens it is deposited into the yolk of the egg and is detectable in the plasma, liver, thymus and skin of hatched chicks (Koutsos et al., 2003a; Surai et al., 2003).

The available literature on the effects of CXN on the immune system of chicks and its effects on the innate immune response of chickens is limited. It is reasonable to expect that if supplemented to hens, the resulting chicks would have CXN available to them *in ovo* and post-hatch that could aid the innate immune response in protecting them from pathogens. However, the effects of CXN on the innate immune response of chicks in the first week of life have not been investigated.

1.5.2 Vitamin E

Vitamin E (**VE**) is a fat-soluble vitamin of plant origin, known for its antioxidant (Ryan et al., 2010) and immunomodulatory properties (Boa-Amponsem et al., 2000). There are eight molecules with VE activity found in nature, these are alpha (**α**), beta (**β**), gamma (**ϒ**), and delta (**δ**) tocopherols and their tocotrienes (Figure 1.4; Jensen et al., 2006); of those eight, α-tocopherol (**ATOC**) has the greatest bioactivity (Jensen et al., 2006). Alpha-tocopherol may exist as one of eight possible stereoisomers which are RRR, RRS, RSS, RSR, SRR, SSR, SRS, and SSS, however only RRR-α-tocopherol exists in nature

(Figure 1.5; Jensen et al., 2006). Commercial synthetic vitamin E (all-*rac*-α-tocopherol; **SVE**) is an equal (racemic) mixture of all eight ATOC stereoisomers, seven of which have lower bioactivities than the stereoisomer which occurs naturally, RRR-α-tocopherol (Natural vitamin E, **NVE**; Jensen et al., 2006). Therefore the effectiveness of supplementing chickens with SVE may differ from when NVE is supplemented in equal amounts.

The presence of tocopherols in cellular membranes confers on them an important role in the maintenance of cell membrane integrity (Combs and Scott, 1977). Tocopherols are chain-breaking antioxidants which function by donating a hydrogen atom from the phenolic ring. The result is a lipid hydroperoxide and a resonance-stabilized antioxidant radical which is stable enough not to propagate the chain reaction (Figure 1.6; El-Agamey et al., 2004). The antioxidant capacity of ATOC is well documented. Supplementing the diets of broilers with 50 or 100 IU/kg ATOC increased the total antioxidant capacity of serum and may do so by modulating the expression of genes of pro-oxidizing proteins and enzymes (Xiao et al., 2011). Supplementing 30 month old rats with 30,000 mg SVE/kg diet inhibited production of H_2O_2, lipid peroxidation and DNA oxidation and increased levels of glutathione peroxidase and CAT in exercised muscles (Ryan et al., 2010). Similarly, hen supplemented dietary 160 mg/kg SVE up-regulated antioxidant enzymes SOD and CAT in the brains of newly hatched chicks (Lin et al., 2005); and supplementation of 400 IU/day of SVE for 28 days reduced oxidative stress in humans and increased antioxidant status relative to a non-supplemented state (Lee and Wan, 2000).

The effectiveness of ATOC as an antioxidant may depend on its supplementation level. For example, in broiler breeder hens the plasma antioxidant status increased linearly when SVE was supplemented at levels from zero to 160 mg/kg diet; the resulting chicks had greater brain antioxidant enzyme activity when the hens were supplemented 160 mg/kg than in chicks hatched from hens fed up to 80 mg SVE/kg feed (Lin et al., 2005). Conversely it was found that supplementing laying hens with levels of NVE greater than 120 mg/kg diet resulted in a pro-oxidant effect of NVE rather than an antioxidant effect (Chen et al., 1998). When NVE was present at very high (250 IU/day) levels in humans it took on pro-oxidant characteristics, resulting in increased oxidation activity in the plasma (Pearson et al., 2006). Furthermore, oxidized metabolites of ATOC are capable of reacting with molecular oxygen and producing superoxide and singlet oxygen (Crisostomo et al., 2007).

The effect of ATOC supplementation on the immune response to a variety of diseases has been well documented and may be the result of increased ATOC content in immune cells, which was observed in the neutrophils of dairy cows (Weiss et al., 2009). Natural vitamin E (37 mg/ml) increased T-cell division and production of IL-2 in rats (Adolfsson et al., 2001; Han et al., 2006); and high supplementation of SVE in rats (2,500 mg/kg) resulted in the greatest phagocytic activity of alveolar macrophages (Moriguchi et al., 1990). Furthermore, SVE supplementation (2,500 IU/day) in cows increased phagocytic capacity of neutrophils relative to the same supplementation level of NVE but had no effect on phagocytic activity (Weiss et al., 2009). When SVE was supplemented in

excess (25 to 200 IU/kg) of the NRC (1994) VE recommendation (10 IU/kg) to the diet of broiler chicks there was an increase in antibody titres after vaccination with killed or live IBV, or sheep red blood cells (**SRBC;** Leshchinsky and Klasing, 2001). Additionally, LPS challenge of chicks resulted in a 1.5-fold decrease in liver ATOC levels when chicks were fed a diet high in omega-3 fatty acids supplemented with 25 IU/kg and had access to feed within 5.5 hrs post hatch, which may indicate a protective effect of ATOC during LPS challenge (Gonzalez et al., 2011).

Tocopherols are passively absorbed with dietary fats (Sontag and Parker, 2002) with the aid of bile salts and pancreatic juices in the small intestine (Gallo-Torres, 1970). However, bile-activated lipase did not contribute to the absorption of ATOC in its alcohol form which was absorbed in the presence of bile salts and fatty acids (Knarreborg et al., 2004). Thus pancreatic enzymes must be required for lipid hydrolysis but not specifically for ATOC absorption, suggesting that the role of bile in tocopherol absorption may be limited to emulsification. As chickens do not possess a lymphatic system, ATOC is transported in portomicrons to the liver via the portal system and circulated throughout the body similarly to carotenoids (Walzem, 1996). In rats, liver tocopherol receptors are highly selective for ATOC and may even increase in number during times when dietary ATOC is inadequate (Behrens and Madere, 1987); it is unknown whether this is also true of birds.

Age may factor into the absorption of tocopherols from the GIT. Knarreborg et al., (2004) found that ileal absorption of ATOC was greater in chicks older than

21 days than in chicks 14 days and younger, but that age did not affect γ-tocopherol absorption. The absorption of ATOC may also decrease after a certain age; serum ATOC status of dogs decreased as dogs aged from five to 10 years of age by nearly 50% (Stowe et al., 2006). The type of dietary fat may also affect the absorption of ATOC, with unsaturated fats facilitating ATOC absorption to a greater degree than saturated fats (Knarreborg et al., 2004).

Despite equal absorption of the various tocopherols from the GIT, NVE possesses the greatest bioactivity because it is the preferred substrate of α-tocopherol transport protein (Traber and Kayden, 1989), however there are no data to support its existence in chickens. If it does exist in chickens it may be expressed in only a few tissues as it is in rats (Sato et al., 1993). Furthermore, cows fed NVE had greater plasma ATOC levels than cows fed SVE despite diets being formulated to the same vitamin E activity (2,500 IU/day; Weiss et al., 2009). Supplementation of ATOC results in increased tissue levels of ATOC in humans (Princen et al., 1995). In rats, at the beginning of ATOC supplementation the highest amounts of ATOC were found in the spleen, followed by the adipose tissue followed by the lung followed by the liver followed by the plasma (Jensen et al., 2006). However, there may be differences in deposition of NVE and SVE. Rats supplemented with equal amounts NVE and SVE up to 100 mg/kg diet had greater amounts of NVE deposited but supplementation of 200 mg/kg diet resulted in no differences between NVE and SVE sources (Jensen et al., 2006). Similarly, SVE concentrations in the egg and blood plasma increased with dietary SVE supplementation up to 120 mg SVE/kg diet (Lin et al., 2005).

Information describing mechanisms of transport and deposition of ATOC into the egg yolk was not found. Alpha-tocopherol is probably transported into eggs with very low density lipoprotein as reviewed by Moran (1987), similarly to carotenoids. When SVE was supplemented to broiler breeders there was an increased concentration of ATOC in the yolk of eggs (Lin et al., 2005). The transfer of ATOC from the egg to the chick occurs very efficiently (Surai and Sparks, 2001); though the chick's liver content of ATOC decreased rapidly post-hatch (Surai et al., 1998).

The immunomodulatory and antioxidant properties of ATOC are well documented. However, data describing the effect of maternal VE supplementation on the post-hatch innate function of broiler chicks is not available, nor is data comparing the effects of NVE vs. SVE on the early innate immune response in broiler chicks.

1.5.3 L-Carnitine

L-Carnitine (**LC**; Figure 1.7) is required for the metabolism of fat and carbohydrates (Stephens et al., 2007). It may be obtained from the diet or via endogenous synthesis. The first step of LC synthesis is the methylation of lysine resulting in trimethyllysine, which is further oxidized to ΄Υ-butyrobetaine by trimethyllysine aldolase and 4-N-trimethylaminobutryaldehyde dehydrogenase. The ΄Υ-butyrobetaine is hydroxylated by ΄Υ-butryobetaine dioxygenase resulting in LC (Figure 1.8; Vaz and Wanders, 2002). Although it is best known for its role in fatty acid metabolism and beta-oxidation (Jakobs and Wanders, 1995), LC also acts as a buffer for excess acetyl-CoA (Childress et al., 1966). Without the aid of

LC and carnitine palmitoyltransferase, mitochondria would be impermeable to fatty acids (Fritz, 1955; Fritz and Marquis, 1965). Carnitine palmitoyltransferase catalyzes the esterification of LC with the fatty acid. The acylcarnitine is then transported across the inner mitochondrial membrane by carnitine acylcarnitine translocase while at the same time free LC is transported from the inner mitochondrial membrane to the outer mitochondrial membrane (Pande, 1975). In this manner LC is recycled.

L-carnitine deficiency may lead to disease and conditions such as cardiomyopathy (di San Filippo et al., 2008). However, plasma levels of LC sharply decrease over the course of gestation in humans without any apparent repercussions (Ringseis et al., 2010). It is unclear if this is the case in other mammalian and non-mammalian species. For example, chickens lay eggs multiple days in a row and must deposit all of the nutrients required by developing chicks into each egg laid. The effect of egg production on hen LC stores has not been determined, however during early lactation dairy cows are in a state of negative energy balance, and there is an up-regulation of LC biosynthesis and uptake (Schlegel et al., 2012). Broiler breeder hens are feed restricted to control their body weight in an effort to maintain egg production (Sun et al., 2006).The peroxisome proliferator-activated receptor α, which regulates the organic cation transporters (**OCTN**) which transport LC, is upregulated suggesting that the uptake of LC would be increased in feed restricted broiler breeder hens as well (Madsen and Wong, 2011).

Although LC is endogenously synthesized, it may be supplemented in poultry to increase feed efficiency and carcass yield (Rezaei et al., 2008). Supplementation of LC may also increase its levels in eggs. Supplementation of 125 ppm LC to hens reduced their body weights, did not affect egg production or egg traits throughout production, but resulted in increased yolk content of LC (Zhai et al., 2008). Even low (25 ppm) levels of LC supplementation result in increased levels of egg LC (Peebles et al., 2007). L-carnitine levels in neonatal mammals are relatively low but increase substantially during post-natal development. For example, LC levels in neonatal rat pups increased from birth to d11 after which serum levels stabilized (Ling et al., 2012). This increase in tissue LC levels was attributed to increased expression of LC transporters, OCTN 1, 2 and 3 in the heart, kidney and intestine (Ling et al., 2012). Again, LC status in chicks has not been reported and as opposed to many mammals which switch from a carbohydrate energy source *in utero* to a fatty acid energy source when they are nursing (Ling et al., 2012), chicks are supplied energy via fatty acids *in ovo* and must adapt to a carbohydrate rich energy source post hatch (Vieira and Moran, 1999), so chick LC requirement may be different than that of neonatal mammals.

L-carnitine is known to possess antioxidant and immunomodulatory properties, although it has been reported that when present at levels greater than 3 mM (*in vitro*) it is cytotoxic (Li et al., 2012). The immunomodulatory properties are probably largely due to its antioxidant properties because of the production of ROS by the innate immune system during inflammation. As an antioxidant LC

may increase the expression of antioxidant enzymes CAT and SOD in human hepatocytes challenged with H_2O_2 (Li et al., 2012) or it could act as a chelator of iron or as a free radical scavenger (Gülçin, 2006). Evidence for the link between the antioxidant capacity and immunomodulatory properties of LC is provided by the response of rats to intra-peritoneal (**i.p.**) LPS injection (Abd-Allah et al., 2009). Lipopolysaccharide is an outer wall component of gram negative bacteria such as *E. coli*, and is a PAMP so it triggers an inflammatory response in the tissues where it has been detected (Kannaki et al., 2010). When LC was injected i.p. (500 mg/kg body weight), it was able to reduce the production of MDA in rat testis as well as the production of nitric oxide (**NO**) and IL-2, thereby inhibiting the inflammatory response (Abd-Allah et al., 2009). Similarly, *in vitro* exposure of splenocytes to LC suppressed the expression of LPS induced cytokines IL-6 and IL-1β (Fortin et al., 2009).

The immunomodulatory properties of LC have been demonstrated in rats where supplementation of LC to aged rats (24 months old) resulted in restored neutrophil mobility (chemotaxis) and phagocytic ability to levels observed in 2 month old rats (Izgüt-Uysal et al., 2004). The mechanism by which this occurred is unknown, but it was hypothesized that the antioxidant effects of LC may have stabilized membrane fluidity which aided cellular motility (Izgüt-Uysal et al., 2004). Despite evidence for LC acting as an antioxidant and immuno-modulator in other species, there are no reports on the effect that LC might have as an antioxidant or immuno-modulator in poultry. Furthermore, given that hen supplementation of LC results in increased egg levels of LC, it is possible that

chick immune function could be influenced via the maternal diet supplementation of LC.

1.6 CONCLUSIONS

Liveability and wellness of the post-hatch chick begins with the hen's diet and her age. First week mortality is lowest in chicks from middle aged hens, which could be a reflection on the development of the chick's innate immune response on which it relies during the first week of life. The hen deposits all nutrients required for embryonic development into the egg prior to laying it, if there is inadequate deposition of a nutrient into the egg, chick development will likely suffer. However, it is possible to increase the deposition of nutrients which are immunomodulatory and antioxidative. Increasing the availability of immunomodulatory and antioxidant nutrients to chicks could possibly enhance the development of the chicks' innate immune response, benefitting them in the first week post-hatch.

The following chapters investigate the effects of hen age and the three nutrients discussed in the literature review on chick post-hatch innate immune function. It has been established that each of these nutrients are immunomodulatory and antioxidant, and furthermore that when supplemented to hens, are deposited into eggs. The ability of each of these ingredients to influence indices of chick post-hatch innate immune function was measured *ex vivo* by *E. coli* bactericidal capacity of chick phagocytes, phagocytic capacity and activation and oxidative burst response at one and four days of age. The *E. coli* bactericidal capacity is a general indicator of the innate immune function, while the

phagocytic capacity and activation and oxidative burst are specific mechanisms of the innate immune function. Phagocytic capacity is an indicator of how many *E. coli* bioparticles each phagocyte has ingested and phagocytic activation is an indicator of how many phagocytes contain at least one *E. coli* bioparticle. Oxidative burst is a measure of oxidative products produced by phagocytes over the course of a 20 minute assay. Both the phagocytosis parameters and oxidative burst are measured via fluorescence on a flow cytometer, The greater the fluorescence measured the greater the number of *E. coli* bioparticles ingested, or the greater the number of active phagocytes or the greater the production of oxidative products.

Canthaxanthin was supplemented to hens at one of three levels, 0, 6 or 12 ppm throughout their production cycle. It was hypothesized that chick post-hatch innate immune function would increase with increasing hen age and canthaxanthin supplementation level (Chapter 2). A second group of hens were supplemented with either synthetic vitamin E or natural vitamin E formulated to equivalent activity levels (40 IU/kg). It was hypothesized that chick post-hatch innate immune function would increase as hens aged and be greater in chicks hatched from hens supplemented with NVE than in chicks hatched from hens supplemented with SVE (Chapter 3). A third group of hens was supplemented with either 0 or 50 ppm L-carnitine. It was hypothesized that chick post-hatch innate immune function would increase with increasing hen age and maternal LC supplementation (Chapter 4). The most consistent effect observed in the main trial was a dramatic decrease in chick innate immune function (as measured by *E. coli*

bactericidal capacity). Upon reflection it was determined that an incubator temperature effect could have been observed because all the eggs in the main trial were incubated under the same conditions regardless of egg size. Given the existence of literature supporting that incubator temperature is affected by egg size it was deemed prudent to follow up on this effect. Eggs were obtained from commercial Ross 708 broiler breeder flocks of three hen ages reflective of those used in the main trial). The eggs were subjected to one of four experimental temperatures (36.0, 36.5, 37.0 or 37.5°C) from day 15 of incubation to hatch. It was hypothesized that chicks from younger hens would have a greater innate immune response than chicks from older hens and that reducing the incubator temperature from 37.5 to 36.5°C would reduce the differences in innate immune function observed between chicks from young and old hens (Chapter 5).

The overall hypotheses of this research is that supplementing hens with antioxidant nutrients would increase the innate immune function of the post-hatch chick and that chick post-hatch innate immune function would be greatest in chicks from middle aged hens.

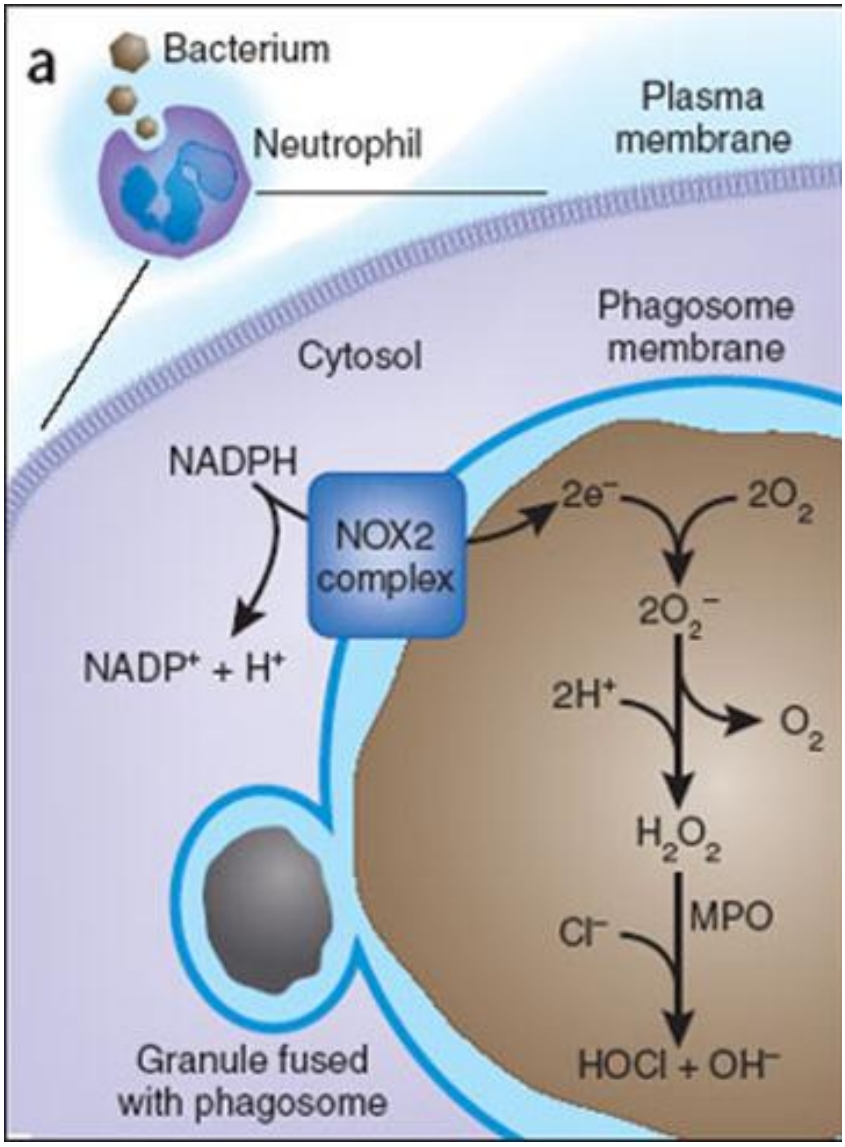

Figure 1.1. The production of reactive oxygen species by a mammalian neutrophil. NADPH oxidase (NOX2) is activated on the phagosomal membrane, and electrons are transported from NADPH across the membrane to oxygen to generate superoxide radicals (O_2^-), which dismutate to hydrogen peroxide (H_2O_2). Degranulation releases myeloperoxidase (MPO), which generates hypochlorous acid (HOCl).
From: Winterburn, 2008

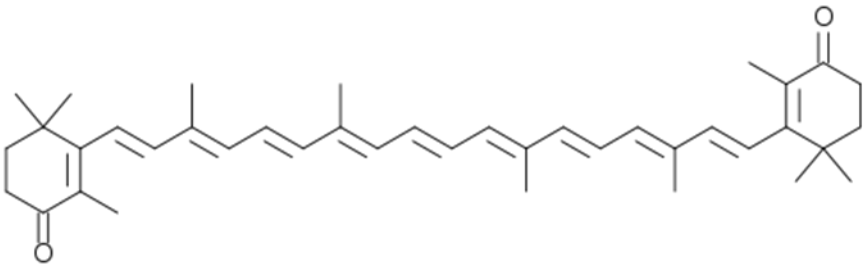

Figure 1.2. Canthaxanthin (a xanthophyll).
From: http://en.wikipedia.org/wiki/File:Canthaxanthin.svg

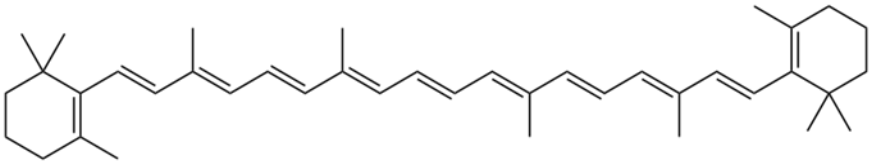

Figure 1.3. Beta-carotene (a carotene)
From: http://en.wikipedia.org/wiki/File:Beta-Carotin.svg

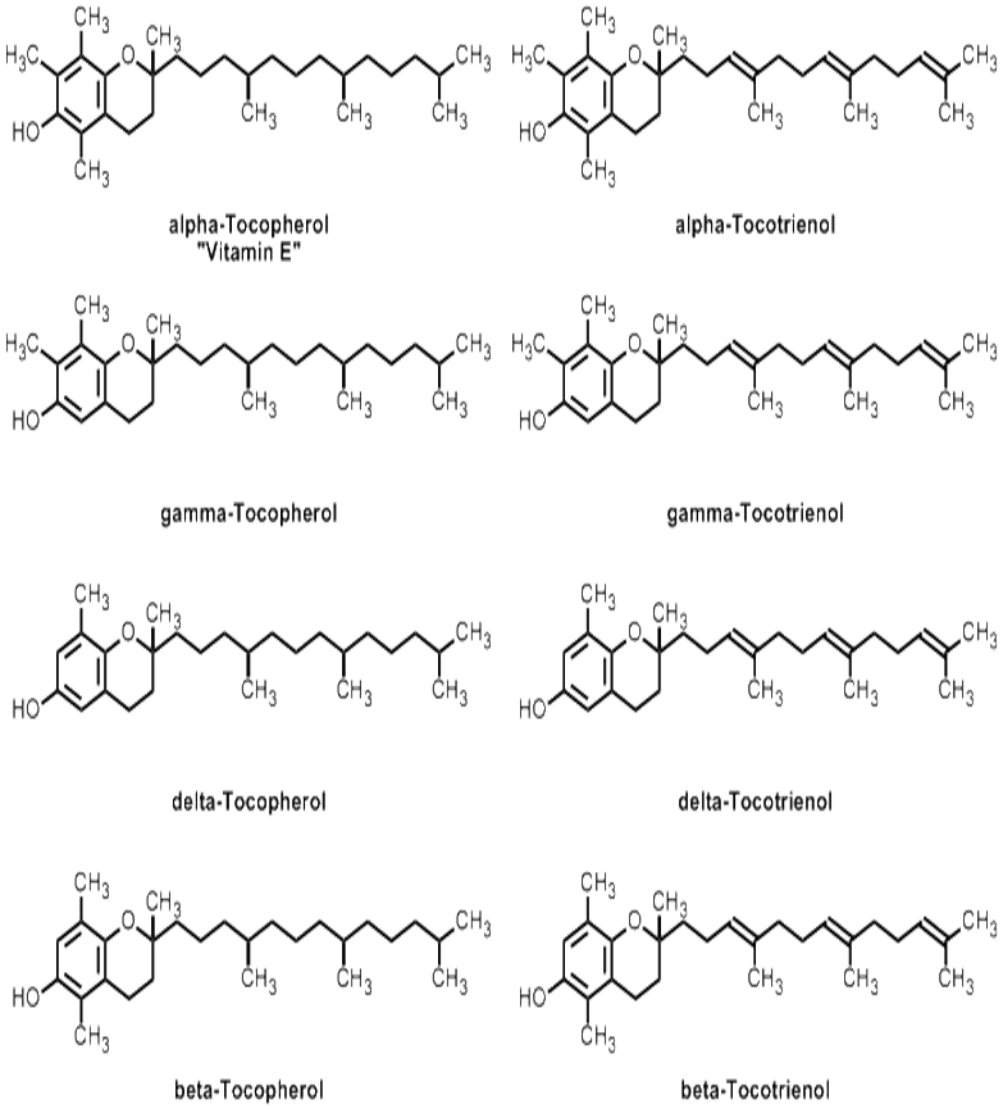

Figure 1.4. Forms of vitamin E
From: http://www.vita-dose.com/structure-of-vitamin-e.html. Accessed: Sept. 18, 2012

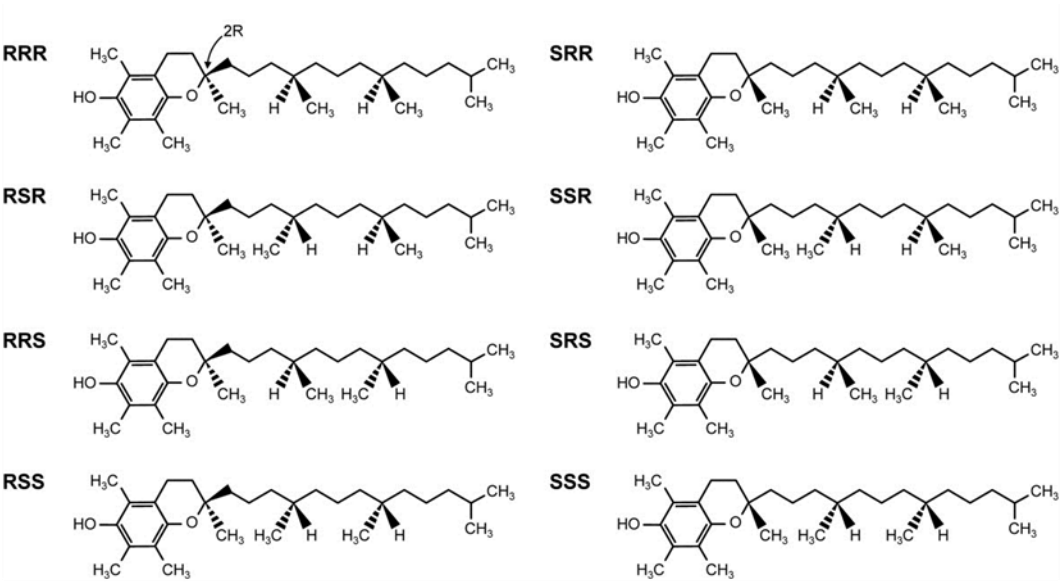

Figure 1.5. Stereoisomers of alpha-tocopherol
From: Biesalski, 2009

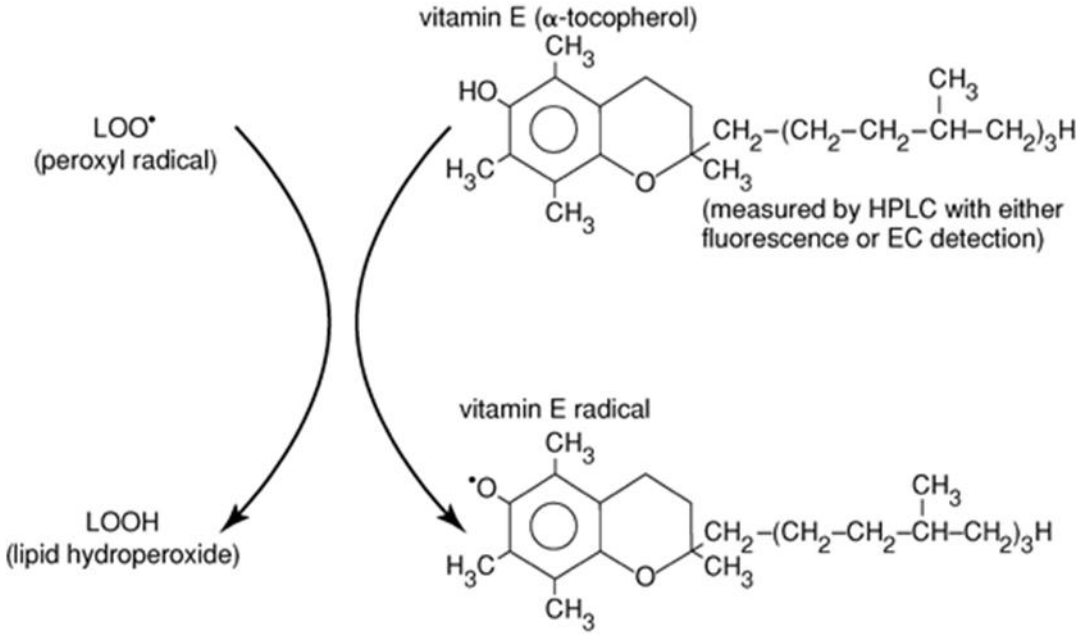

Figure 1.6. Antioxidant mechanism of alpha-tocopherol against lipid peroxides
From: Hall and Bosken, 2009

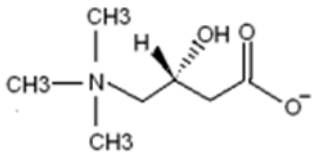

Figure 1.7. L-carnitine
From: analytical-lab.com. Accessed Sept. 18, 2012

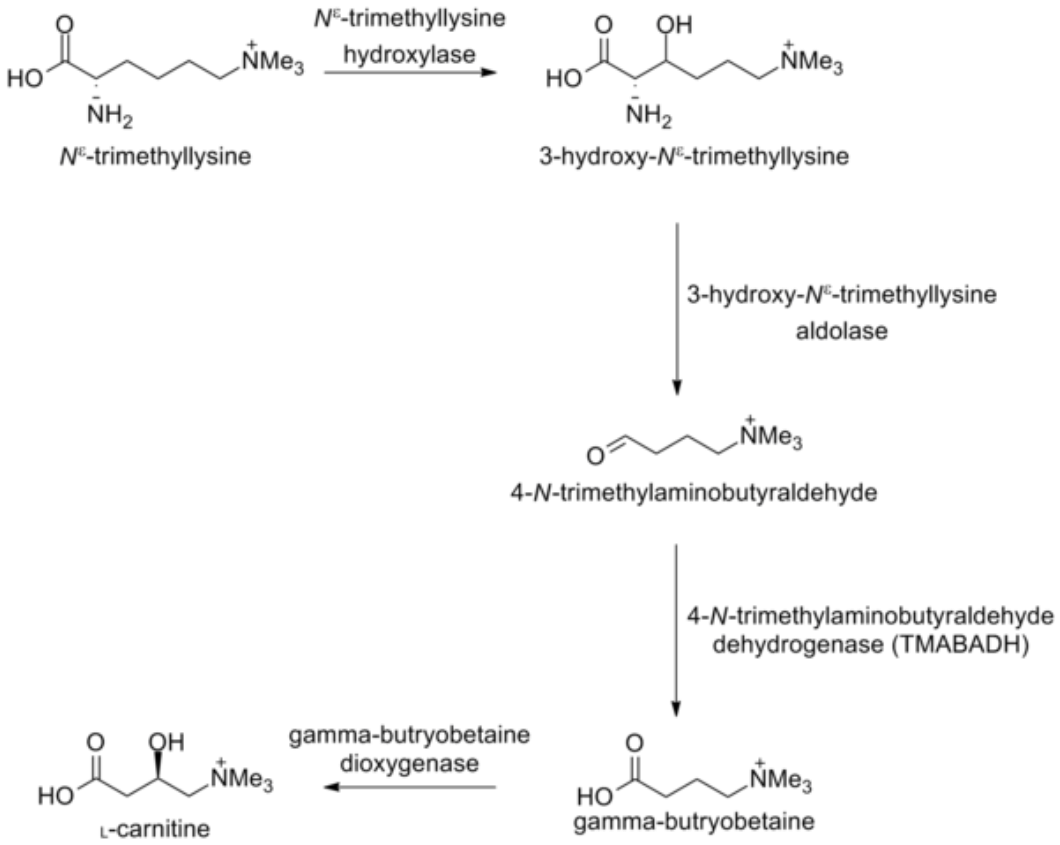

Figure 1.8. L-carnitine synthesis in humans.
From: http://en.wikipedia.org/wiki/Carnitine_biosynthesis. Accessed: Dec. 3, 2012

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2. THE EFFECT OF MATERNALLY SUPPLEMENTED CANTHAXANTHIN AND HEN AGE ON INDICES OF CHICK POST-HATCH INNATE IMMUNE FUNCTION

2.1 INTRODUCTION

Carotenoids are a diverse group of fat soluble, highly pigmented molecules found in nature and are responsible for the colourful feather displays of some avian species and bright colours of plants and flowers (Kull and Pfander, 1995). Carotenoids are known immuno-modulators and supplementation of various carotenoids has shown that they enhance aspects of the immune function. In rats dietary β-carotene (0.2% wt/wt) and canthaxanthin (**CXN**;0.2% wt/wt) each increased the activity of rat B and T lymphocytes in response to phytohaemagglutinin, concanavalin A and lipopolysaccharide (**LPS**;Bendich and Shapiro, 1986). Additionally, when chicks were fed lutein (40 ppm) indices of inflammation, such as changes in plasma haptoglobin, body weight, spleen and bursa weights, were minimized in response to *Salmonella typhimurium* LPS challenge (Koutsos et al., 2006). Maternal and direct supplementation of CXN to chicks resulted in the incorporation of CXN into immune tissues including plasma, thymus, bursa and liver (Koutsos et al, 2003a). Studies investigating the effect of CXN on immune function indices in chicks are few and focus on direct dietary supplementation to chicks beyond the first week of life (Haq et al., 1995; Haq et al., 1996; Selveraj et al., 2006; Koutsos et al., 2006).

The effect of hen age has predominantly been studied for its effect on growth and market age weight, and largely ignores the effect of hen age on

physiological systems such as the immune system in the chick which contribute to its liveability and well-being. First week chick mortality decreased as hens approached 38 weeks of age and then increased slightly to 60 weeks of age (Yassin et al., 2009). Reasons for increased first week mortality among chicks from young and old hens have not specifically been investigated but there are published data which could explain high first week mortality in chicks from young and old hens. Latour et al., (1998) found that chicks hatched from 36 and 64 week old broiler breeder hens were smaller than chicks hatched from 51 week old hens, yet had greater residual yolk sac weights, suggesting that chicks from young and old hens mobilized less fat *in ovo* than chicks from middle aged hens. Increased first week mortality of chicks from older hens could also be the result of earlier hatching times of large eggs and increased risk of dehydration from extended time spent in the hatcher (Suarez et al., 1997). Eggs from young hens have a lower proportion of yolk and a greater proportion of albumen than eggs from older hens (Ulmer-Franco et al., 2010). Although chick hatch weight is relative to egg weight (Lourens et al., 2009), a lower proportion of yolk results in a lower amount of yolk fat available to the chick (Yadgary et al., 2010). In addition to those challenges, the post-hatch chick is exposed to microbes, both pathogenic and non-pathogenic regardless of hen age. It has been established that an activated immune response diverts nutrients away from anabolism towards catabolism (Benson et al., 1993) and chicks which are dehydrated or weaker because of lower nutrient availability may not be strong enough to withstand the additional stress. It is also possible that chicks from older hens have a more

developed immune response than chicks from younger hens which could contribute to greater liveability during the first week.

In the first days post-hatch a chick's immune system is immature (Bar-Shira et al., 2003; Crhanova et al., 2011). The gut associated acquired immune function in chicks requires two weeks to develop (Bar-Shira et al., 2003), but many changes occur by four days of age. During the first four days of life antimicrobial peptides were the predominant form of protection in the chick's gastrointestinal tract (**GIT**), but thereafter there was increased production of pro-inflammatory cytokines and a transition to a cell-mediated response (Crhanova et al., 2011), reflecting the maturation of the innate response in the GIT.

The objectives of the current research were three-fold: To determine the effect of maternal supplementation of CXN on indices of chick post-hatch innate immune function; to determine the effect of hen age on chick post-hatch innate immune response; and to determine how the innate immune response changed as chicks aged. It was hypothesized that chick post-hatch innate immune function would increase with increasing levels of maternal CXN supplementation and hen age.

2.2 MATERIALS AND METHODS

2.2.1 Experiment 1

2.2.1.1 *Animals.*

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with the Canadian Council of Animal Care (1993) guide. Ross 308 broiler breeder hens were raised in floor

pens from the day of hatch, and managed to maintain growth curves as recommended by Ross 308 Breeder performance objectives (Aviagen, 2007) until 21 weeks of age when they were transferred to individual cages (48 x 42 x 45 cm with sloped floors). Photostimulation and dietary treatments (n=45 hens per treatment) were initiated at 22 weeks of age. They were fed a standard commercial broiler breeder basal diet supplemented with either 0 (Control), 6 or 12 ppm CXN (Table 2.1). Individual body weights were recorded weekly, averaged by treatment, and compared to the Aviagen Ross 308 target body weight curve to determine daily feed allotment (Aviagen, 2007). Egg production was recorded daily; for each hen the data were summarized on a weekly basis. At three hen ages corresponding with Early, Mid and Late production (31 to 33, 45 to 47 and 57 to 59 weeks of age respectively) the hens were artificially inseminated with 0.5 ml of pooled semen from Ross 308 roosters. At each of the three breeder ages given above, hens were inseminated starting three weeks prior to the first egg collection. Insemination was performed weekly until the final week of the last egg collection at each age. The settable eggs (eggs which had a single yolk, and were neither mis-shapen nor cracked) were stored for up to 7 days between 16 to 18°C and 70 to 80% relative humidity (**RH**). The settable eggs were placed in the incubator at weekly intervals at 37.5°C and 56% RH. The resulting chicks were weighed and feather sexed at hatch. Ten eggs per treatment at each sampling time were sampled to determine whole egg, yolk and albumen weights. Wet shell weight was determined by subtracting the yolk and albumen weights from the whole egg weight. Yolk and albumen from these eggs were separated and stored

at -20°C until analysis for CXN content by HPLC. Plasma was collected from 15 hens per treatment at each hen age and stored at -20°C for subsequent total antioxidant capacity determination.

After hatching, chicks were separated by maternal treatment. Twenty four male chicks per maternal treatment from the first hatch within each production phase were placed in Petersime battery brooders for the innate immune function assay sampling. At one and four days of age, 12 chicks were weighed and bled via decapitation. The whole blood from 8 chicks/maternal treatment was used for the innate immune function assays. These procedures were repeated for the second hatch of each production period, and the data from the two hatches were pooled.

Fifty chicks per maternal treatment from the third hatch were feather sexed, male chicks were separated by maternal treatment and placed in Specht cages for a three week grow out trial. Ten chicks per maternal treatment were weighed, bled and dissected for liver collection at each of 0, 7, 14, and 21 days of age. The blood was centrifuged at 1,000 x g at 4°C for 10 minutes and plasma collected for total antioxidant capacity analysis. Livers were stored at -20°C until analysis of liver CXN content.

2.2.1.2 *Innate Immune Function Indices.*

Whole blood collected from the chicks was used to determine *E. coli* bactericidal activity (**EBC**), to assess the number of cells containing at least one *E. coli* (phagocyte activation) and the average number of *E. coli*/cell (phagocytic capacity) and oxidative burst (**OB**) *ex vivo*.

2.2.1.3 *E. coli Bactericidal Capacity Assay.*

This procedure was conducted using sterile instruments in a biosafety cabinet. Two days before sampling a stock solution of *E. coli* Epower (ATCC #51813 Epower; cat # 0791E7, Microbiologics) was made by aseptically dissolving 1 pellet of *E. coli* equilibrated to room temperature in 40 mL of sterile PBS (7.4 1x cat # 10010-072, Gibco) warmed to 40°C. The solution was incubated at 37°C for 30 minutes and stored at 4°C. A working solution of *E. coli* was made by diluting 2 ml of the stock solution to 10 ml with sterile phosphate buffered saline (**PBS**) (7.4 1x cat # 10010-072, Gibco). On day of sampling an aliquot of 40 µL of heparinized whole blood was deposited into a sterile Eppendorf tube. The blood was diluted with 400 µL of CO_2 independent media (cat # 18045-070, Gibco) containing 4 mM of L-glutamine (cat # 25030-149, Gibco). Two time-zero (control) plates were plated with a solution containing 10 µL of the *E. coli* solution and 100 µL of PBS, using 50 µL of the solution for each plate. Then 40 µL of the *E. coli* working solution was added to each sample and mixed. The *E. coli* - blood mixture was incubated at 41°C for 90 minutes, at which time the solutions were mixed again and 50 µL of was removed and plated on a trypic soy agar (cat# CA81000-271, VWR) medium plate. The inoculated plates were incubated at 37°C overnight and the colonies counted in the morning. Bactericidal capacity was inferred by converting colonies grown on the sample plate to a percentage of colonies grown on the control plate and subtracting from 100, using the equation; $100 - ((1-(CFU_{sample}/CFU_{control}))*100)$.

2.2.1.4 *Phagocytosis Assay.*

Phagocytic capacity and activation were measured in this assay. Phagocytic capacity was a measure of the number of *E. coli* bioparticles that were phagocytized per phagocyte and phagocytic activation was a measure of the number of phagocytic cells containing at least one *E. coli* bioparticle present in the sample. *E. coli* fluorescent bioparticles (K12 strain, cat # E-2864, Invitrogen) were reconstituted in 500 µl of sterile PBS (7.4 1x cat # 10010-072, Gibco) and 2 mM sodium azide (cat # 190381000, Fisher Scientific). The solution was then thoroughly mixed with a vortex and sonicated for 10 minutes and kept at 4°C until needed. Using sterile technique 40 µL of heparinized whole blood was aliquoted to a sterile Eppendorf tube and diluted with 800 µL of a media containing 97% CO_2 independent media (cat # 18045-070, Gibco), 1% penicillin/streptomycin (cat # 15140-148, Gibco), and 2% L-glutamine (cat # 25030-149, Gibco). This solution was mixed to create a uniform suspension, of which 66 µL was pipeted into a new sterile Eppendorf tube. To this solution was added 250 µL of a working solution of bioparticles consisting of 249.17 µl of 92% CO_2 independent media (cat # 18045-070, Gibco), 2% glutamine (cat # 25030-149, Gibco), 5% fetal calf serum (cat# 10437-028, Gibco), 1% penicillin/streptomyocin (cat # 15140-148, Gibco) to which was added 0.83 µl to create a suspension of 1:100 cell:particle ratio of reconstituted bioparticles. The tubes were covered with aluminum foil to further protect the bioparticles from light and incubated at 41°C for 15 minutes and then placed on ice for 5 minutes to end phagocytosis. The tubes were centrifuged at 10,600 x g for 10 minutes and the supernatant poured

off leaving only the pellet. The following step was repeated twice: The blood solution was washed with 300 µL of cooled (4°C) CO_2 independent media (cat # 18045-070, Gibco) containing 5% fetal calf serum (cat# 10437-028, Gibco) and 1% pen/strep (cat # 15140-148, Gibco), spun at 3,800 x g for 8 minutes and then the supernatant poured off. Three hundred µL of room temperature sterile lysis buffer (4.15 g ammonium chloride, 0.84 g $NaHCO_3$, 1 ml 0.5 M EDTA, and 1,000 mL of deionized distilled water) was added, the solution was then centrifuged at 110 x g for 5 minutes and the supernatant poured off. Then 300 µl of -20°C methanol was added and the resulting solution was incubated on ice for 5 minutes and then centrifuged at 6,800 x g for 5 minutes and the methanol (cat# 439193, Sigma) was then poured off. The remaining pellet was re-suspended in 1,000 µL of sterile wash buffer (0.5 g BSA (cat #A7906, Sigma) and 1 mL 0.5 M EDTA in 1,000 mL of Hanks Balanced Salt Solution pH 7.4 (cat # H6648, Sigma)) and transferred to tubes for analysis on a FacsCalibur Flow Cytometer (BD Sciences). The data were analyzed using CellQuest software (BD Sciences).

2.2.1.5 *Oxidative Burst Assay.*

Approximately 1 ml of heparinized whole blood was centrifuged at 960 x g for 10 minutes to separate the cells from the plasma, and the plasma was removed and discarded. The remaining cells were diluted with 1000 µL of 1% BSA (cat #A7906, Sigma) in PBS (7.4 1x cat # 10010-072, Gibco) so that the ratio of blood to BSA/PBS was 1:2. The solution was layered over a density gradient (histopaque 1.119d, cat #1119-1, Sigma) and then centrifuged at 420 x g for 30 minutes, any remaining plasma that was present was discarded and the

cloudy layer of mononuclear cells and the clear layer above the red blood cells were collected and transferred to a new tube. This solution was then washed with 1% BSA (cat #A7906, Sigma) in PBS (7.4 1x cat # 10010-072, Gibco) (filled to top of 15 ml conical tube) and centrifuged at 165 x g for 10 minutes. The resulting supernatant was poured off and discarded and the remaining pellet was resuspended in 1000 µL wash buffer (0.5 g BSA (cat #A7906, Sigma) and 1 mL 0.5 M EDTA in 1,000mL of Hanks Balanced Salt Solution pH 7.4 (cat # H6648, Sigma)) and then transferred to a facscan tube. Ten µL of 10 µg/ml Dichlorofluorescin diacetate (cat #D-6883, Sigma) in 95% Ethanol was added and then the samples were incubated in a 37°C water bath for 5 minutes. Two hundred µL of the solution was transferred to a new set of tubes labelled 0 min and set on ice. To the remaining solution was added 10 µL of 100 µg/ml phorbol 12-myristate 13-acetate (cat # BML-PE160-0001, Enzo Life Sciences) (1 ml DMSO, 9 ml RPMI 1640 medium (cat #R7509, Sigma) and then incubated again. Five minutes later another 200 µL of the solution was transferred to a set of tubes labelled 5 minutes and set on ice, this process was repeated at 10, 15 and 20 minutes of incubation. The tubes were protected from light and processed on a FacsCalibur Flow Cytometer, data were processed using CellQuest software (BD Sciences). Data were presented as a ratio of each time point (5, 10, 15 and 20) against time zero.

2.2.1.6 *Determination of Egg, Liver and Feed Content of Canthaxanthin.*

Determinations of egg liver and feed content of CXN were made by weighing 0.5 g of sample of egg or liver and 1 g of feed into a 13 x 100 screw top

test tube. To the sample, 1 ml of HPLC grade water, 2.5 ml of 10% butylated hydroxytoluene in ethanol (w/v), and 1 ml of ethanol (to reflect amount of ethanol in the standard) were added. The contents were mixed on a vortex for 1 minute. Two ml of 2.5% NaCl (w/v) were added and the contents again mixed for 1 minute. Finally, 2 ml of dicholoromethane were added to extract the CXN. The tubes were again mixed on a vortex for 1 minute and then centrifuged at 430 x g for 3 minutes. The resulting upper layer was discarded and the bottom organic layer was transferred to a crimp top HPLC vial and 25 µL was injected. Samples were analyzed using a Shimadzu LC 20AT pump, SP DM20A Diode array detector, and Sil-10 AF autosampler, with a Zorbax Exlipse XBC-C18 column (5 *u*m x 4.6 mm x 150 mm) (Agilent, Mississauga, ON). The solvent was a mixture of dichloromethane, methanol and acetonitrile (50:25:25 v/v) and the flow rate was 1 ml/min. Retention time was compared to a canthaxanthin standard (Sigma) and peaks were collected and integrated using Shimadzu's class VP software, EZStart 7.4 SP1 (Shimadzu, Kyoto, Japan).

2.2.1.7 *Total Plasma Antioxidant Capacity.*

To determine plasma total antioxidant capacity (**AOC**) an antioxidant assay kit from Cayman Chemicals Ltd (cat#: 709001) was used. The antioxidant capacity of the sample was compared to the antioxidant capacity of Trolox, a water-soluble tocopherol analogue and was quantified as millimolar Trolox equivalents (Koracevic et al., 2001).

2.2.2 Experiment 2

White Leghorn laying hens, 44 weeks of age, were individually housed in battery cages and were fed one of three diets (n=14), 0 ppm CXN (control), 6 ppm CXN, or 12 ppm CXN. Feed and water were available *ad libitum*. Egg samples were taken at days 0, 2, 4, 7, 9, 11, 14, 18, 21, 25, 28, 35 and 40. Whole egg and combined albumen and yolk weights were recorded. Yolk and albumen were homogenized and a portion was weighed and kept for analysis of CXN content via HPLC. Samples were frozen at -20°C until HPLC analysis.

2.2.3 Statistical Analysis

Individual hens or chicks were considered experimental units. Immune function data were analyzed using Proc Mixed of SAS 9.2 (SAS Institute, 2008) as a 3-way ANOVA with hen age, hen treatment and chick age as the main effects. Hen and chick production data were analyzed as 2-way ANOVA with hen age and maternal treatment as the main effects using Proc Mixed (SAS Institute, 2008). Differences were determined using PDIFF of LSMeans and were considered significant at $p \leq 0.05$ (Steel and Torrie, 1960). Production data which are typically expressed as a percentage were $\log_{10}$ transformed for analysis to satisfy requirements for ANOVA, but are presented as a percentage.

2.3 RESULTS AND DISCUSSION

2.3.1 Hen and Egg Production Traits

Hen body weight increased as hens aged (Table 2.2), and was depressed by CXN treatment; this did not appear to be due to feed allocation. Settable egg production (Table 2.2) decreased as hens aged, in accordance with published

literature (Gibson et al., 2008), and was greatest in the 6 ppm hens relative to Control and 12 ppm hens which did not differ at each of the hen ages observed. Similarly, when egg production over the duration of the experiment was considered hens supplemented with 6 ppm CXN laid a greater number of total eggs and settable eggs than either the Control or 12 ppm treatment ($p = 0.0504$; Table 2.2). The effect of maternally supplemented CXN is not commonly reported in the literature, but CXN supplementation (6 ppm) to Chinese Three Yellow broiler breeder hens did not affect total or settable egg production (Zhang et al., 2011). It is possible that CXN supplementation affects different strains of chickens differently, which could explain why a difference in egg production was observed in the current research but not in the published research. Canthaxanthin supplementation did not affect egg traits (Table 2.3). Egg, yolk, wet shell weights and proportion of shell weight increased as hens aged, while the proportion of albumen decreased (Table 2.3). There was a nearly significant hen age by treatment interaction ($p = 0.0533$), which showed that eggshell proportion decreased with increased CXN supplementation level among eggs from Early and Late hens, but increased with increasing CXN supplementation level among eggs from Mid hens. Increased egg and yolk weight as hens age has been well documented (Tůmová and Gous, 2012: Ulmer-Franco et al., 2010) as has decreased albumen proportion (Ulmer-Franco et al., 2010; Hamidu et al., 2007).

Hen age did not affect CXN deposition on a per gram of yolk or whole egg basis (Table 2.3). As hypothesized, higher levels of supplementation resulted in greater amounts of CXN being deposited into the egg (Table 2.3) in Experiment 1.

However, the deposition rate was not proportional to diet content as eggs from 12 ppm hens did not contain twice the amount of CXN as eggs from 6 ppm hens. Egg content of CXN doubled when feed content of CXN (3, 6, 12, and 24 ppm) was doubled in previous work with broiler breeders (Surai et al., 2003). However, when dietary astaxanthin, a carotenoid pigment similar to CXN, was supplemented to humans at levels of 0, 2 or 8 mg/d, plasma levels did not increase in a manner proportional to dietary astaxanthin (Park et al., 2010).

In Experiment 2, the deposition rate of CXN into eggs increased with increasing supplementation level and plateaued around day 28 (Figure 2.1). At 42 days of supplementation the CXN content of eggs collected in Experiment 2 were comparable to levels observed in eggs from Experiment 1 (Table 2.3). The egg deposition of CXN observed in this trial is comparable to that observed in other trials. Surai et al., (2003) reported that supplementing hens with 6, or 12 ppm CXN for 35 days resulted in yolk contents of 14.75 and 28.89 ug/g yolk respectively, which reflects the deposition rate observed if the current data is adjusted to reflect yolk content rather than egg content. It is likely that the egg CXN content of the Ross 308 broiler breeder hens used in Experiment 1 also reached a plateau prior to egg sampling as the hens had been on the experimental diets for seven weeks at time of sampling.

2.3.2 Hen Plasma Total Antioxidant Capacity

Hen AOC was unaffected by CXN supplementation (Table 2.4). This was very surprising because CXN is reportedly a very potent lipid-soluble antioxidant (Palozza and Krinsky, 1992, Zhao et al., 1998; Rengel et al., 2000), and when

CXN was supplemented to Chinese Three Yellow breeder hens at 6 ppm the hens had a significantly greater serum antioxidant capacity than non-supplemented hens (Zhang et al., 2011). Our hens were on a restricted feeding program as is customary for broiler breeder hens and were fed only once per day in the morning. Plasma samples were collected prior to their daily feed allocation so a fasting plasma sample was collected. When mice were fasted for 18 hours, levels of various antioxidant enzymes were decreased in comparison to antioxidant enzyme levels of *ad libitum* fed mice (Di Simplicio et al., 1997), although no dietary antioxidant was supplemented. Furthermore, antioxidant status follows a diurnal variation peaking at night (or dark period) in chicks (Albarrán et al., 2001). The fasting state and diurnal patterns could be why differences in plasma antioxidant status were not observed between treatments. Hen AOC decreased as hens aged from Photostimulation to Mid before reaching a plateau (Table 2.4). As rats aged from 6 to 22 months antioxidant enzyme activities (catalase and superoxide dismustase) decreased (Valls et al., 2005). It is possible that reduced enzyme activities contributed to the reduced hen AOC as hens aged.

2.3.3 Hatch Data

Maternal supplementation of CXN did not affect hatchability, nor did hen age (Table 2.5). The lack of a hen age effect was interesting because it is well known that hatchability decreases as hens age (Fasenko et al., 1992; Yassin et al., 2008) (Table 2.5). However, the cited studies reported hatch of fertile as about 90%, while the current data reports hatch of set eggs. Male chick hatch weight increased as hens aged but at each of the hen ages observed, chicks from 6 ppm

hens had the lowest hatch weight relative to chicks from control and 12 ppm hens (Table 2.5). Similarly, female chick hatch weight increased with hen age, but among chicks from Mid hens, hatch weight of chicks from 12 ppm hens was the greatest while that of chicks from 6 ppm hens was the lowest and among chicks from Late hens, chicks from 6 ppm hens had the lowest hatch weight relative to chicks from Control and 12 ppm hens which did not differ (Table 2.5). The increased chick hatch weight with hen age is well documented (McNaughton et al., 1978; Sklan et al., 2003), and coincides with the increases in egg sizes.

2.3.4 Indices of Innate Immune Function

Chick EBC decreased dramatically as hens aged (Table 2.6). Decreased inflammatory response in chicks as hens age has not been reported in the literature. However a similar observation of the effect of hen age on progeny inflammatory response was made in turkey poults from turkey hens aged 33 and 55 weeks by Schaefer et al., (2006), although the authors were unable to determine why this happened. In the current research it could be related to incubation temperature (Ardia et al., 2010; Oznurlu et al., 2010), which affects immune function development, and egg size, which influences incubation temperature (Lourens et al., 2006). Decreased incubation temperature reduced *E. coli* bactericidal capacity of tree swallow nestlings (*Tachycineta bicolor*; Ardia et al., 2010) and increased incubation temperature inhibited the development of the bursa and thymus and reduced numbers of peripheral lymphocytes in broiler chicks (Oznurlu et al., 2010). Lourens et al., (2006) showed that larger eggs (embryos) produced greater amounts of metabolic heat and, if incubated under the

same conditions as smaller eggs, experienced a greater incubation temperature. The eggs in the current research were incubated under the same conditions regardless of egg size, which increased as hens aged. It is possible that the eggs from Early hens, which were smaller then eggs from Mid or Late hens, experienced an incubation temperature that was more conducive to the development of the innate immune response.

Canthaxanthin content of eggs in this study increased with increasing maternal supplementation level but increased availability of CXN to chicks did not result in a greater innate response, as chicks from 6 ppm hens had the greatest EBC. Koutsos et al., (2006) supplemented broiler chicks with 40 ppm lutein and reported that indices of inflammation in response to LPS challenge (changes in plasma haptoglobin, Zn, Fe and Cu levels, and body weight) were blunted compared to non-supplemented chicks. When the cited and current research are considered together, carotenoid exposure may aid in reducing morbidity and improving clearance of the pathogen, however, different carotenoids may be required in different amounts to achieve this. *Ex vivo E. coli* bactericidal capacity increased from day one to day four in chicks indicating a maturation of the immune system. Data describing the maturation of the chick's innate immune system are limited and focus on the development of the gut associated lymphoid tissue, but it has been reported that infiltration of functional immune cells into the gastrointestinal tract increases between one and four days of age (Bar-Shira et al., 2003).

Phagocytosis data from day old chicks from Early hens were lost and therefore not reported, this also affected the statistical analysis of the data. Hen supplementation of CXN had no effect on phagocytic capacity or phagocyte activation in chicks (Table 2.6). Supplementation of 0, 25, or 50 ppm lutein to Cobb broiler chicks also did not affect macrophage phagocytic capacity (Selveraj et al., 2006). When the results of Selveraj et al., (2005) and the current research are considered together, it is possible that carotenoids do not affect phagocytosis. Phagocytic capacity and phagocyte activation of chicks was greatest in chicks hatched from Mid hens. Phagocytic capacity did not differ between day one chicks and day four chicks from Mid hens, while it decreased as chicks aged in chicks from Late hens (Table 2.6). Phagocytic activation increased as chicks aged in chicks from both Mid and Late hens (Table 2.6). The ability of chick phagocytes to engulf *E. coli* either did not change (Mid) or decreased as chicks aged (Late), while the proportion of phagocytic cells increased as chicks aged. This may indicate that there are a greater number of mature phagocytes present in day four chicks and when considered with EBC indicate that the chick's immune system is maturing (Table 2.6).

Hen age and treatment interacted to affect OB in day one chicks (Table 2.7). No treatment differences in OB were observed in day one chicks from Early or Mid hens, but in chicks from Late hens, 6 ppm CXN resulted in the greatest OB. *Ex vivo* OB did not increase during the course of the assay in chicks from Early hens, but increased with time in chicks from Mid and Late hens; the extent of the increase was greatest in chicks from Mid hens. Four day old chicks from

Young hens had the greatest oxidative burst compared to OB of day four chicks from Mid or Late hens which did not differ from each other (Table 2.7). According to Zhao et al., (1998), when rat peritoneal macrophages were activated *in vitro* to produce an OB, CXN had the greatest ability to quench reactive oxygen species and inhibit the OB relative to bixin, β-carotene and lutein. Evidence of OB inhibition by CXN was not observed in the current research.

2.3.5 Chick Growth Trial

Hen dietary CXN did not affect chick body weight or gain (Table 2.8), although it nearly decreased day zero body weight of chicks from 6 ppm hens (p= 0.0641), this effect was not reflective of egg weight. Growth performance of chicks from Chinese Three Yellow broiler breeder hens supplemented with 6 ppm CXN also was not affected (Zhang et al., 2011); neither was the growth and performance of chicks from Indian River broiler breeders supplemented with 4 ppm CXN affected (Haq et al., 1996). These studies combined with the current research provide strong evidence that hen-supplemented carotenoids do not affect chick performance. Chick body weight at hatch increased as hens aged, but day seven, day 14 and day 21 chick body weights were lowest among chicks from Early hens and did not differ between chicks from Mid and Late hens (Table 2.8). Similarly, day seven, day 14 and day 21 average daily gain was lowest in chicks from Early hens while not differing between chicks from Mid and Late hens. Chick body weight at hatch was reported to increase with hen age from 26 to 50 weeks, plateauing thereafter (Sklan et al., 2003), which agrees with the observations made in this research.

CXN was not detected in the livers of chicks at hatch from Control hens at any hen age (Table 2.9), but in the 6 and 12 ppm treatment groups, there was a decline in chick liver CXN at hatch from Early to Mid hen ages, with no further decline thereafter (Table 2.9). This interaction is interesting because egg CXN content was not affected by hen age and may indicate that CXN was being deposited in greater amounts in other tissues. Although, an effect of hen age was not reported, Koutsos et al., (2003a) found that CXN from the maternal diet was incorporated into the bursa, thymus, skin and plasma, in addition to the liver. It is possible that either more egg CXN was being used by embryos from Mid and Late hens than by embryos from Early hens. This could be related to embryo size and possible heat stress.

Larger embryos produce greater amounts of metabolic heat affecting the actual incubation temperature to which they are exposed (Lourens et al., 2006). Incubating eggs at 43.5°C resulted in embryos experiencing heat stress *in ovo* and weak chicks at hatch (Ande and Wilson, 1981). To the author's knowledge there are no published data investigating the relationship between heat stress and oxidative stress *in ovo,* but exposing White Leghorn cockerels to heat stress resulted in increased oxidative stress (Mujahid et al., 2007) and it is possible that heat stressed embryos would also experience increased oxidative stress. Eggs in the current study were incubated under the same conditions (37.5°C and 56% RH) at each of the three hen ages observed, without consideration of egg size and subsequently, embryo size. It is possible that the embryos from Mid and Late hens (which were larger than those from Early hens) in the current research were

exposed to greater oxidative stress caused by heat stress than embryos from Early hens. Therefore, CXN could have been used up as an antioxidant resulting in lower levels of the carotenoid at hatch. The literature reporting the effects of stress on carotenoid status is limited, but in humans stress results in a transient decrease in carotenoid antioxidants (Darvin et al., 2008).

A three way interaction between hen age, treatment and chick age on chick AOC was observed (Table 2.10). As chicks from Early hens aged, the chick AOC increased among chicks from Control and 6 ppm hens, but remained constant in chicks from 12 ppm hens. Overall chick AOC remained constant as chicks from Mid hens aged, but when maternal treatments were considered AOC of chicks from Control and 12 ppm hens peaked at day 14 and day seven respectively before decreasing to day zero levels; while AOC of chicks from 6 ppm Mid hens remained constant. Among chicks from Late hens, chick AOC decreased as chicks aged, although the pattern of decrease differed by maternal treatment. Antioxidant capacity of chicks from Control Late hens was constant from day zero to day 14 and then decreased at day 21. Plasma total antioxidant capacity of chicks from 6 ppm Late hens decreased from day zero to day seven and then remained constant; while that of chicks from 12 ppm Late hens steadily declined as chicks aged. These observations were unexpected as chicks were not subjected to further dietary treatment, and may reflect changes in antioxidant enzyme activities or plasma antioxidant molecule concentrations, such as uric acid (Cohen et al., 2007). During embryonic development the activities of antioxidant enzymes not only changed daily within specific tissues (heart, lung, brain,

kidney), but also differed between tissues (Surai, 1999). It is possible that as chicks mature the activities of antioxidant enzymes continue to fluctuate.

Chick AOC after day zero was not expected to be affected by maternal CXN, however it was expected that as maternal CXN level increased day zero AOC would also increase. Day zero AOC of chicks from Early hens was not affected by maternal CXN supplementation, but did increase with increasing level of maternal CXN supplementation among chicks from Mid and Late hens. Broiler breeder supplementation of 6 or 12 ppm CXN reduced the lipid oxidation product malondialdehyde in day one chick livers (Surai et al., 2003). Further when Chinese Three Yellow broiler breeder hens were supplemented with 6 ppm CXN, the resulting chicks had lower serum malondialdehyde levels at one and seven days of age, and greater superoxide dismutase activity at one day of age (Zhang et al., 2011). Total antioxidant capacity was unaffected by chick age among chicks from non-supplemented and 6 ppm supplemented hens (Zhang et al., 2011). However, the published literature does not account for the effect of hen age in addition to hen supplemented CXN. The current research indicates that hen age affects how CXN affects chick AOC post-hatch. Upon reflection, it would have been wise to include measurements of individual antioxidant enzymes and plasma uric acid concentration and oxidative products which would have allowed for a more accurate understanding of the chick's antioxidant status as it aged from day zero to day 21.

2.4 CONCLUSIONS

In conclusion, hen supplementation with CXN decreased hen body weight. Supplementation of 6 ppm CXN increased settable egg production and as hypothesized, higher levels of supplementation resulted in greater amounts of CXN being deposited into the egg and chick liver. Hen plasma total antioxidant capacity was not affected by CXN supplementation nor did it affect hatchability or chick performance.

As hypothesized, chicks from hens supplemented with CXN appeared to have a more robust innate immune response than chicks from non-supplemented hens. However, greater levels of maternal CXN did not increase chick innate immune function as chicks from 6 ppm hens had the greatest EBC and day one OB. Chicks from older hens had a less robust immune response as indicated by EBC, which could be related to incubator temperature; and chick innate immune function increased as chicks aged, indicating that the chick's immune system is maturing during the first four days of life. Chick day zero plasma total antioxidant capacity increased with increasing dietary level of CXN in chicks from Mid and Late hens. The profile of chick plasma total antioxidant capacity as chicks aged differed by hen age and treatment, but the reason is unclear and may be made clearer as information on the activities of antioxidant enzymes as chicks age becomes available. The current data support that CXN is immuno-modulatory and suggest that hen age affects chick innate immune function as well as chick plasma total antioxidant capacity.

Table 2.1. Ingredients and calculated nutrient content of canthaxanthin diets

Ingredient	%
Calculated Inclusion	
Corn	36.6
Soybean meal	15.8
Wheat	34.1
Calcium carbonate	7.8
Dicalcium phosphate	1.44
Salt	0.4
L-lysine	0.04
Methionine hydroxy analogue, 95%[1]	0.2
Vitamin E premix[2]	0.8
Inorganic trace mineral premix[3]	0.1
Canola oil	0.5
Avizyme 1302[4]	0.05
Broiler Breeder premix[5]	1.0
Canola oil	1.3
Canthaxanthin[6]	
Calculated Nutrient Profile	
ME (kcal/kg)	2,882
CP (%)	15
Calcium (%)	3
Available P (%)	0.37
Lysine (%)	0.73
Vitamin E (IU/kg)	54
Sodium (%)	0.19

[1] Novus International, Inc.
[2]Vitamin E premix provided 40 IU/kg diet as dl-alpha-tocopehyl acetate
[3] Inorganic trace mineral premix supplied the following per kg of feed: $CuSO_4 \cdot 5H_2O$, 40 mg; $MnSO_4$, 380 mg; $ZnSO_4$, 295 mg
[4] Danisco
[5] Vitamin/Mineral premix supplied the following per kg of feed: Vitamin A, 12,500 IU; Vitamin D_3, 3,100 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; choline, 2.7 mg; iron, 0.0 mg; zinc, 100 mg; manganese, 87 mg; copper, 15 mg; iodine, 1.665 mg; selenium, 0.3 mg
[6] Canthaxanthin was supplemented as Carophyll-Red (DSM Nutritional Products) at levels of 0, 6 or 12 ppm.
Analyzed levels of canthaxanthin were 0, 7.62 and 15.84 ppm for each respective diet

Table 2.2. Effect of hen age and dietary canthaxanthin on hen production parameters

		Body Weight	Settable Eggs[4]	Total Eggs	Total Settable Eggs
Hen Age[1]	Treatment[2]	Kg	%[5]		
Early		3.15[c]	87.40[a]		
Mid		3.34[b]	67.00[b]		
Late		3.52[a]	53.33[c]		
SEM		0.029	0.044		
Treatment[2]					
	0 ppm	3.38[a]	68.68[b]	188[ab]	183
	6 ppm	3.31[b]	72.27[a]	198[a]	190
	12 ppm	3.33[b]	66.77[b]	180[b]	175
SEM		0.029	0.044	12.38	12.48
Interaction					
Hen Age * Treatment[3]					
Early	0 ppm	3.16	88.12		
Early	6 ppm	3.14	88.36		
Early	12 ppm	3.15	85.72		
Mid	0 ppm	3.40	66.80		
Mid	6 ppm	3.30	70.25		
Mid	12 ppm	3.31	63.94		
Late	0 ppm	3.57	51.13		
Late	6 ppm	3.47	58.21		
Late	12 ppm	3.51	50.63		
SEM		0.051	0.076		
P-Values					
Age		<0.0001	<0.0001		
Treatment		<0.0001	<0.0001	0.0229	0.0504
Age*Treatment		0.0932	0.1822		

[abc] Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM Nutritional Products)

[3] n = 45 hens per dietary treatment

[4] Settable eggs: eggs were considered settable provided they were not deemed to be too large, cracked nor misshapen

[5] Data $\log_{10}$ transformed to meet ANOVA assumptions, but presented as percentage

Table 2.3. Effect of hen age and dietary canthaxanthin on egg traits

Hen Age[1]	Treament[2]	Egg Weight	Yolk Weight	Proportion Yolk	Albumen Weight	Proportion Albumen[4]	Wet Shell Weight[4]	Proportion Wet Shell[4]	Egg Canthaxanthin	
		g	g	%[5]	g	%[5]	g	%[5]	µg/g yolk	µg/egg
Early		56.64[c]	16.67[c]	30.24[b]	32.53[b]	56.73[a]	7.44[b]	13.14[b]	24.23	213.33
Mid		64.06[b]	18.85[b]	30.29[b]	35.15[a]	54.17[ab]	10.06[a]	15.16[a]	22.92	235.56
Late		67.24[a]	20.87[a]	31.17[a]	35.90[a]	52.86[b]	10.47[a]	15.57[a]	22.03	440.79
SEM		2.24	0.92	0.017	1.88	0.025	1.68	0.039	12.31	260.14
Treatment[2]										
	0 ppm	63.32	18.80	30.35	34.72	54.48	9.80	15.48	0[c]	0[c]
	6 ppm	62.24	18.64	30.85	34.70	55.00	8.90	14.15	28.57[b]	372.28[b]
	12 ppm	62.37	18.95	31.17	34.17	54.27	9.25	14.83	39.89[a]	533.20[a]
SEM		2.24	0.93	0.017	1.88	0.025	1.68	0.039		287.05
Interaction										
Hen Age * Treatment (ppm)[3]										
Early	0	57.90	16.85	29.63	32.71	53.03	8.34	14.40	0	0
Early	6	56.67	16.75	30.79	32.45	56.66	7.47	13.18	29.53	260.50
Early	12	55.67	16.41	30.31	32.42	57.50	6.88	12.19	42.45	395.29
Mid	0	64.74	18.66	29.65	36.88	56.23	9.25	14.12	0	0
Mid	6	62.92	18.29	30.02	34.50	54.00	10.19	15.98	29.75	313.95
Mid	12	64.51	19.59	31.20	34.08	52.26	10.89	16.54	38.29	408.53
Late	0	67.33	20.89	31.77	34.56	51.19	11.93	17.04	0	0
Late	6	67.44	20.88	31.73	37.14	54.35	9.46	13.92	26.44	542.38
Late	12	66.95	20.83	32.01	36.01	53.05	10.16	14.94	38.92	795.79
SEM		3.87	1.60	0.030	3.26	0.043	2.91	0.068	17.10	364.70
P-Values										
Age		<0.0001	<0.0001	0.0212	0.0016	0.0192	0.0010	0.0009	0.2889	0.3783
Treatment		0.5829	0.8083	0.4338	0.8079	0.7699	0.5698	0.7116	<0.0001	0.0285
Age * Treatment		0.8475	0.6109	0.7751	0.2511	0.2381	0.3734	0.0533	0.4794	0.6285

[a,b,c,d] Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

ND – Not detectable

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM Nutritional Products)

[3] n = 10 eggs per treatment

[4] Shell weights include shell membrane

[5] Data $\log_{10}$ transformed to meet ANOVA assumptions, but presented as percentage

Table 2.4.The effect of hen age and maternal canthaxanthin on hen total plasma antioxidant capacity

Hen Age[1]	Treatment[2]	Antioxidant Capacity (mM Trolox Equivalents)
Photostimulation		3.24[a]
Early		2.46[b]
Mid		1.65[c]
Late		1.98[c]
SEM		0.45
	0 ppm	2.55
	6 ppm	2.29
	12 ppm	2.15
SEM		0.39
Interaction		
Age * Treatment[3]		
Photostimulation	0 ppm	3.37
Photostimulation	6 ppm	3.12
Photostimulation	12 ppm	3.22
Early	0 ppm	2.48
Early	6 ppm	2.50
Early	12 ppm	2.41
Mid	0 ppm	2.28
Mid	6 ppm	1.59
Mid	12 ppm	1.09
Late	0 ppm	2.08
Late	6 ppm	1.97
Late	12 ppm	1.88
SEM		0.78
P-Values		
Age		<0.0001
Treatment		0.1265
Age * Treatment		0.4724

[abc]Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column
[1]Hen ages are defined as production stages. Photostimulation = 23 wks; Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks
[2]Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM Nutritional Products)
[3] n = 10 hens per treatment

Table 2.5. Effect of hen age and maternal canthaxanthin on hatchability and chick hatch weight

		Hatchability[4]	Male Hatch Weight	Female Hatch Weight
Hen Age[1]	Treatment[2]	%	g	g
Early		78	40.41[c]	40.30[c]
Mid		76	44.78[b]	44.94[b]
Late		76	46.92[a]	46.97[a]
SEM		0.030	0.53	0.51
	0 ppm	75	44.55[a]	44.18[b]
	6 ppm	79	42.77[b]	43.31[c]
	12 ppm	77	44.79[a]	44.72[a]
SEM		0.030	0.53	0.51
Interaction				
Hen Age * Treatment[3]				
Early	0 ppm	73	40.27[de]	40.02[e]
Early	6 ppm	82	39.95[e]	40.20[e]
Early	12 ppm	80	41.01[d]	40.70[e]
Mid	0 ppm	75	45.65[b]	44.88[c]
Mid	6 ppm	77	43.13[c]	43.96[d]
Mid	12 ppm	76	45.57[b]	45.98[b]
Late	0 ppm	77	47.73[a]	47.64[a]
Late	6 ppm	77	45.23[b]	45.79[b]
Late	12 ppm	73	47.80[a]	47.49[a]
SEM		0.052	0.92	0.88
P-Values				
Age		0.5793	<0.0001	<0.0001
Treatment		0.3796	<0.0001	<0.0001
Age * Treatment		0.4016	0.0036	0.0095

[abcde] Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

[1] Hen ages are defined as production stages: Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM Nutritional Products)

[3] n = 50 chicks per treatment

[4] Data $\log_{10}$ transformed to meet ANOVA assumptions, but presented as percentage

Table 2.6. Effect of hen age and maternal canthaxanthin on *E. coli* bactericidal and phagocytic capacities of chicks[1]

			E. coli Bactericidal Capacity	Phagocytic Capacity[5,6]	Phagocyte Activation[5,7]
			%[4]	Mean Fluorescent Unit[8]	
Hen Age[1]	Treatment[2]	Chick Age			
Early			54.92[a]	.	.
Mid			29.12[b]	144.25[a]	38.15[b]
Late			12.24[c]	114.78[b]	27.33[a]
SEM			5.45	8.40	2.52
	0 ppm		29.18[b]	.	.
	6 ppm		36.87[a]	.	.
	12 ppm		30.22[b]	.	.
SEM			5.45	.	.
		Day 1	26.59[b]	.	.
		Day 4	37.60[a]	117.24	36.13
SEM			4.45	.	.
Interactions					
Hen Age * Treatment					
Early	0 ppm		57.44	.	.
Early	6 ppm		58.95	.	.
Early	12 ppm		48.37	.	.
Mid	0 ppm		20.06	151.78	41.41
Mid	6 ppm		39.13	139.31	34.33
Mid	12 ppm		28.16	141.65	38.70
Late	0 ppm		10.03	110.45	27.96
Late	6 ppm		12.54	115.73	27.75
Late	12 ppm		14.14	118.15	26.28
SEM			9.44	14.52	4.35
Hen Age * Chick Age					
Early		Day 1	47.79	.	.
Early		Day 4	62.05	102.56[c]	34.87[b]
Mid		Day 1	24.13	142.43[a]	32.20[bc]
Mid		Day 4	34.10	146.06[a]	44.09[a]
Late		Day1	7.83	126.46[b]	25.24[d]
Late		Day 4	16.64	103.10[c]	29.42[c]
SEM			7.70	12.77	3.83
Treatment * Chick Age					
0 ppm		Day 1	20.46	.	.
0 ppm		Day 4	37.90	113.33	36.17
6 ppm		Day 1	34.57	.	.
6 ppm		Day 4	39.18	118.58	36.99
12 ppm		Day 1	24.73	.	.
12 ppm		Day 4	35.71	119.81	35.22
SEM			7.71	12.78	3.83
Hen Age * Treatment * Chick Age					
Early	0 ppm	Day 1	45.15	.	.
Early	0 ppm	Day 4	69.73	90.45	34.98
Early	6 ppm	Day 1	53.81	.	.
Early	6 ppm	Day 4	64.09	109.83	34.98
Early	12 ppm	Day 1	44.41	.	.
Early	12 ppm	Day 4	52.34	107.41	36.50
Mid	0 ppm	Day 1	11.78	154.29	37.52

Mid	0 ppm	Day 4	28.34	149.26	45.30
Mid	6 ppm	Day 1	38.25	134.96	24.02
Mid	6 ppm	Day 4	40.01	143.65	44.64
Mid	12 ppm	Day 1	22.37	138.03	35.06
Mid	12 ppm	Day 4	33.94	145.26	42.35
Late	0 ppm	Day 1	4.44	120.63	25.82
Late	0 ppm	Day 4	15.63	100.27	30.10
Late	6 ppm	Day 1	11.64	129.20	24.13
Late	6 ppm	Day 4	13.43	102.26	31.36
Late	12 ppm	Day 1	7.43	129.54	25.76
Late	12 ppm	Day 4	20.86	106.76	26.81
SEM			13.34	22.06	6.62
P-Values					
Hen Age			<0.0001	<0.0001	<0.0001
Treatment			0.0171	.	.
Chick Age			<0.0001	.	.
Hen Age * Treatment			0.2400	0.2346	0.0996
Hen Age * Chick Age			0.1694	0.0018	0.0029
Treatment * Chick Age			0.1672	.	.
Hen Age * Treatment * Chick Age			0.7765	0.6039	0.2611

[abcd] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 12 per treatment

[4] Data log transformed to meet ANOVA assumptions, but presented as %

[5] Data for D1 chicks from Early hens lost

[6] Phagocytic capacity is an indication of the number of *E. coli* bioparticles phagocytized by each cell.

[7] Phagocyte activation is an indication of the number of cells which contain at least one *E. coli* bioparticles

[8] Fluorescence of treated cells used to estimate phagocytic capacity and activation in comparison to fluorescence of untreated cells.

Table 2.7. Effect of hen age and maternal canthaxanthin on oxidative burst response in chicks

			Oxidative Burst	
			Mean Fluorescence Unit[5]	
			Chick Age	
Hen Age[1,3]	Treatment[2,3]	OB Time	Day 1	Day 4
Early			0.87[c]	5.57[a]
Mid			5.66[a]	2.97[b]
Late			2.03[b]	3.30[b]
SEM			0.94	1.52
	0 ppm		2.42	5.57
	6 ppm		3.06	2.97
	12 ppm		3.08	3.30
SEM			0.94	1.52
		5	0.70[d]	3.29
		10	2.21[c]	3.49
		15	3.59[b]	4.60
		20	4.92[a]	4.41
Interactions			1.08	1.76
Hen Age * Treatment				
Early	0 ppm		1.21[cd]	4.15
Early	6 ppm		0.14[d]	5.38
Early	12 ppm		1.50[cd]	7.18
Mid	0 ppm		6.03[a]	3.33
Mid	6 ppm		5.11[ab]	3.27
Mid	12 ppm		5.86[a]	2.30
Late	0 ppm		0.03[d]	2.31
Late	6 ppm		4.22[b]	4.44
Late	12 ppm		1.83[c]	3.16
SEM			1.62	2.64
Hen Age * OB Time				
Early		5	0.32[f]	5.35
Early		10	0.51[f]	4.81
Early		15	1.07[ef]	6.86
Early		20	1.59[ef]	5.27
Mid		5	1.38[ef]	2.30
Mid		10	4.66[c]	2.78
Mid		15	6.97[b]	3.34
Mid		20	9.66[a]	3.44
Late		5	0.39[f]	2.21
Late		10	1.45[ef]	2.87
Late		15	2.74[de]	3.60
Late		20	3.53[cd]	4.53
SEM			1.08	3.05
Treatment * OB Time				
0 ppm		5	0.36	2.37
0 ppm		10	1.55	2.93
0 ppm		15	3.28	3.67
0 ppm		20	4.50	4.10
6 ppm		5	0.61	2.54
6 ppm		10	2.77	3.95
6 ppm		15	3.75	5.90
6 ppm		20	5.12	5.06
12 ppm		5	1.12	4.95

12 ppm		10	2.30	3.59
12 ppm		15	3.75	4.22
12 ppm		20	5.15	4.09
SEM			1.87	3.05
Hen Age * Treatment * OB Time[4]				
Early	0 ppm	5	0.55	3.01
Early	0 ppm	10	0.76	3.15
Early	0 ppm	15	1.40	4.69
Early	0 ppm	20	2.13	5.77
Early	6 ppm	5	0	2.47
Early	6 ppm	10	0	5.33
Early	6 ppm	15	0	8.79
Early	6 ppm	20	0.31	4.92
Early	12 ppm	5	0.93	10.59
Early	12 ppm	10	1.14	5.94
Early	12 ppm	15	1.88	7.09
Early	12 ppm	20	2.25	5.11
Mid	0 ppm	5	0.87	2.29
Mid	0 ppm	10	4.10	3.31
Mid	0 ppm	15	8.04	3.96
Mid	0 ppm	20	11.10	3.77
Mid	6 ppm	5	1.54	2.46
Mid	6 ppm	10	5.65	2.83
Mid	6 ppm	15	5.81	3.74
Mid	6 ppm	20	7.43	4.02
Mid	12 ppm	5	1.73	2.16
Mid	12 ppm	10	4.22	2.21
Mid	12 ppm	15	7.04	2.31
Mid	12 ppm	20	10.44	2.53
Late	0 ppm	5	0	1.82
Late	0 ppm	10	0	2.32
Late	0 ppm	15	0.39	2.38
Late	0 ppm	20	0.27	2.74
Late	6 ppm	5	0.81	2.71
Late	6 ppm	10	3.03	3.67
Late	6 ppm	15	5.49	5.17
Late	6 ppm	20	7.56	6.22
Late	12 ppm	5	0.71	2.10
Late	12 ppm	10	1.53	2.62
Late	12 ppm	15	2.32	3.26
Late	12 ppm	20	2.76	4.64
SEM			1.65	2.47
P-Values			3.24	5.28
Hen Age			<0.0001	0.0014
Treatment			0.2941	0.3088
OB Time			<0.0001	0.3600
Hen Age * Treatment			<0.0001	0.1989
Hen Age * OB Time			<0.0001	0.9248
Treatment * OB Time			0.9927	0.6297
Hen Age * Treatment * OB Time			0.2020	0.8823

[abcdef] Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

[1]Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2]Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM Nutritional Products)

[3] Values reported are average OB over length of assay

[4] n = 12 chicks per maternal treatment
[5] Oxidative products quantified via fluorescence intensity

Table 2.8. Effect of hen age and maternal canthaxanthin on weekly chick body weight and gain

		Body Weight				Average Daily Gain		
		g				g/bird/day		
		D0	D7	D14	D21	Week 1	Week 2	Week 3
Hen Age[1]	Treatment[2]							
Early		41.04[c]	129.67[b]	352.10[b]	655.23[b]	12.68[b]	33.37[b]	53.02[b]
Mid		45.03[b]	144.34[a]	411.75[a]	788.85[a]	14.24[a]	35.46[a]	60.09[a]
Late		47.25[a]	149.26[a]	392.21[a]	806.61[a]	14.59[a]	33.81[ab]	62.14[a]
SEM		1.56	8.11	33.10	126.86	1.17	3.85	7.00
	0 ppm	45.27	140.31	380.76	754.92	13.65	31.01	58.79
	6 ppm	43.41	142.18	396.81	791.64	14.11	37.43	59.39
	12 ppm	44.65	140.78	378.49	704.13	13.75	34.20	57.07
SEM		1.56	8.16	33.31	126.86	1.17	3.87	7.04
Interaction								
Hen Age * Treatment[3]								
Early	0 ppm	40.75	122.85	336.66	696.23	11.75	28.65	55.29
Early	6 ppm	40.39	131.18	414.14	719.10	12.99	32.78	52.08
Early	12 ppm	41.99	134.99	391.47	550.36	13.30	31.70	51.69
Mid	0 ppm	45.94	144.66	414.14	771.12	14.28	37.91	59.35
Mid	6 ppm	43.46	146.60	426.63	847.38	14.69	38.68	65.04
Mid	12 ppm	45.70	141.76	399.67	748.06	13.74	35.70	55.88
Late	0 ppm	49.11	153.44	355.51	797.10	14.92	33.55	61.73
Late	6 ppm	46.39	148.75	394.47	808.45	14.65	34.93	61.04
Late	12 ppm	46.26	145.60	385.49	813.98	14.21	34.11	63.65
SEM		2.71	14.10	57.54	219.72	2.03	6.68	12.16
P-Values								
Hen Age		<0.0001	0.0003	0.0046	0.0436	0.0069	0.0096	0.0342
Treatment		0.0641	0.8823	0.4674	0.3656	0.6898	0.4977	0.7771
Hen Age * Treatment		0.2412	0.3107	0.8825	0.7402	0.4662	0.8221	0.6158

[abc] Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

[1]Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2]Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM)

[3] n = 10 chicks per maternal treatment

Table 2.9. Effect of hen age and maternal canthaxanthin on weekly chick liver weight and canthaxanthin content

		Liver Weight				D0 Liver Canthaxanthin
		g				µg/g
		D0	D7[4]	D14	D21	D0
Hen Age[1]	Treatment[2]					
Early		1.00[b]	.	12.78[b]	22.34[b]	76.39[a]
Mid		1.16[a]	5.54	15.05[a]	25.61[a]	52.29[b]
Late		1.15[a]	5.62	14.32[a]	23.25[b]	56.44[b]
SEM		0.59	0.67	1.05	1.48	11.66
	0 ppm	1.13	5.78	13.31[b]	23.94	0[c]
	6 ppm	1.09	5.38	15.01[a]	24.02	55.75[b]
	12 ppm	1.08	5.59	13.83[b]	23.23	140.35[a]
SEM		0.059	0.82	1.05	1.48	11.67
Interaction						
Hen Age * Treatment[3]						
Early	0 ppm	1.04	.	11.93	22.50	0[e]
Early	6 ppm	1.02	.	13.62	22.28	70.56[c]
Early	12 ppm	0.93	.	12.80	22.23	169.58[a]
Mid	0 ppm	1.16	5.81	14.06	27.11	0[e]
Mid	6 ppm	1.16	5.24	15.97	26.22	40.90[d]
Mid	12 ppm	1.15	5.57	15.13	23.50	126.94[b]
Late	0 ppm	1.18	5.74	13.94	22.20	0[e]
Late	6 ppm	1.10	5.51	15.45	23.58	55.78[cd]
Late	12 ppm	1.16	5.60	13.57	23.98	124.52[b]
SEM		0.10	1.16	1.82	2.57	20.16
P-Values						
Hen Age		<0.0001	0.8176	0.0002	<0.0001	<0.0001
Hen Treatment		0.2523	0.6179	0.0073	0.4899	<0.0001
Hen Age * Hen Treatment		0.2418	0.9114	0.7992	0.0617	0.0102

[abcde] Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM)

[3] n = 10 chicks per maternal treatment

[4] Livers from seven day old chicks not collected from Early hens.

Table 2.10. The effect of hen age and maternal canthaxanthin on chick total plasma antioxidant capacity

			Antioxidant Capacity
			mM Trolox Equivalents
Hen Age[1]	Treatment[2]	Chick Age	
Early			2.40[b]
Mid			2.69[a]
Late			2.17[c]
SEM			0.18
	0 ppm		2.37[b]
	6 ppm		2.29[b]
	12 ppm		2.61[a]
SEM			0.19
		D0	2.66[a]
		D7	2.36[bc]
		D14	2.44[b]
		D21	2.22[c]
SEM			0.21
Interactions			
Hen Age * Chick Age			
Early		D0	2.20[ef]
Early		D7	2.45[cde]
Early		D14	2.19[ef]
Early		D21	2.78[abc]
Mid		D0	2.67[bc]
Mid		D7	2.72[bc]
Mid		D14	2.84[ab]
Mid		D21	2.55[bcd]
Late		D0	3.11[a]
Late		D7	1.91[f]
Late		D14	2.30[de]
Late		D21	1.34[g]
SEM			0.36
Hen Age * Treatment			
Early	0 ppm		2.67[b]
Early	6 ppm		2.46[bc]
Early	12 ppm		2.09[de]
Mid	0 ppm		2.21[cd]
Mid	6 ppm		2.61[b]
Mid	12 ppm		3.26[a]
Late	0 ppm		2.23[cd]
Late	6 ppm		1.78[e]
Late	12 ppm		2.48[bc]
SEM			0.31
Treatment * Chick Age			
	0 ppm	D0	2.23[bc]
	6 ppm	D0	2.77[a]
	12 ppm	D0	2.89[a]
	0 ppm	D7	2.30[bc]
	6 ppm	D7	2.10[c]
	12 ppm	D7	2.68[a]
	0 ppm	D14	2.71[a]
	6 ppm	D14	2.01[c]
	12 ppm	D14	2.62[ab]

	0 ppm	D21	2.15[c]
	6 ppm	D21	2.26[bc]
	12 ppm	D21	2.25[c]
SEM			0.36
Hen Age * Treatment * Chick Age[3]			
Early	0 ppm	D0	2.4[fghijkl]
Early	0 ppm	D7	2.72[cdefghi]
Early	0 ppm	D14	2.41[fghijkl]
Early	0 ppm	D21	3.12[bcd]
Early	6 ppm	D0	2.18[ijkl]
Early	6 ppm	D7	2.65[cdefghijk]
Early	6 ppm	D14	2.12[jklm]
Early	6 ppm	D21	2.89[bcdef]
Early	12 ppm	D0	2.01[klmn]
Early	12 ppm	D7	1.99[klmn]
Early	12 ppm	D14	2.04[jklmn]
Early	12 ppm	D21	2.31[ghijkl]
Mid	0 ppm	D0	1.95[lmn]
Mid	0 ppm	D7	1.89[lmno]
Mid	0 ppm	D14	2.90[bcdefg]
Mid	0 ppm	D21	2.10[jklm]
Mid	6 ppm	D0	2.81[cdefgh]
Mid	6 ppm	D7	2.39[efghijkl]
Mid	6 ppm	D14	2.72[cdefghi]
Mid	6 ppm	D21	2.52[efghijk]
Mid	12 ppm	D0	3.25[bc]
Mid	12 ppm	D7	3.88[a]
Mid	12 ppm	D14	2.89[bcdef]
Mid	12 ppm	D21	3.02[bcde]
Late	0 ppm	D0	2.62[defghij]
Late	0 ppm	D7	2.28[hijkl]
Late	0 ppm	D14	2.81[cdefgh]
Late	0 ppm	D21	1.23[op]
Late	6 ppm	D0	3.30[abc]
Late	6 ppm	D7	1.27[p]
Late	6 ppm	D14	1.18[p]
Late	6 ppm	D21	1.37[nop]
Late	12 ppm	D0	3.40[ab]
Late	12 ppm	D7	2.18[ijkl]
Late	12 ppm	D14	2.92[bcde]
Late	12 ppm	D21	1.42[mnop]

SEM	0.62
P-Values	
Hen Age	<0.0001
Treatment	0.0014
Chick Age	0.0004
Hen Age * Treatment	<0.0001
Hen Age * Chick Age	<0.0001
Treatment * Chick Age	0.0002
Hen Age * Treatment * Chick Age	<0.0001

[abcdefghijklmnop] Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

[1]Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2]Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM Nutritional Products)

[3] n = 10 chicks per maternal treatment

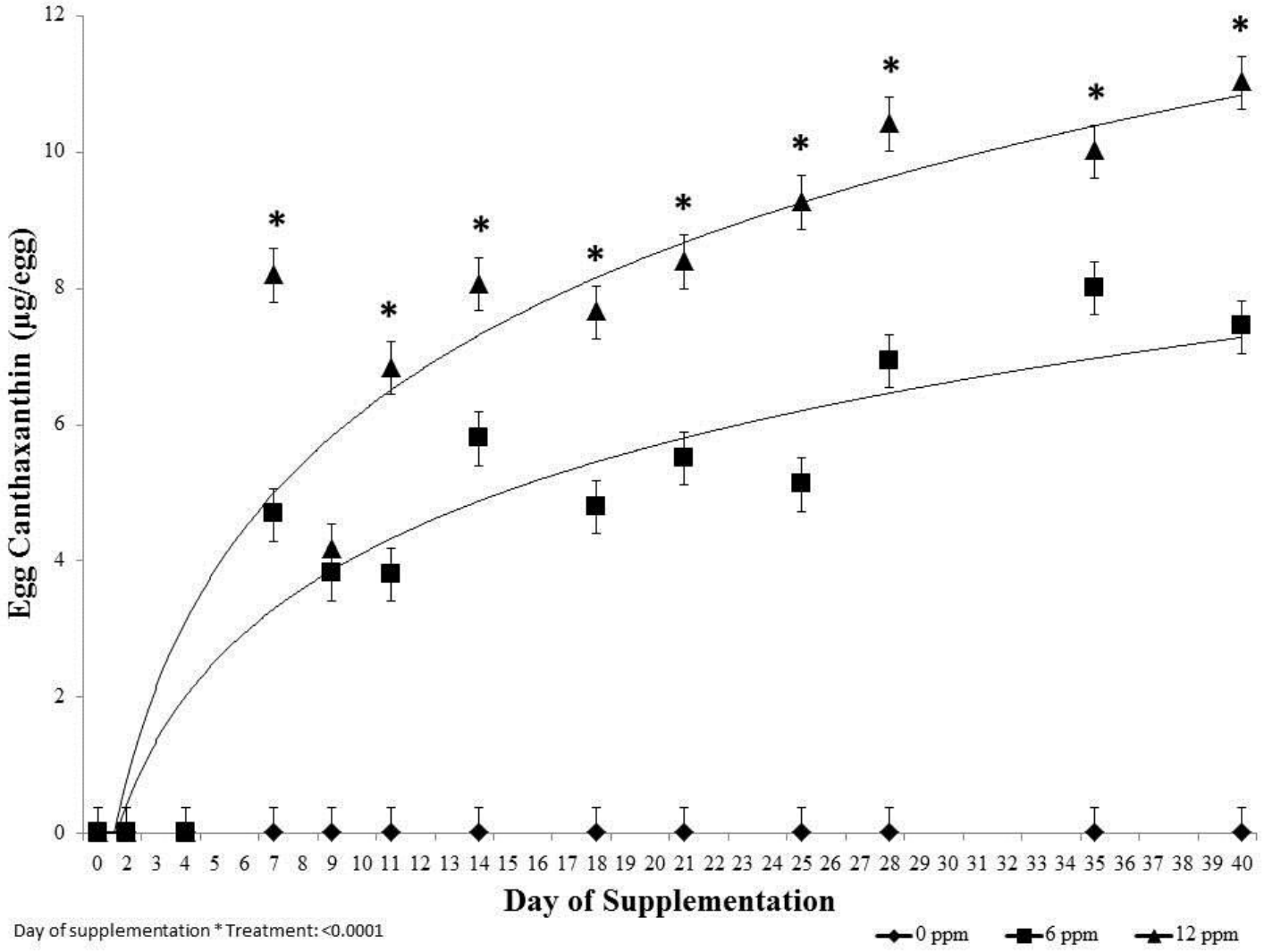

Figure 2.1. Deposition rate of canthaxanthin into eggs
* Indicates differences ($p < 0.0001$) between treatments 6 ppm and 12 ppm
Diets supplemented with 0, 6 or 12 ppm Canthaxanthin as Carophyll Red (DSM Nutritional Products)

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3. THE EFFECT OF MATERNALLY SUPPLEMENTED VITAMIN E AND HEN AGE ON INDICES OF CHICK POST-HATCH INNATE IMMUNE FUNCTION

3.1 INTRODUCTION

Vitamin E (**VE**) is a fat-soluble vitamin of plant origin, known for its antioxidant (Ryan et al., 2010), and immunomodulatory properties (Boa-Amponsem et al., 2000). In nature there are eight different vitamin E molecules; of those eight, α-tocopherol (**ATOC**) has the greatest bioactivity (Jensen et al., 2006). Alpha-tocopherol has eight possible stereoisomers which are RRR, RRS, RSS, RSR, SRR, SSR, SRS, and SSS (Jensen et al., 2006). Commercial synthetic vitamin E (**SVE**) is a racemic (equal) mixture of all eight ATOC stereoisomers seven of which have lower bioactivities from the stereoisomer which occurs naturally, RRR-α-tocopherol (**NVE**; Weiser et al., 1996). Therefore at equal levels of dietary inclusion (in grams) the effectiveness of SVE supplementation may differ from that of NVE supplementation (Hidiroglou et al., 1992). It is also possible that differential effects of SVE vs. NVE may also be observed when equal activity levels (IU) are supplemented as in lactating sows and suckling piglets natural sources of RRR-α-tocopherol were preferentially deposited into tissues compared to synthetic sources of RRR-α-tocopherol (Lauridsen et al., 2002).

The immuno-modulatory properties of VE have been well documented. In aged mice supplemental VE increased naïve T-cell division and production of the pro-inflammatory cytokine IL-2 (Han et al., 2006; Adolfsson., 2001). When SVE

was supplemented in excess of the NRC (1994) VE recommendation to the diet of broiler chicks there was an increase in antibody titres after vaccination with killed or live infectious bronchitis virus, or sheep red blood cells (**SRBC**; Leshchinsky and Klasing, 2001). However, moderate levels (25 to 50 IU/kg diet) of SVE were more effective at enhancing the antibody response to killed and live infectious bronchitis virus, and SRBC in chickens than higher levels (100 to 200 IU/kg of diet); it was hypothesized that this was the result of the pro-oxidant effects of VE at high concentrations (Leshchinsky and Klasing, 2001).

As an antioxidant, vitamin E is a potent scavenger of free radicals and reactive oxygen species (**ROS**). As such, it plays an important physiological role in the maintenance of cell membrane integrity (Howard et al., 2011) and may affect the innate immune response which includes the production of ROS. Supplemented dietary VE was also capable of upregulating the antioxidant enzymes superoxide dismutase and catalase in the brains of newly hatched chicks (Lin et al., 2005). However, when VE is present at very high levels in humans it no longer functions as an antioxidant, but rather takes on pro-oxidant characteristics, resulting in increased oxidation activity in the plasma (Pearson et al., 2006). In rats, high doses of SVE (all-*rac*-α-tocopherol acetate) equivalent to 1,000 mg/kg diet reduced antioxidant enzyme activity (Eder et al., 2002).

The effect of hen age on chick post-hatch innate immune function has not been studied, but there is evidence which indicates that it is worth investigating. For example, first week chick mortality decreased as hens approached 38 weeks of age and then increased slightly to 60 weeks of age (Yassin et al., 2009). The

causes of increased first week mortality associated with old hen ages could be related to egg size and associated hatching times. Egg size increases as hens age and increased first week mortality among chicks from older hens could be the result of earlier hatching times of large eggs and increased risk of dehydration (Suarez et al., 1997). First week mortality could also be related to the ability of embryos to utilize fatty acids *in ovo.* Chicks from 36 and 64 week old hens had greater residual yolk sac weights than chicks from 51 week old hens, suggesting that chicks from young and old hens were less able to mobilize fatty acids *in ovo* (Latour et al., 1998). In addition to those challenges the post-hatch chick, regardless of hen age, is exposed to pathogenic and non-pathogenic microbes and potentially subject to immune challenge.

Data describing the differential effects of SVE vs. NVE on chick post-hatch innate immune function and the effect of hen age on chick post-hatch innate immune function are not available. The objective of this study was to determine if maternally supplemented NVE and hen age affected indices of innate immune function (*Escherichia coli* bactericidal capacity, phagocytosis and oxidative burst) and plasma total antioxidant capacity in chicks differently than SVE. The hypotheses were that chicks hatched from hens supplemented with NVE would exhibit stronger *ex vivo* indicators of innate immune cell function than chicks hatched from hens supplemented with SVE at equivalent levels of supplementation; and that chick innate immune function would increase as hens age.

3.2 MATERIALS AND METHODS

3.2.1 Animals

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with the Canadian Council of Animal Care (1993) guide. For animal rearing and sampling schedule refer to Chapter 2, Experiment 1. Hens (n = 45 per treatment) were fed a standard commercial broiler breeder diet supplemented with either 40 IU/kg of all-*rac*-α-tocopherol (SVE) or RRR-α-tocopherol (NVE; Table 3.1). At each of the three hen ages observed (Early: 31 to 33 wks; Mid: 45 to 47 wks; and Late: 57 to 59 wks) 10 eggs per treatment were sampled for whole egg, yolk and albumen weight. Yolk and albumen from these eggs were kept for analysis of egg ATOC content and were stored at -20°C until analysis via HPLC. Plasma was collected from 10 hens per treatment at each hen age for determination of total plasma antioxidant capacity. Fertile eggs were collected and set once per week at each hen age. Whole blood from male chicks from the first hatch at each hen age was collected for *ex vivo* determination of innate immune function indices which included *Esherichia coli* bactericidal capacity, phagocytic capacity and activation and oxidative burst response at one and four days of age. This was repeated for the second hatch and the data was pooled. Male chicks from the third hatch of each hen age were grown out to 21 days without further dietary treatment. On day zero, day seven, day 14 and day 21 10 chicks per maternal treatment 10 chicks per maternal treatment were weighed and sampled for total plasma antioxidant capacity and liver weight.

3.2.2 Innate Immune Function Indices

Whole blood collected from the chicks was used to determine *Escherichia coli* (***E. coli***) bactericidal capacity (**EBC**), to assess the number of cells containing at least one *E. coli* (phagocyte activation), the average number of *E. coli*/cell (phagocytic capacity) and oxidative burst response (**OB**) *ex vivo*. Assay conditions were as described in Chapter 2, Experiment 1.

3.2.3 Plasma Total Antioxidant Capacity Assay

Plasma was collected form 10 chicks per maternal treatment at day zero, day seven, day 14 and day 21 for plasma total antioxidant capacity (**AOC**). Assay conditions were as described in Chapter 2, Experiment 1.

3.2.4 Determination of Egg and Feed α-Tocopherol

Egg yolk and feed samples were analyzed via HPLC for α-tocopherol content. Two grams of egg yolk or feed were transferred to a 16 x 100 mm screw top vial, 2 ml of HPLC-grade water were added and the contents were mixed on a vortex. One ml of methanol and 1 ml of 33% Methanolic KOH (v/w) were added and the mixture was shaken vigorously for 2 minutes. The samples were than saponified overnight (about 16 hrs) at 70°C. The samples were cooled to room temperature and then centrifuged at 850 x g for 10 minutes. The supernatant was removed to a new set of tubes, and the samples were washed one time with 2 ml of water and 1 ml of methanol, shaken vigorously for 2 minutes and re-centrifuged at 850 x g for 10 minutes. The supernatant was combined with that in the new tubes. To the combined supernatents, 2 ml of acetone:chloroform 3:7 (v/v) was added, the samples were shaken vigorously for two minutes and then

centrifuged at 850 x g for 10 minutes. The bottom organic layer was removed to a new set of tubes, and the remaining upper layer was extracted a second time with 2 ml of acetone:chloroform 3:7 (v/v), shaken vigorously for 2 minutes and respun at 850 x g for 10 minutes. The bottom organic layer was combined with the bottom organic layer from the first extraction. The extract was washed with 2 ml of a 1% NaCl solution. The samples were vigorously shaken for 2 minutes and spun at 850 x g for 10 minutes. The bottom organic layer was transferred to a 7 ml screw top vial, and from this 0.5 ml was transferred to a disposable culture tube and dried in a 40°C water bath under a stream of N_2. The dried extract was reconstituted in 0.2 ml of hexane, mixed on a vortex and spun at 850 x g for 10 minutes. The extract was transferred to a crimp top HPLC vial fitted with a 300 µl polyspring insert, and 25 µl was injected. Samples were analyzed at room temperature with a Varian Pro Star Autosampler 410 and Pump 210 (Walnut Creek, California),a SUPELCOSIL LC-Diol column (5 um x 4.6 mm x 250 mm) from Sigma (Belleforte, PA) and a Supelcosil LC-DIOL guard column. Alpha-tocopherol was detected on a Shimadzu RF-535 Fluorescence HPLC Monitor with the excitation wavelength set at 296 nm and the emittance wavelength set at 330 nm. The solvent was 99.4% hexane and 0.6% isopropanol, at a flow rate of 1 ml/min. Tocopherol isomers were determined by comparing retention times to standards (α, β and ϒ, and δ tocopherols) obtained from Sigma. Peaks were collected and integrated using Varian Galaxy Software.

3.2.5 Statistical Analysis

Individual hens or chicks were considered experimental units. Data were analyzed using Proc Mixed of SAS 9.2. Production data were analyzed as 2-way ANOVA and immune function and chick antioxidant capacity data were analyzed as a 3-way ANOVA with hen age, hen treatment and chick age as the main effects. Differences were determined using PDIFF of LSMeans and were considered significant at $p \leq 0.05$ (Steel and Torrie, 1960). Production data which are typically expressed as a percentage were $\log_{10}$ transformed to meet the requirements for ANOVA analysis, but were presented as a percentage.

3.3 RESULTS AND DISCUSSION

3.3.1 Hen and Egg Production Traits

Hen body weight (Table 3.2) increased as hens aged, but was consistently about 8% lower than the target body weight (Aviagen, 2007) at each hen age observed, and hens supplemented with NVE had lower body weights than hens supplemented with SVE. Production of hen housed settable eggs (Table 3.2) decreased as hens aged, but among Early and Late hens was 7 and 8% greater than target, respectively. Mid hen production met target settable egg production (Aviagen, 2007). Settable egg production was lower in hens supplemented with NVE than in hens supplemented with SVE, but total egg numbers and total settable egg numbers were not different between the two treatment groups (Table 3.2). It is accepted that hen egg production decreases as hens age (Tůmová et al., 2012; Gibson et al., 2008) and has been attributed to photorefractoriness (Lewis et al., 2003). Why NVE would decrease settable egg production relative to SVE is

unknown. Egg weight increased as hens aged (Table 3.3), as has often been reported (Tůmová et al., 2012; Ulmer-Franco et al., 2010; Lillpers and Wilhemson, 1993; Garlich et al., 1984), and was not affected by treatment. Yolk, albumen and shell weights were all greatest in eggs from Late hens and lowest in eggs from Early hens (Table 3.3), but were unaffected by treatment. An effect of VE form on egg composition has not been reported previously, but it has been reported frequently that egg weight and yolk increase with hen age (Tůmová and Gous, 2012: Ulmer-Franco et al., 2010). Proportions of yolk, albumen, and wet shell weights were not affected by hen age or treatment (Table 3.3), however published data report that the proportion of albumen decreased and yolk proportion increased in eggs as broiler breeders aged (Ulmer-Franco et al., 2010).

Alpha-tocopherol content of eggs from SVE hens decreased as hens aged, while decreasing from Early to Mid hen ages and then remaining unchanged from Mid to Late hen ages in eggs from NVE hens (Table 3.3). These observations have not been documented in the literature and are difficult to explain. One possible reason may be the preference for NVE by α-tocopherol transport protein (**α-TTP**) which is found in mammals. Of the eight distinct ATOC stereoisomers, only RRR-α-tocopherol is found in nature and is preferentially retained in tissues; however α-TTP will also bind the isomers configured so that the 2nd carbon is in the R configuration; so RRR, RRS, RSS, and RSR are retained by the body (Weiser et al., 1996). Although support for the existence of α-TTP in chickens could not be found in the literature, there are data which suggest the existence of a discriminatory process with regards to ATOC in chickens (Cortinas et al., 2004).

Diets in this study were formulated to provide the hens with the same VE activity (54 IU/kg feed, although analyzed levels were 82 and 84 IU/kg for the SVE and NVE diets respectively), so theoretically there should have been equal amounts of 2-R stereoisomers present in the diet. However in lactating sows and suckling piglets natural sources of RRR-α-tocopherol are preferentially deposited into tissues compared to synthetic sources of RRR-α-tocopherol (Lauridsen et al., 2002). This does not explain the relatively sharp decline in ATOC content of eggs from SVE hens, but a similar decrease has been observed in the serum of dogs as they aged from six to ten years (Stowe et al., 2006). Why egg SVE would decrease as hens age has not been determined. Intestinal absorption of ATOC increased as rats aged from four to 24 months of age (Hollander and Dadufalza, 1989). If intestinal absorption of ATOC also increases as hens age it is possible that SVE stereoisomers are metabolised more quickly, or perhaps there is a shift in tissue deposition of SVE as hens age.

There was neither a treatment nor a hen age effect on hen AOC (Table 3.4). The lack of effect could be explained by the timing of sample collection. Samples were always collected in the morning prior to feeding the hens, and as the hens were fed only once per day the samples reflected a fasting state, and plasma levels of dietary antioxidants would therefore be at their lowest (Di Simplicio et al., 1997). However, Brenes et al., (2008) did not observe an increase in serum antioxidant activity when the diet was supplemented with 200 mg/kg of α-tocopheryl acetate and fed *ad libitum* to broilers, indicating that the lack of treatment effects observed in the current research may not be the result of a

fasting plasma sample. The same observation was made by Lin et al. (2005), who found that supplementing the diet with 40 mg SVE/kg (59.6 IU/kg) did not affect plasma antioxidant capacity in Native Tawainese chicks relative to birds that were not supplemented with SVE. They observed increased antioxidant capacity when supplemental SVE was 80 mg/kg, indicating that the level of dietary supplementation (SVE: 73.82 mg/kg = 82.02 IU/kg diet; and NVE: 56.36 mg/kg = 84.12 IU/kg diet) in the current study may have been below the threshold for increased antioxidant capacity. The effect of NVE, on AOC in broiler breeder hens has not yet been reported in the literature, nor has a comparison of the effects of NVE vs. SVE on hen AOC been reported.

3.3.2 Hatch Data

Dietary VE did not affect hatchability (Table 3.5), nor was the hatchability of eggs from White Leghorn hens supplemented with 6.6, 66 or 132 mg of SVE affected (Tengerdy and Nockels, 1973). However, in turkeys embryonic injection of a water-soluble form of NVE (d-α-tocopheryl polyethylene glycol-1000 succinate) at levels greater than 20 IU resulted in reduced hatchability (Gore and Qureshi, 1997). In that study maternal VE deposited in the egg was unaccounted for. Hatchability was lowest in eggs from Late hens which was expected as hatchability decreases as hens age (Fasenko et al., 1992; Yassin et al., 2008). Male chick hatch weight was nearly affected by a hen age by hen treatment interaction ($p = 0.0547$). It increased linearly as NVE hens aged, while peaking in chicks from Mid SVE hens relative to Early and Late SVE hens. There were no treatment effects observed for female chick hatch weight (Table 3.5), and as

expected chick hatch weight significantly increased as hens aged regardless of chick sex, similarly to data reported in the literature (Ulmer-Franco et al., 2010; McNaughton et al., 1978).

3.3.3 Innate Immune Function Indices

As hens aged chick EBC decreased, but in chicks hatched from Mid hens those hatched from NVE hens had nearly greater EBC ($p = 0.0548$; Table 3.6) than chicks hatched from SVE hens, which is interesting because the ATOC content of eggs from hens of this age was not different. The ATOC assay did not differentiate between stereoisomers of ATOC but it has been reported that synthetic stereoisomers of ATOC are deposited into the eggs of hens supplemented with SVE (Piironen et al., 1991). If present in the egg, these synthetic stereoisomers of ATOC may have been less well utilized or absorbed by the chick embryo, and therefore not as potent when cells of the chick's immune system were challenged with *E. coli ex vivo*. To the author's knowledge there are no published data describing the effect of VE on the development of the chick's immune system *in ovo.*

Ex vivo E. coli bactericidal capacity increased as chicks aged, but decreased as hens aged (Table 3.6). However it decreased to a greater extent in day one chicks than in day four chicks suggesting that as the chick's immune system matures it is less affected by hen age. Decreased efficacy of innate immune function in chicks as hens aged has not been reported before in the literature (although it has consistently been observed by our research group, see Chapters 2, 4, and 5) and has been reported in turkeys. The inflammatory

response to LPS of turkey poults decreased as turkey hens aged (33 wks vs. 55 wks;Schaefer et al., 2006), although the authors were not able to determine the cause. The decrease in EBC in chicks as hens aged is difficult to explain and may be related to incubator conditions. Eggs were incubated under the same incubation conditions at each of the three hen ages, and it is possible that incubator conditions played a role in the development of the chicks' immune function as decreased incubation temperature reduced the innate immune response in tree swallow nestlings (*Tachycineta bicolor*; Ardia et al., 2009). Larger eggs produce larger chicks (Ulmer-Franco et al., 2010), and larger chicks produce more metabolic heat (Lourens et al., 2006) which would affect the environmental conditions in an incubator. It is possible that there is an incubation temperature range that optimizes the development of the chick's immune system. Eggs from Early hens in the current study could have experienced an incubator temperature more conducive to the development of the immune system. While to achieve the optimal incubation temperature for immune development of the larger eggs from older hens the incubator temperature would have to be adjusted, most likely downwards. *E. coli* bactericidal capacity increased from day one to day four in chicks, likely indicating the maturation of the chicks' immune system as the gut associated immune system has shown significant increases in pro-inflammatory cytokines from day one to day four (Bar-Shira et al., 2003).

Phagocytic capacity and activation data for day one chicks from Early hens were lost. Treatment affected neither phagocytic capacity (the number of bacterial cells internalized by phagocytes) nor phagocytic activation (the number

of active phagocytes; Table 3.6); this could be a contributing reason for so few treatment effects on EBC. There was a nearly significant interaction (p=0.0610) of hen age and chick age on *ex vivo* phagocytic capacity. Without the day one data it is difficult to fully interpret it, but the decrease in phagocytic capacity as hens aged from Mid to Late was greater for day four chicks than day one chicks. Similarly to phagocytic capacity, phagocytic activation decreased as hens aged from Mid to Late (Table 3.6). Supplemental SVE (greater than 110 mg VE/kg diet) increased the phagocytosis of opsonized SRBC in three week old broilers (capacity), but did not increase the yield (activation) of macrophages (Konjufca et al., 2004). Data reported on the effects of VE on chick immune function focus on chicks that are older than one week (Niu et al., 2009; Leshchinsky and Klasing, 2001; Friedman et al., 1998) giving the immune function in those studies a period of time to mature; conversely, the chicks in the current research were one or four days of age. A comparison of the effect of maternal SVE vs. NVE on phagocyte capacity and activation in broiler chicks has not been made prior to this research. The increase in EBC from one to four days of chick age, in conjunction with the increase in phagocyte activation during the same time indicates an increase in maturity of the immune system.

Synthetic VE nearly increased OB in day one chicks (Table 3.7; p = 0.0557). Although not reported in chicks less than a week old, *in ovo* injection of 10 IU water soluble NVE resulted in increased nitric oxide production by four week old chick macrophages challenged with LPS *in vitro* (Gore and Qureshi, 1997). Furthermore, it has been demonstrated in day 21 Cobb 500 broiler

chickens that supplementing SVE at levels greater than NRC recommendations resulted in enhanced OB of heterophils against *Eimeria* challenge (Perez-Carbajal et al., 2010). Day one oxidative burst was affected by hen age and OB progression (minutes; Table 3.7). The magnitude of OB was highest initially, and increased over the course of the assay to the greatest extent in chicks from Mid hens, while the magnitude of OB in chicks from Early hens increased slightly from 0 to 20 minutes and did not change in chicks from Late hens. Natural VE increased OB in day four chicks from Mid and Late hens, but not in day four chicks from Early hens (Table 3.7). Increased OB in chicks may reflect the ability of vitamin E to maintain cell membrane integrity (Howard et al., 2011), allowing the oxidative enzymes NADPH oxidase and nitric oxide synthase to maintain their function. In consideration of the reported data and the current data it is possible that exposure to NVE *in ovo* increases the bactericidal abilities heterophils in post-hatch chicks as measured by EBC and day four OB.

3.3.4 Chick Growth Trial

Maternal treatment did not affect chick body weight at any chick age (Table 3.8), nor chick average daily gain (**ADG**; Table 3.8), but high levels of SVE supplementation to rats (Moriguchi et al,. 1990) and broilers (Niu et al., 2009) also did not affect ADG. Day zero chicks hatched from Late hens were heavier than chicks from Early hens and day seven chicks from Late hens were nearly heavier than chicks from Early hens ($p = 0.0570$) as is generally expected and has been shown repeatedly (Nangsuay et al., 2011; and Ulmer-Franco et al.,

2010). Chick week one ADG increased as hens aged from Early to Mid and then did not change from Mid to Late hens (Table 3.8).

Livers from day seven chicks from Early hens were not collected. Maternal NVE decreased day 21 liver weight in chicks from Mid hens, but not in chicks from Early or Late hens (Table 3.9). In previous studies supplemental SVE did not affect liver weight (Brenes et al., 2008), so it is likely that vitamin E supplementation did not affect liver weight and that the effect that was observed was due to an unusually large liver in the SVE sample set. Liver weight at day of hatch increased as hens aged reflecting the increased body weight of chicks as hens aged.

A hen age by treatment by chick age interaction affected chick AOC (Figure 3.1). As chicks from Early hens aged chick AOC increased, while in chicks from Mid hens chick AOC was greatest at day 14 and in chicks from Late hens chicks from SVE hens had the greatest AOC at day 14 and AOC of chicks from NVE hens decreased as chicks aged. Among chicks from Early hens the AOC increased more dramatically in chicks from NVE hens then in chicks from SVE hens. Hen treatment did not affect chick AOC in chicks from Mid hens and in chicks from Late hens AOC of chicks from NVE hens decreased more dramatically than in chicks from SVE hens. Chicks were not subjected to further dietary treatment so the fluctuation in chick AOC as they aged may reflect fluctuations in antioxidant enzyme activities. Surai (1999) reported that during embryonic development the activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase fluctuate greatly by day and tissue.

It is possible that as a chick matures the activity levels of antioxidant enzymes continue to fluctuate. This should be considered in future investigations in chick AOC.

Perhaps the most interesting observation is that chick AOC on day of hatch did not reflect egg levels of ATOC. Where egg levels of ATOC in eggs from SVE hens decreased as hens aged, day zero AOC of chicks from SVE hens did not change as hens aged. Conversely, where ATOC egg levels of eggs from NVE hens remained relatively constant as hens aged, chick day zero AOC did not change in chicks from Early or Mid hens, but increased dramatically in chicks from Late hens. This is a perplexing observation. In hindsight, measurements of specific antioxidant enzyme activity and yolk sac and liver ATOC content may have been beneficial. Measuring yolk sac and liver levels of ATOC would have determined ATOC not yet absorbed but available, and ATOC absorbed respectively. It would have been interesting to determine if those two measurements reflected yolk levels of ATOC or the chick AOC.

3.4 CONCLUSIONS

Egg ATOC content from SVE hens decreased as hens aged, while ATOC content of eggs from NVE hens remained relatively constant, affecting the availability of VE to embryos during development and subsequent chicks. However, despite the differences in VE availability, chick phagocytic capacity and phagocyte activation were unaffected. However, hen supplemented NVE nearly increased *ex vivo E. coli* bactericidal capacity and did increase the oxidative burst of day four chicks. Indicating that NVE exposure *in ovo* may

increase chick early innate immune function. Despite the differential deposition of SVE vs. NVE into eggs, maternal VE form did not differentially affect chick plasma total antioxidant capacity.

Hen age affected chick innate immune indices more prominently than hen VE. As hens aged chick innate immune function as measured by *E. coli* bactericidal capacity decreased; and increased as chicks aged, the latter most likely indicating maturation of the chicks' immune system. The results of the current study do not offer an explanation for the effect of hen age on indices of chick early innate immune function and the effect of hen age warrants further investigation.

In this study, relatively high levels of VE were supplemented to hens, much greater than what is required for maximizing growth as determined by the NRC (1994). Vitamin E form did affect indices of innate immune function, but inconsistently and did not affect chick performance. Although it is common to supplement chicken diets with a relatively high level of VE (40 IU/kg diet), the high level of supplementation (approximately 80 IU/kg diet as measured), may have masked maternal treatment effects observed on chick innate immune function indices, and it is possible that at lower levels an effect of VE form would have been observed.

Table 3.1. Ingredients and calculated nutrient content of vitamin E diets

Ingredient	%
Calculated Inclusion	
Corn	36.6
Soybean meal	15.8
Wheat	34.1
Calcium carbonate	7.8
Dicalcium phosphate	1.44
Salt	0.4
L-lysine HCl	0.04
Methionine hydroxy analogue, 95%[1]	0.2
Vitamin E premixes[2]	
Inorganic Trace Mineral Premix[3]	0.1
Canola oil	0.5
Avizyme 1302	0.05
Broiler Breeder premix[4]	1.0
Calculated Nutrient Profile	
ME (kcal/kg)	2,882
CP (%)	15
Calcium (%)	3
Available P (%)	0.37
Lysine (%)	0.73
Vitamin E[5] (IU/kg)	54
Sodium (%)	0.19

[1] Novus International, Inc.

[2] Vitamin E supplemented as Vitamin E 5000 IU/kg in the control diet at 0.8% (40 IU/kg diet), and as RRR-α-tocopherol (Vitamin E 405 from ADM) included at 0.0099% (40.1 IU/kg)

[3] Inorganic trace mineral premix supplied the following per kg of feed: $CuSO_4 \cdot 5H_2O$, 40 mg; $MnSO_4$, 380 mg; $ZnSO_4$, 295 mg

[4]Vitamin/Mineral premix supplied the following per kg of feed: Vitamin A, 12,500 IU; Vitamin D_3, 3,100 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; choline, 2.7 mg; iron, 0.0 mg; zinc, 100 mg; manganese, 87 mg; copper, 15 mg; iodine, 1.665 mg; selenium, 0.3 mg

[5]Analyzed vitamin E in feed: Synthetic vitamin E (SVE): 73.82 mg/kg (82.02 IU/kg) diet; and Natural vitamin E (NVE): 56.36 mg/kg (84.12 IU/kg) diet

Conversion factor: SVE mg/0.9 IU; NVE mg/0.67 IU (Dietary Supplement Ingredient Database – USDA, 2009)

Table 3.2. Effect of hen age and maternal vitamin E on hen production parameters

		Body Weight	Hen Housed Settable Eggs[4]	Total Eggs	Total Settable Eggs
Hen Age[1]	Treatment[2]	Kg	%[5]		
Early		3.18[c]	91.54[a]		
Mid		3.39[b]	72.29[b]		
Late		3.57[a]	58.85[c]		
SEM		0.032	0.063		
	SVE	3.40[a]	76.69[a]	187	184
	NVE	3.36[b]	71.76[b]	177	175
SEM		0.026	0.052	14.12	14.01
Interaction					
Hen Age * Treatment[3]					
Early	SVE	3.20	95.36		
Early	NVE	3.15	87.72		
Mid	SVE	3.42	74.9		
Mid	NVE	3.37	69.67		
Late	SVE	3.58	59.8		
Late	NVE	3.57	57.89		
SEM		0.045	0.089		
P-Values					
Age		<0.0001	<0.0001		
Treatment		0.0112	0.0001	0.1945	0.1945
Hen Age*Treatment		0.3881	0.5617		

[abc] Means with differing letters are different ($p \leq 0.05$) within effect

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Synthetic vitamin E (SVE) diet contained 54 IU/kg supplemented SVE, Natural vitamin E (NVE) diet contained 40 IU/kg supplemented NVE with no SVE supplementation

[3] n = 45 per treatment

[4] Settable eggs were eggs which had a single yolk, and were not misshapen nor cracked

[5] Data log transformed to meet ANOVA assumptions, but presented as percentage

Table 3.3. Effect of hen age and maternal vitamin E on egg traits

		Egg Weight	Yolk Weight	Proportion Yolk[4]	Albumen Weight	Proportion Albumen[4]	Shell Weight[5]	Proportion Wet Shell[4,5]	α-Tocopherol
		g	g	%	g	%	g	%	μg/g yolk
Hen Age[1]	Treatment[2]								
Early		57.54[b]	17.04[c]	29.36[b]	32.74[b]	56.89	8.07[b]	14.00	176.19
Mid		66.37[a]	19.96[b]	29.71[ab]	36.58[a]	55.14	10.23[ab]	15.46	131.98
Late		68.05[a]	21.28[a]	30.86[a]	36.27[a]	53.52	10.97[a]	15.99	115.78
SEM		2.38	1.05	0.020	2.22	0.034	2.35	0.052	16.86
	SVE	64.01	19.44	29.94	34.72	54.48	10.30	15.93	141.67
	NVE	63.96	19.41	30.01	35.68	55.88	9.22	14.38	140.97
SEM		1.94	0.86	0.016	1.81	0.028	1.92	0.043	13.77
Interaction									
Hen Age * Treatment[3]									
Early	SVE	58.39	17.31	29.34	32.71	56.03	8.37	14.88	198.58[a]
Early	NVE	56.68	16.78	29.38	32.77	57.75	7.14	13.11	153.81[b]
Mid	SVE	65.53	19.40	29.18	36.88	56.23	9.25	14.98	135.47[bc]
Mid	NVE	67.22	20.52	30.24	36.29	54.04	10.41	15.94	128.50[c]
Late	SVE	68.12	21.63	31.31	34.56	51.19	11.93	17.91	90.97[d]
Late	NVE	67.97	20.92	30.41	37.98	55.86	9.07	14.07	140.60[bc]
SEM		3.36	1.48	0.028	3.14	0.048	3.31	0.074	23.85
P-Values									
Age		<0.0001	<0.0001	0.0873	0.0014	0.1571	0.0453	0.1866	<0.0001
Treatment		0.9545	0.9296	0.7926	0.2928	0.2623	0.2614	0.1462	0.9172
Age * Treatment		0.3619	0.1638	0.3643	0.1391	0.1445	0.2577	0.1226	0.0001

[a,b,c,d] Means within column with differing letters are different ($p \leq 0.05$)

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Synthetic vitamin E (SVE) diet contained 40 IU/kg supplemented SVE, Natural vitamin E (NVE) diet contained 40 IU/kg supplemented NVE with no SVE supplementation

[3] n = 10 eggs per treatment

[4] Values analyzed as $\log_{10}$ to meet ANOVA assumptions, but presented as percentage

[5] Shell weights include shell membrane

Table 3.4. Effect of hen age and maternal vitamin E on hen total plasma antioxidant capacity

		Antioxidant Capacity (mM Trolox Equivalents)
Hen Age[1]	Treatment[2]	
Photostimulation		2.58
Early		2.30
Mid		2.32
Late		2.02
SEM		0.67
	SVE	2.39
	NVE	2.22
SEM		0.48
Interaction		
Hen Age * Treatment[3]		
Photostimulation	SVE	2.38
Photostimulation	NVE	2.78
Early	SVE	2.49
Early	NVE	2.11
Mid	SVE	2.53
Mid	NVE	2.12
Late	SVE	2.17
Late	NVE	1.86
SEM		0.95
P-Values		
Age		0.4177
Treatment		0.4692
Age * Treatment		0.5644

[1] Hen ages are defined as production stages. Photostimulation = 23 wks; Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] SVE diet contained 54 IU/kg supplemented SVE, NVE diet contained 54 IU/kg supplemented NVE with no SVE supplementation

[3] n = 10 hens per treatment

Table 3.5. Effect of maternal vitamin E and hen age on hatchability and chick hatch weight

		Hatchability[4]	Male Hatch Weight	Female Hatch Weight
Hen Age[1]	Treatment[2]	%	g	g
Early		79.97[a]	40.75[c]	40.21[c]
Mid		78.52[a]	45.46[b]	45.21[b]
Late		71.77[b]	48.30[a]	47.64[a]
SEM		0.043	0.88	0.63
	SVE	74.63	44.64	44.18
	NVE	78.87	45.04	44.53
SEM		0.035	0.74	0.52
Interaction				
Hen Age * Treatment[3]				
Early	SVE	77.35	40.36	40.02
Early	NVE	82.60	41.14	40.40
Mid	SVE	76.64	45.74	44.88
Mid	NVE	80.39	45.18	45.55
Late	SVE	69.90	47.82	47.64
Late	NVE	73.63	48.78	47.65
SEM		0.060	1.25	0.89
P-Values				
Hen Age		0.0169	<0.0001	<0.0001
Treatment		0.0634	0.1621	0.1767
Hen Age * Treatment		0.9422	0.0547	0.5953

[a,b,c,d] Means within column with differing letters are different ($p \leq 0.05$) within treatment

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Synthetic vitamin E (SVE) diet contained 40 IU/kg supplemented SVE, Natural vitamin E (NVE) diet contained 40 IU/kg supplemented NVE with no SVE supplementation

[3] n = 50 chicks per treatment

[4] Data $\log_{10}$ transformed to meet ANOVA assumptions, but presented as percentage

Table 3.6. Effect of hen age and maternal vitamin E on *ex vivo E. coli* bactericidal and phagocytic capacities of chicks

			E. coli Bactericidal Capacity[4]	Phagocytic Capacity[5,6]	Phagocyte Activation[5,7]
Hen Age[1]	Treatment[2]	Chick Age	%	Mean Fluorescent Unit[8]	
Early			56.60	.	.
Mid			24.68	153.84[a]	41.51[a]
Late			10.25	121.36[b]	27.38[b]
SEM			0.18	9.37	3.19
	SVE		30.06	.	.
	NVE		31.06	.	.
SEM			0.15	.	.
		Day 1	21.58	.	.
		Day 4	39.44	121.28	34.61
SEM			0.15	.	.
Interactions					
Hen Age * Treatment					
Early	SVE		57.44	.	.
Early	NVE		55.76	.	.
Mid	SVE		20.09	160.41	41.89
Mid	NVE		29.27	147.28	41.12
Late	SVE		12.63	118.84	28.44
Late	NVE		7.86	123.89	26.33
SEM			0.25	13.23	4.50
Hen Age * Chick Age					
Early		Day 1	43.54[b]	.	.
Early		Day 4	69.66[a]	99.37	32.49
Mid		Day 1	17.74[c]	154.74	38.93
Mid		Day 4	31.62[b]	152.95	44.09
Late		Day 1	3.46[d]	131.21	27.51
Late		Day 4	17.03[c]	111.52	27.25
SEM			0.25	14.28	4.86
Treatment * Chick Age					
	SVE	Day 1	20.47	.	.
	SVE	Day 4	39.64	121.96	36.66
	NVE	Day 1	22.69	.	.
	NVE	Day 4	39.24	120.60	32.57
SEM			0.21	11.81	4.02
Hen Age * Treatment * Chick Age[3]					
Early	SVE	Day 1	45.15	.	.
Early	SVE	Day 4	69.73	99.52	33.62
Early	NVE	Day 1	41.93	.	.
Early	NVE	Day 4	69.59	99.22	31.36

Mid	SVE	Day 1	11.83	162.75	37.99
Mid	SVE	Day 4	28.34	158.07	45.80
Mid	NVE	Day 1	23.64	146.74	39.86
Mid	NVE	Day 4	34.90	147.82	42.39
Late	SVE	Day 1	4.44	129.40	26.31
Late	SVE	Day 4	20.83	108.28	30.56
Late	NVE	Day 1	2.49	133.02	28.70
Late	NVE	Day 4	13.22	114.76	23.95
SEM			0.36	20.16	6.86
P-Values					
Hen Age			<0.0001	<0.0001	<0.0001
Treatment			0.7693	.	.
Chick Age			<0.0001	.	.
Hen Age * Treatment			0.0548	0.1615	0.7814
Hen Age * Chick Age			0.0010	0.0610	0.0948
Treatment * Chick Age			0.4464	.	.
Hen Age * Treatment * Chick Age			0.9036	0.8782	0.5634

[abcd] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Synthetic vitamin E (SVE) diet contained 40 IU/kg supplemented SVE, natural vitamin E (NVE) diet contained 40 IU/kg supplemented NVE with no SVE supplementation

[3] n = 12 chicks per maternal treatment

[4]Data log transformed to meet ANOVA assumptions, but presented as %

[5] Data for D1 chicks from Early hens lost

[6]Phagocytic capacity is an indication of the number of *E. coli* bioparticles phagocytized by each cell.

[7] Phagocyte activation is an indication of the number of cells which contain at least one *E. coli* bioparticles

[8] Fluorescence of treated cells used to estimate phagocytic capacity and activation in comparison to fluorescence of untreated cells.

Table 3.7. Effect of hen age and maternal vitamin E on oxidative burst response in chicks

			Oxidative Burst	
			Mean Fluorescence Unit[5]	
			Chick Age	
Hen Age[1,3]	Treatment[2,3]	OB Time	Day 1	Day 4
Early			13.48[b]	3.4
Mid			16.91[a]	3.51
Late			11.35[c]	2.75
SEM			1.08	0.86
	SVE		14.32	2.63[b]
	NVE		13.51	3.82[a]
SEM			0.88	0.70
		5	12.36[b]	1.98[b]
		10	13.24[b]	2.88[b]
		15	14.56[a]	3.90[a]
		20	15.49[a]	4.15[a]
Interactions			1.25	0.99
Hen Age * Treatment				
Early	SVE		13.57	3.52[ab]
Early	NVE		13.39	3.32[ab]
Mid	SVE		17.78	2.70[bc]
Mid	NVE		16.04	4.33[a]
Late	SVE		11.60	1.68[c]
Late	NVE		11.10	3.82[ab]
SEM			1.53	1.22
Hen Age * OB Time				
Early		5	12.92[efg]	2.15
Early		10	13.12[df]	2.52
Early		15	13.71[cde]	3.92
Early		20	14.17[cd]	5.08
Mid		5	13.26[de]	1.96
Mid		10	15.46[c]	3.69
Mid		15	18.32[b]	4.56
Mid		20	20.59[a]	3.85
Late		5	10.92[g]	1.82
Late		10	11.15[fg]	2.43
Late		15	11.64[efg]	3.22
Late		20	11.70[efg]	3.53
SEM			2.16	1.72
Treatment * OB Time				
	SVE	5	12.49	1.73
	SVE	10	13.56	2.29
	SVE	15	15.04	3.04
	SVE	20	16.18	3.46
	NVE	5	12.24	2.22
	NVE	10	12.92	3.46
	NVE	15	14.08	4.76
	NVE	20	14.79	4.84
SEM			1.77	1.40
Hen Age * Treatment * OB Time[4]				
Early	SVE	5	12.92	2.36
Early	SVE	10	13.10	2.52
Early	SVE	15	13.76	4.05
Early	SVE	20	14.50	5.14

Early	NVE	5	12.91	1.94
Early	NVE	10	13.13	2.52
Early	NVE	15	13.66	3.79
Early	NVE	20	13.84	5.02
Mid	SVE	5	13.38	1.65
Mid	SVE	10	16.25	2.68
Mid	SVE	15	19.39	3.33
Mid	SVE	20	22.10	3.14
Mid	NVE	5	13.15	2.27
Mid	NVE	10	16.68	4.69
Mid	NVE	15	17.24	5.79
Mid	NVE	20	19.09	4.55
Late	SVE	5	11.18	1.18
Late	SVE	10	11.33	1.69
Late	SVE	15	11.96	1.74
Late	SVE	20	11.93	2.10
Late	NVE	5	10.65	2.45
Late	NVE	10	20.96	3.18
Late	NVE	15	11.32	4.70
Late	NVE	20	11.46	4.95
SEM			3.05	2.43
P-Values				
Hen Age			<0.0001	0.1652
Treatment			0.0557	0.0010
OB Time			<0.0001	<0.0001
Hen Age * Treatment			0.2724	0.0219
Hen Age * OB Time			<0.0001	0.5608
Treatment * OB Time			0.8107	0.6678
Hen Age * Treatment * OB Time			0.9746	0.9741

[abcdefg] Means within a main effect or interaction within a column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Synthetic vitamin E (SVE) diet contained 40 IU/kg supplemented SVE, natural vitamin E (NVE) diet contained 40 IU/kg supplemented NVE with no SVE supplementation

[3] Values reported are average OB over length of assay

[4] n = 12 chicks per maternal treatment

[5] Oxidative products quantified via fluorescence intensity

Table 3.8. Effect of hen age and maternal vitamin E on weekly chick body weight and gain

		Body Weight				Average Daily Gain		
		g				g/chick/day		
Hen Age[1]	Treatment[2]	D0	D7	D14	D21	Week 1	Week 2	Week 3
Early		41.06[c]	124.82[b]	331.87	707.78	11.83[b]	29.06	52.87
Mid		46.23[b]	142.34[a]	385.45	758.55	13.66[a]	35.43	54.16
Late		48.70[a]	151.81[a]	376.61	804.72	14.59[a]	32.24	60.26
SEM		1.37	12.38	46.12	115.78	1.69	5.43	8.20
	SVE	45.27	139.76	366.37	754.92	13.42	32.29	56.94
	NVE	45.40	139.56	362.92	759.11	13.31	32.19	54.59
SEM		1.12	10.11	37.65	95.54	1.38	4.43	6.69
Interactions								
Hen Age * Treatment[3]								
Early	SVE	40.75	122.32	323.09	696.23	11.53	27.64	53.54
Early	NVE	41.37	127.33	340.65	771.12	12.14	30.49	52.19
Mid	SVE	45.94	144.04	398.10	797.41	14.02	36.71	57.29
Mid	NVE	46.52	140.64	372.80	719.32	13.31	34.15	51.03
Late	SVE	49.11	152.91	377.90	745.97	14.70	32.54	59.98
Late	NVE	48.29	150.72	375.32	812.03	14.47	31.94	60.54
SEM		1.93	17.52	65.22	163.74	2.39	7.67	11.60
P-Values								
Hen Age		<0.0001	0.0018	0.0570	0.2274	0.0126	0.0739	0.1518
Treatment		0.8019	0.9670	0.8441	0.9241	0.8651	0.9614	0.4558
Hen Age * Treatment		0.4461	0.7281	0.6064	0.8882	0.6896	0.5560	0.6515

[abc] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Synthetic vitamin E (SVE) diet contained 40 IU/kg supplemented SVE, Natural vitamin E (NVE) diet contained 40 IU/kg supplemented NVE with no SVE supplementation

[3] n = 10 chicks per maternal treatment

Table 3.9. Effect of hen age and maternal vitamin E on weekly chick liver weight

Hen Age[1]	Treatment[2]	Liver Weight g			
		D0	D7[4]	D14	D21
Early		1.06[b]	.	12.41	21.09[b]
Mid		1.18[a]	5.98	13.79	24.23[a]
Late		1.21[a]	5.94	13.76	21.01[b]
SEM		0.078	0.67	1.42	2.13
	SVE	1.13	5.95	13.09	22.70
	NVE	1.17	5.97	13.55	21.52
SEM		0.064	0.67	1.62	1.74
Interaction					
Hen Age * Treatment[3]					
Early	SVE	1.0	.	11.74	21.30
Early	NVE	1.08	.	13.08	20.88
Mid	SVE	1.16	5.98	13.83	26.11
Mid	NVE	1.20	5.97	13.76	22.34
Late	SVE	1.18	5.91	13.71	20.68
Late	NVE	1.24	5.96	13.82	21.34
SEM		0.11	0.95	2.01	3.02
P-Values					
Hen Age		0.0006	0.9042	0.1022	0.0042
Treatment		0.1343	0.9520	0.4321	0.1810
Hen Age * Treatment		0.9371	0.9281	0.5749	0.0968

[ab] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Synthetic vitamin E (SVE) diet contained 40 IU/kg supplemented SVE, Natural vitamin E (NVE) diet contained 40 IU/kg supplemented NVE with no SVE supplementation

[3] n = 10 chicks per maternal treatment

[4] Livers were not collected from d7 chicks from Early hens

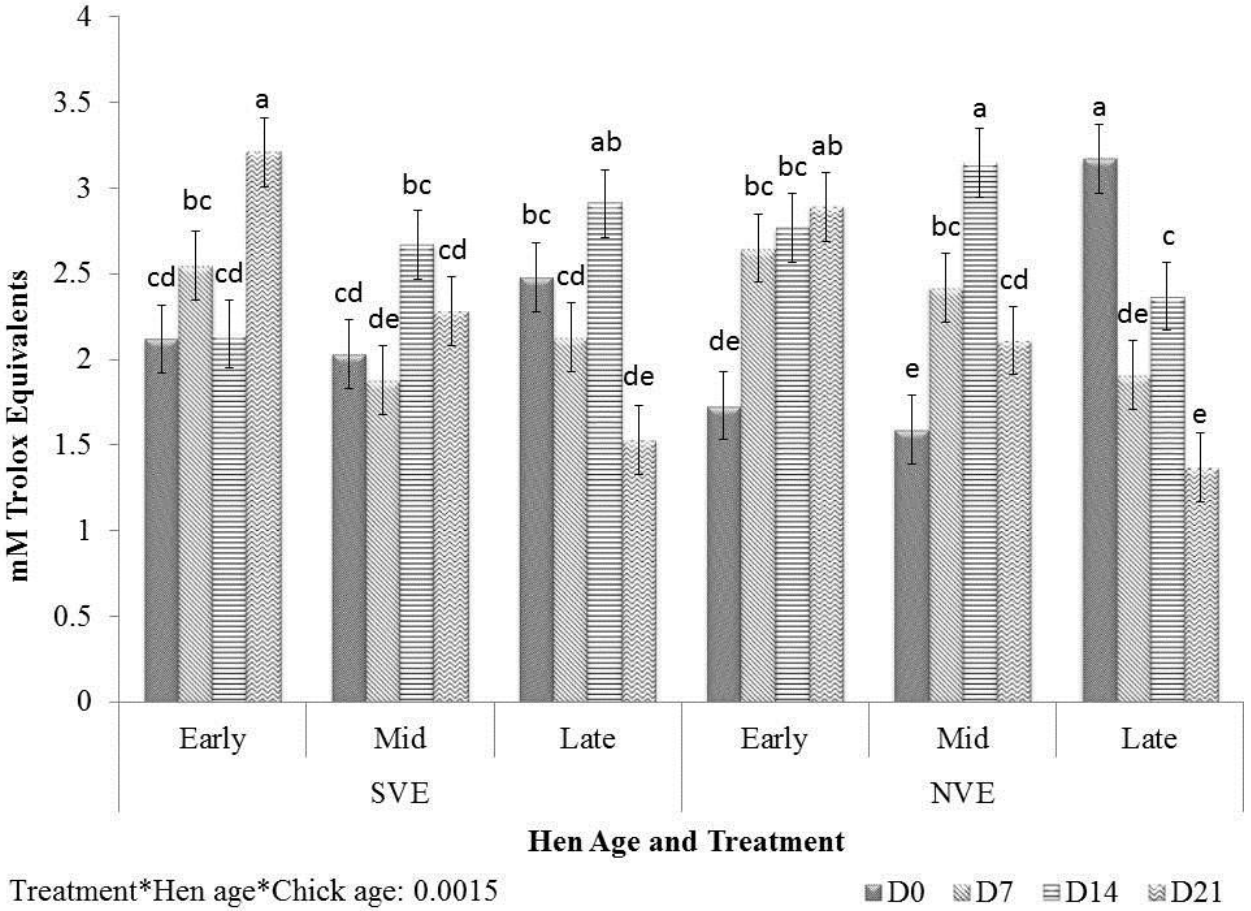

Figure 3.1. Effect of maternal vitamin E[1], hen age[2] and chick age on chick plasma total antioxidant capacity

[abcde] Indicate differences ($p \leq 0.05$) among bars.

[1] Hens supplemented with 54 IU/kg of either all-*rac*-α-tocopherol acetate or RRR-α-tocopherol acetate.

[2] Early: 33 wks; Mid: 47 wks; Late: 59 wks

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4. THE EFFECT OF MATERNALLY SUPPLEMENTED L-CARNITINE AND HEN AGE ON INDICES OF CHICK POST-HATCH INNATE IMMUNE FUNCTION

4.1 INTRODUCTION

L-carnitine (**LC**) is a quaternary ammonium compound involved in energy metabolism as a carrier of fatty acids into the matrix of mitochondria for β-oxidation (Cox and Hoppel, 1973). The primary site of LC synthesis in mammals is the liver from lysine and methionine (Tanphaichitr and Broquist, 1974), although it may also be obtained from the diet (Flanagan et al., 2010). Lysine and methionine are the first and second limiting ingredients in poultry diets (Dunkelgod and Winkelman, 1982), and are routinely supplemented into the diet making LC deficiency unlikely in poultry. However, in neonatal rats dietary LC is important for maintaining tissue LC levels (Flores et al., 1996), suggesting that neonatal LC synthesis is insufficient for optimal growth. Furthermore, when LC was insufficient, plasma free fatty acid content was increased in infants and resulted in increased excretion of medium chain fatty acids (Olson et al., 1989). The egg is a closed system in that the nutrients deposited into the egg by the hen are the only source of nutrition for the developing embryo. If the hen deposits insufficient amounts of LC into the egg, the chick's development *in ovo* could be impaired. If fatty acid metabolism in the embryo is decreased because of insufficient LC availability and valuable fatty acids are excreted rather than incorporated into tissues, the post hatch performance of the chick may be affected. This situation could be avoided by maternal supplementation of LC, which

increased LC deposition into eggs when supplemented to the hen at relatively high levels (125 mg/kg) (Zhai et al., 2008a). Hen age also may affect the fatty acid metabolism of the embryo. Chicks from 36 and 64 week old hens had greater residual yolk sac weights than chicks from 51 week old hens, suggesting that chicks from young and old hens were less able to mobilize fatty acids *in ovo* (Latour et al., 1998). This may be a contributing factor to the increased first week mortality observed in chicks from young and old hens relative to chicks from middle aged hens (Yassin et al., 2009). However, it is logical that immune function would be related to mortality and it is possible that the immune response of chicks from older hens is more developed than it is in chicks from young hens. In this regard, making LC more available to chicks *in ovo* may not only enhance the fatty acid metabolism of chicks, but may also positively affect the innate immune response because of its immuno-modulatory properties.

There is evidence that LC supplementation may result in immunomodulation. Hypertensive rats supplemented with LC at a rate of 4g/L water had reduced blood pressure and levels of inflammatory cytokines (Miguel-Carrasco et al., 2008). Additionally supplementation (50 mg/kg BW) of LC to aged rats (24 months old) resulted in restored neutrophil mobility (chemotaxis) and phagocytic ability to levels observed in 2 month old rats (Izgüt-Uysal et al., 2003). The mechanism by which this occurred is unknown, but it was hypothesized that the antioxidant effects of LC may have stabilized membrane fluidity which aided cellular motility (Izgüt-Uysal et al., 2003). L-carnitine could act as an antioxidant by chelating iron or as a free radical scavenger (Gülçin,

2006). Given the reports of LC to attenuate the production of pro-inflammatory cytokines, and therefore inflammation and symptoms of illness, it is possible that morbidity of post-hatch chicks may be reduced when they are hatched from LC-supplemented hens. The ability of supplemented LC to increase fertility and fatty acid metabolism in poultry (Peebles et al., 2007; Keralapurath et al., 2010; Kidd et al., 2005) and to mitigate the inflammatory response in humans (Abd-Allah et al., 2009; Fattorossi et al., 1993) and rats (Izgüt-Uysal et al., 2004; Flores et al., 1996) has been well documented, but the availability of literature for the effect of LC on the post-hatch innate immune response of chicks is limited. Furthermore, given the possibility that hen age may affect fatty acid metabolism *in ovo* it is possible that the development of the immune system is affected by hen age as well.

The objective of this study was to determine the effect of maternal LC and hen age on the post-hatch innate immune function as measured by *Escherichia coli* (***E. coli***) bactericidal capacity, phagocytic capacity and activation and oxidative burst *ex vivo* in broiler chicks. It was hypothesized that chick post-hatch innate immune function would increase as result of maternal LC supplementation and increasing hen age.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with the Canadian Council of Animal Care (1993) guide. Please refer to Chapter 2, Experiment 1 for animal rearing and experimental design. Hens in this experiment were divided into two treatments

(n=45) consisting of LC supplementation levels of 0 mg/kg or 50 mg/kg diet (Table 4.1). At each of the three hen ages (Early: 31 to 33 wks; Mid: 45 to 47 wks; and Late: 57 to 59 wks) observed, 10 eggs per treatment were collected; whole egg, yolk and albumen weights recorded.

4.2.2 Innate Immune Function Indices

Whole blood collected from the chicks at one and four days of age was used to determine *E. coli* bactericidal activity (**EBC**), to assess the number of cells containing at least one *E. coli* (phagocyte activation) and the average number of *E. coli*/cell (phagocytic capacity) and oxidative burst response (**OB**) *ex vivo*. Assay conditions were as described in Chapter 2, Experiment 1. Phagocyte data collected from d 1 chicks from Early hens were lost.

4.2.3 Plasma Total Antioxidant Capacity Assay

Plasma was collected from 10 hens per treatment at each hen age for determination of total plasma antioxidant capacity. Plasma was also collected from chicks from the third hatch of each hen age for determination of plasma total antioxidant capacity (**AOC**). Ten chicks per maternal treatment were sampled at 0, 7, 14 and 21 days of age. Assay conditions were as described in Chapter 2, Experiment 1.

4.2.4 Statistical Analysis

Individual hens or chicks were considered experimental units. Immune function data were analyzed as a 3-way ANOVA using Proc Mixed with hen age, chick age and treatment as the main effects; and the production data were analyzed as 2-way ANOVAs using mixed models of SAS 9.2 with hen age and

treatment as the main effects. Differences were determined using PDIFF of LSMeans and were considered significant at $P \leq 0.05$ (Steel and Torrie, 1960). Production data which are typically expressed as a percentage were $\log_{10}$ transformed for analysis, but are presented as a percentage.

4.3 RESULTS AND DISCUSSION

4.3.1 Hen and Egg Production Traits

L-carnitine reduced hen body weight (Table 4.2) relative to Control. There are no data describing the effects of LC supplementation on hen body weight in the literature; however 25 mg/kg dietary LC supplementation reduced abdominal fat in broilers (Kidd et al., 2005). This may have been the effect observed in the current research, although without carcass composition measurements this cannot be concluded. Hen body weight increased as hens aged regardless of treatment. This was expected according to the Ross 308 production guide (Aviagen, 2007), however, the average body weight at each of the hen ages observed was 9% lower than target weight. Concomitantly production of settable eggs (Table 4.2) decreased as hens aged; this is commonly observed among broiler breeders and turkeys and is thought to be due to photorefractoriness (Lewis et al., 2003; Siopes, 2005). In addition to reducing hen body weight, LC also reduced settable egg production (Table 4.2), but overall LC supplementation did not change the number of total or settable eggs laid (Table 4.2). L-Carnitine supplementation at 125 mg/kg (Zhai et al., 2008a) or 250 mg/kg (Rezaei et al., 2008) diet did not affect egg production in White Leghorns either.

Egg weight increased as hens aged (Table 4.3), which was expected and agrees with published literature (Ulmer-Franco et al., 2010; Lillpers and Wilhemson, 1993). L-carnitine supplementation did not affect egg component composition in the current research; however, when hens were supplemented with 125 mg LC/kg diet, albumen weight increased (Zhai et al., 2008a). Similarly eggs from hens injected *in ovo* with 2 μmol LC as embryos (Zhai et al., 2008b), as well as eggs from hens supplemented with 50, 100, or 500 mg LC/ kg of diet (Rabie et al., 1997) had increased albumen weights. The data reported by Zhai et al., (2008a,b) and Rabie et al., (1997) were collected from laying hens and the current data is from broiler breeder hens so LC supplementation may affect hen strains differently. As hens aged absolute weights of yolk, albumen and shell increased, but proportionally there were no differences in egg components (Table 4.3). The absolute weight of eggs, yolk, albumen and shell increased in laying hens to about 36 weeks of age and then the weights of egg components plateaued (Anderson et al., 1978), as was observed in the current research. However, the proportions of the yolk, albumen and shell remained unaffected by hen age in the current research while in other research the proportion of albumen decreased and yolk proportion increased in eggs from broiler breeders (Ulmer-Franco et al., 2010).

Hen AOC did not differ with age in Control hens, while dramatically decreasing from Early to Mid hen ages in hens supplemented with LC (Table 4.4). L-carnitine not only is capable of scavenging reactive oxygen species (**ROS**) (Gülçin, 2006), but it may also increase the expression of the antioxidant enzymes catalase and superoxide dismutase (Li et al., 2012). Given the dramatically greater

AOC of Photostimulation (23 weeks) and Early LC hens relative to Control hens, it is possible there was an upregulation of antioxidant enzymes in the LC hens. However, the activities of specific antioxidant enzymes were not measured in the current research, so this is strictly speculation. It is generally accepted that increased oxidative stress is associated with increasing age. The status and activities of some antioxidant enzymes (superoxide dismutase and catalase) decreased with age in rats (Valls et al., 2005). However, in the current research, the AOC of Control hens was unaffected by age. The AOC of LC hens was high at Photostimulation and in Early hens, but decreased to the same level as Control hens at Mid and Late ages. It appears that the antioxidative properties of LC decreased in hens as they aged.

4.3.2 Hatch Data

Hatchability was not affected by hen age (Table 4.5), and was low compared to published data (Molenaar et al., 2011; Lourens et al., 2006; Reis et al., 1997), but was increased by LC supplementation. Zhai et al. (2008a) did not find an effect of LC on hatchability when hens were supplemented with 125 mg LC/kg diet or from hens injected *in ovo* with 1 or 2 μmol LC; but Keralpurath et al., (2010) found that injecting LC at 49.6 μmol/egg *in ovo* delayed hatch and increased hatchability in broilers. L-carnitine is required for the β-oxidation of fatty acids (Friedman and Fraenkel, 1955), but chick may embryos have a limited capacity to synthesize it as was observed in neonatal rats (Flores et al., 1996). Supplementing hens with LC resulted in increased levels of LC in the yolk and also smaller yolk sacs in hatched chicks; it was theorized that the greater

availability of LC in the yolks of eggs from LC supplemented hens may have favoured fat utilization by developing embryos resulting in the smaller yolk sacs at hatch (Zhai et al., 2008a, b). Greater use of fat by embryos supplemented with LC, and therefore availability of energy, could have contributed to the greater hatchability observed in the current research when hens were supplemented with LC. This effect may have been exacerbated because of the low overall hatchability; however evidence for this possibility could not be found in the literature. Chick hatch weight increased as hens aged (Table 4.5). Maternal LC nearly increased male chick hatch weight ($p = 0.0545$) and increased female chick hatch weight. When Ross 308 broiler breeder hens were supplemented with 25 mg LC/kg diet there was no maternal treatment effect on chick hatch weight (Kidd et al., 2005), the difference in maternal treatment effects could be a reflection of maternal LC supplementation level.

4.3.3 Indices of Chick Innate Immune Function

Hen supplemented LC did not affect the EBC of chicks, but there was an interactive effect of hen age and chick age (Table 4.6). *Ex vivo E. coli* bactericidal capacity of chicks decreased more consistently among day four chicks than day one chicks as hens aged, (Table 4.6). Among day one chicks the EBC decreased substantially as hens aged from Early to Mid, but then was unchanged as hens aged from Mid to Late. The decrease in chick EBC as hens age has been observed multiple times by our research group (see chapters 2 and 3) and is difficult to explain. Decreased efficacy of innate immune function of chicks as hens age has not been reported before in the literature, but has been reported in turkey poults.

The inflammatory response of turkey poults, as measured by plasma haptoglobin response to *Salmonella* lipopolysaccharide (**LPS**) also decreased as turkey hens aged (33 wks vs 55 wks; Schaefer et al., 2006), although the inflammatory response was not measured in post-hatch poults, but rather in 66-d-old poults.

Incubator conditions could also have been a factor in chick immune function development. In the current research eggs were incubated under the same incubation conditions at each of the hen ages without consideration for egg size, which increased as hens aged, or the possibility that larger embryos might produce greater metabolic heat during incubation effectively altering the incubation temperature (Lourens et al., 2006). An eggshell temperature of 37.8°C resulted in the greatest hatchability (Joseph et al., 2006), although this may not necessarily be the temperature required to optimize immune function development *in ovo*. In tree swallow nestlings (*Tachycineta bicolor*) lowering the incubation temperature resulted in reduced ability of nestlings to kill *E. coli ex vivo* (Ardia et al., 2010). It is possible that the incubation temperature experienced by the embryos from Young hens was better suited for immune function development than the incubation temperature experienced by the embryos from the Mid and Late hens.

Phagocytosis data collected for day one chicks hatched from Early hens were lost, affecting the statistical analysis of the results. Phagocytic capacity was greatest in chicks hatched from Mid hens and decreased more dramatically among day four chicks as hens aged from Mid to Late than among day one chicks (Table 4.6). Although phagocytic capacity of day four chicks from Early hens did not

differ from that of day four chicks from Late hens, without the data from day one chicks from Early hens it cannot be determined if phagocytic capacity decreased as chicks aged in chicks from Early hens. There was a nearly significant interaction ($p = 0.0620$) between hen age and treatment on phagocytic capacity (Table 4.6) which indicated that LC supplementation had more of a stabilizing effect on phagocytic capacity in chicks as hens aged from Mid to Late, although without the data from the Early hen day one chicks, the interaction may not be interpreted accurately.

Ex vivo phagocyte activation was also affected by a hen age by chick age interaction (Table 4.6). Phagocyte activation increased as chicks from Mid hens aged, while not changing as chicks from Late hens aged. It is interesting that the number of active phagocytes increased while phagocytic capacity remained the same as chicks from Mid hens aged, but that phagocyte activation remained the same while phagocytic capacity of chicks from Late hens decreased as chicks aged. Although literature describing the changes in specific innate cell activities as chicks age in the early days of life are limited, there are several reports of various aspects of the gut associated immune system maturing from day one to day four of the chicks life. During the first week of life the predominant mechanism of protecting the gastrointestinal tract was antimicrobial peptides, after which a cell-mediated response became more prevalent (Crhanova et al., 2011; Bar-Shira and Friedman, 2006). Also during the first week the production of pro-inflammatory cytokines (IL-1β, IL-8 and K203) required for chemo-attraction of phagocytic cells increased (Bar-Shira and Friedman, 2006).

Additionally, there is significant development of the acquired immune system during the first two weeks of life, as measured by the production of Chicken IL-2 and Chicken IFN-ϒ (Bar-Shira et al., 2003). The production of pro-inflammatory cytokines was not measured in chicks in the current study, but the increase in EBC and phagocytic activation observed in D4 chicks could be explained by increased levels of pro-inflammatory cytokines. In addition, the increased number of active phagocytes and increased EBC at day four are indicative that the chick's innate immune system is maturing from day one to day four. Also interesting is that phagocytic ability (capacity and activation) was greatest in chicks hatched from Mid hens, while innate immune function as indicated by EBC was moderate in chicks from hens of this age, but was greatest in chicks from Early hens. The current data suggest that a mechanism other than phagocytosis, such as antimicrobial peptides, is more prevalent in chicks from Early hens than in chicks from older hens. Published literature on chick innate immune function development does not take hen age into account; the current research strongly suggests that the development of chick immune function is affected by hen age.

The OB increased dramatically over 20 minutes in chicks from Mid hens while not changing in chicks from Early or Late hens (Table 4.7). The OB of day one chicks increased more in chicks from Control Mid hens than chicks from LC Mid hens relative to chicks from Early hens. Conversely, the decline in OB was greater in chicks from Control hens as hens aged Mid to Late than in chicks from LC hens (Table 4.7). The OB of day four chicks was affected by a hen age by treatment interaction. The OB of day four chicks from Control hens decreased as

hens aged while that of chicks from LC hens increased as hens aged from Mid to Late (Table 4.7). As with day one chicks, OB of day four chicks increased with increasing time of the assay (Table 4.7). In young (2 months) and old (24 months) rats LC supplementation (50 mg/kg) decreased the production of superoxide anion by exudate cells in response to carrageenan injection (Izgüt-Uysal et al., 2003), and when phorbol myristate acetate stimulated human polymorphonucleocytes (**PMN**) were incubated with 50 μg/ml LC the production of ROS was inhibited. Furthermore storing the human PMNs in the presence of LC at increasing concentrations up to 200 μg/ml minimized their activity thereby maintaining their integrity, and therefore their functionality (Fattorossi et al., 1993). The literature suggests that dietary LC supplementation reduced the production of ROS in stimulated cells; which was not observed in the current research. The ability of LC to scavenge and quench ROS is thought to stabilize cellular membrane fluidity and aid in chemotaxis of cells (Izgüt-Uysal et al., 2003). It is possible, given the ability of LC to maintain cellular membrane integrity and fluidity of human PMNs, that LC had a positive effect on the integrity of the cellular membrane of the chicks' phagocytes enabling them to be more active in producing ROS.

4.3.4 Chick Growth Trial

Treatment did not affect chick body weight or average daily gain at any chick age (Table 4.8). Chick body weight increased with hen age at chick ages day zero and day seven, but was unaffected by hen age thereafter (Table 4.8). Willemsen et al., (2008) reported that by day seven chick body weight was

unaffected by hen age which was not observed in the current research but the current research agrees with published data in that beyond seven days of age hen age did not affect chick body weight. Chick gain increased with increasing hen age during day zero to day seven of age (Table 4.8). As LC is required for β-oxidation of fatty acids, one might expect to see differences in performance as a result of LC supplementation. Chicks supplemented with 50, 100 or 150 mg LC/kg diet had greater body weights and reduced fat deposition compared to non-supplemented chicks (Rabie et al., 1997); but chicks in the current study were not supplemented with LC post-hatch, and would have had to rely on any dietary LC naturally present and endogenous synthesis of LC. As increased gains among chicks from LC supplemented hens were not observed, it is likely that all of the maternal LC available to the chick was used *in ovo* or that LC remaining in the yolk sac was insufficient to yield any observable differences.

At hatch chick liver weight increased to a greater extent from Early to Mid hen ages in chicks from LC hens than chicks from Control hens (Table 4.9); there was no further increase from the Mid to Late hen ages in either hen dietary group. At 14 days of age chick liver weight was lowest in chicks from Early hens, and not different in chicks from Mid or Late hens, but was greater in chicks from LC hens relative to chicks from Control hens. Day 21 liver weights of chicks from Control hens were greatest in chicks from Mid hens, while day 21 liver weights from LC hens did not differ as hens aged. The reason that maternal LC resulted in increased day 14 chick liver weight is unknown. Likewise, the reason for the increased day 21 chick liver weight in chicks from Control Mid hens is unknown.

A hen treatment by hen age by chick age interaction was observed affecting chick AOC (Figure 4.1). Regardless of treatment, as chicks from Early hens aged AOC was lowest at day 14, but increased to day 21. As chicks from Mid Control hens aged AOC only increased at day 14, while chick AOC from Mid LC hens was lowest at day 14. Chick AOC of chicks from Late Control hens peaked at day 14, but ultimately decreased to day 21 while chick AOC of chicks from Late LC hens generally decreased to day 21. Maternal LC supplementation increased AOC in chicks hatched from Mid hens. This is a complex data set which is difficult to interpret as there are no published data on the effect of LC on chick AOC. The patterns of chick AOC observed as hens aged are similar to what has been observed by our research group with other antioxidant ingredients (see Chapters 2 and 3). The chicks in this study were fed standard broiler starter diets with no further dietary treatment. Any effect that supplemental LC had would be residual from the hens' diet and it is logical to expect that beyond the first week maternal LC would not have an effect. Day zero chick AOC was unaffected by hen age, but the profile of AOC as chicks aged differed with hen age. Independent of an antioxidant supplemented diet, the activities of antioxidant enzymes changed with age in rats (Valls et al., 2005), however those data are for young and old rats and do not describe antioxidant enzyme activities over a short time period. During embryonic development of chicks the activities of antioxidant enzymes (glutathione peroxidase, superoxide dismutase and catalase) changed daily, the profiles of which differed by tissue (liver, brain, yolk sac

membrane, kidney, muscle, lung and heart) (Surai, 1999). It is possible that antioxidant enzyme activities continue to fluctuate in chicks as they mature.

4.4 CONCLUSIONS

In conclusion, L-carnitine supplementation reduced hen body weight and increased hatchability at each of the hen ages investigated. Maternal supplementation of LC did not affect chick performance. Maternal supplementation of LC may have benefits such as increased hatchability as observed in the current research, and reduced abdominal fat (Xu et al., 2003). In addition, the results of the current research suggest that although it may not greatly increase the activity of the chicks' innate immune system, some immunomodulation as a result of hen LC supplementation was observed. Maternal LC supplementation did not affect EBC, but it may have had a stabilizing effect on phagocytic capacity and day one OB. However, maternal LC supplementation reduced the OB response of day four chicks. These results support that LC is immuno-modulatory and demonstrate this effect in chicks for the first time. It may be worthwhile to investigate the effect of LC on chick innate immune function when directly supplemented to chicks.

Chick innate immune function as measured by *ex vivo E. coli* bactericidal capacity decreased as hens aged, the reason for which was not apparent and warrants further investigation. Hen age also affected the profile of chick plasma total antioxidant capacity, but the reason for why is unknown and may be made clearer as information on the activities of antioxidant enzymes as chicks age becomes available.

Table 4.1. Diet composition and calculated nutrient profile of L-carnitine diets

Ingredient	%
Calculated Inclusion	
Corn	36.6
Soybean meal	15.8
Wheat	34.1
Calcium carbonate	7.8
Dicalcium phosphate	1.44
Salt	0.4
L-lysine	0.04
Methionine hydroxy analogue, 95%[1]	0.2
Vitamin E[2]	0.8
Inorganic trace mineral premix[3]	0.1
Canola oil	0.5
Avizyme 1302[4]	0.05
Broiler Breeder premix[5]	1.0
L-Carnitine[6]	
Calculated Nutrient Profile	
ME (kcal/kg)	2,882
CP (%)	15
Calcium (%)	3
Available P (%)	0.37
Lysine (%)	0.73
Vitamin E[3] (IU/kg)	54
Sodium (%)	0.19

[1]Novus International, Inc.
[2]Vitamin E supplemented as Vitamin E 5,000 IU/kg, 40 IU/kg diet supplemented
[3] Inorganic trace mineral premix supplied the following per kg of feed: $CuSO_4 \cdot 5H_2O$, 40 mg; $MnSO_4$, 380 mg; $ZnSO_4$, 295 mg
[4]Enzyme obtained from Danisco
[5]Vitamin/Mineral premix supplied the following per kg of feed: Vitamin A, 12,500 IU; Vitamin D_3, 3,100 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; choline, 2.7 mg; iron, 0.0 mg; zinc, 100 mg; manganese, 87 mg; copper, 15 mg; iodine, 1.665 mg; selenium, 0.3 mg
[6]L-Carnitine supplemented at 0 or 50 mg/kg diet

Table 4.2. Effect of hen age and maternal L-carnitine on hen production parameters

		Body Weight	Hen Housed Settable Eggs	Total Eggs/hen	Total Settable Eggs/hen
Hen Age	Treatment	Kg	%		
Early		3.19[c]	91.06[a]		
Mid		3.39[b]	69.67[b]		
Late		3.57[a]	56.50[c]		
SEM		0.036	0.066		
	Control	3.40[a]	74.25[a]	178.72	177.50
	L-carnitine	3.37[b]	70.56[b]	170.15	168.45
SEM		0.029	0.055	14.17	14.21
Interaction					
Hen Age * Treatment[3]					
Early	Control	3.20	92.92		
Early	L-carnitine	3.17	89.20		
Mid	Control	3.42	72.46		
Mid	L-carnitine	3.36	66.87		
Late	Control	3.58	57.37		
Late	L-carnitine	3.56	55.63		
SEM		0.050	0.094		
P-Values					
Age		<0.0001	<0.0001		
Treatment		0.0231	0.0061	0.2326	0.2089
Age*Treatment		0.6025	0.4978		

[abc] Means with differing letters are different ($p \leq 0.05$) within effect

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 45 per treatment

[4] Data log transformed to meet ANOVA assumptions, but presented as percentage

Table 4.3. Effect of hen age and maternal L-carnitine on egg traits

		Egg Weight	Yolk Weight	% Yolk[4]	Albumen Weight	% Albumen[4]	Wet Shell Weight[5]	% Wet Shell[5]
Hen Age[1]	Treatment[2]	g	g		g		g	
Early		57.83[c]	16.81[c]	29.09	33.20[b]	57.41	7.82[b]	13.60
Mid		65.46[b]	19.39[b]	29.69	36.54[a]	55.74	9.53[ab]	14.72
Late		69.31[a]	21.30[a]	30.80	37.06[a]	53.61	10.96[a]	15.74
SEM		2.78	0.99	0.022	2.60	0.035	2.36	0.053
	Control	64.01	19.44	30.35	34.72	54.48	9.85	15.30
	L-carnitine	64.39	18.88	29.37	36.49	56.69	9.02	14.07
SEM		2.27	0.81	0.18	2.12	0.028	1.93	0.043
Interaction								
Hen Age * Treatment[3]								
Early	Control	58.39	17.31	29.63	32.71	56.03	8.37	14.44
Early	L-carnitine	57.26	16.31	28.55	33.70	58.78	7.26	12.76
Mid	Control	65.53	19.40	29.65	36.88	56.23	9.25	14.27
Mid	L-carnitine	65.40	19.38	29.73	36.21	55.26	9.81	15.17
Late	Control	68.12	21.63	31.77	34.56	51.19	11.93	17.20
Late	L-carnitine	70.49	20.96	29.83	39.55	56.02	9.98	14.27
SEM		3.94	1.40	0.031	3.68	0.049	3.33	0.075
P-Values								
Age		<0.0001	<0.0001	0.0703	0.0087	0.1101	0.0349	0.2458
Treatment		0.7438	0.1708	0.1344	0.1006	0.1274	0.3872	0.3097
Age * Treatment		0.4359	0.6010	0.4325	0.0904	0.2320	0.5581	0.2690

[abc] Means within column with differing letters are different ($p \leq 0.05$)

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 10 eggs per treatment

[4] Data log transformed to meet ANOVA assumptions, but presented as percentage

[5] Shell weights include shell membrane

Table 4.4. Effect of maternal L-carnitine on hen total plasma antioxidant capacity

Hen Age[1]	Treatment[2]	Antioxidant Capacity (mM Trolox Equivalents)
Photostimulation		3.14[a]
Early		2.79[ab]
Mid		2.25[b]
Late		2.14[b]
SEM		0.75
	Control	2.32
	L-carnitine	2.84
SEM		0.53
Interaction		
Hen Age * Treatment[3]		
Photostimulation	Control	2.31[bc]
Photostimulation	L-carnitine	3.97[a]
Early	Control	2.42[bc]
Early	L-carnitine	3.16[ab]
Mid	Control	2.46[bc]
Mid	L-carnitine	2.04[c]
Late	Control	2.10[bc]
Late	L-carnitine	2.18[bc]
SEM		1.06
P-Values		
Age		0.0328
Treatment		0.0544
Age * Treatment		0.0467

[abc]Means with differing letters are different ($p \leq 0.05$) within effect or interaction in a column

[1] Hen ages are defined as production stages. Photostimulation = 23 wks; Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 10 hens per treatment

Table 4.5. Effect of hen age and maternal L-carnitine on hatchability and chick hatch weight

		Hatchability[4]	Male Hatch Weight	Female Hatch Weight
Hen Age[1]	Treatment[2]	%	g	g
Early		77.11	40.85[c]	40.58[c]
Mid		78.01	46.16[b]	45.27[b]
Late		76.07	48.06[a]	47.83[a]
SEM		0.031	0.59	0.59
	Control	74.63[b]	44.79	44.18[b]
	L-carnitine	79.50[a]	45.26	44.94[a]
SEM		0.025	0.48	0.48
Interaction				
Hen Age * Treatment[3]				
Early	Control	77.35	40.50	40.02
Early	L-carnitine	81.96	41.21	41.15
Mid	Control	76.64	45.89	44.88
Mid	L-carnitine	81.76	46.43	45.66
Late	Control	69.90	47.98	47.64
Late	L-carnitine	74.78	48.15	48.02
SEM		0.43	0.84	0.83
P-Values				
Age		0.7657	<0.0001	<0.0001
Treatment		0.0354	0.0545	0.0017
Age * Treatment		0.0863	0.6806	0.4707

[a,b,c,d] Means within column with differing letters are different ($p \leq 0.05$) within treatment

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 50 per treatment

[4]Data log transformed to meet ANOVA assumptions, but presented as percentage

Table 4.6. Effect of hen age and maternal L-carnitine on *E. coli* bactericidal and phagocytic capacities of chicks

			E. coli Bactericidal Capacity	Phagocytic Capacity[5,6]	Phagocyte Activation[5,7]
			%[4]	Mean Fluorescent Unit[8]	
Hen Age[1]	Treatment[2]	Chick Age			
Early			42.92[a]	.	.
Mid			5.61[b]	154.13[a]	41.90[a]
Late			1.65[b]	118.68[b]	29.51[b]
SEM			0.19	9.56	3.54
	Control		15.85	.	.
	L-carnitine		17.61	.	.
SEM			0.16	.	.
		Day 1	7.89[b]	.	.
		Day 4	25.57[a]	124.73	38.09
SEM			0.15	.	.
Interactions					
Hen Age * Treatment					
Early	Control		44.65	.	.
Early	L-carnitine		6.06	.	.
Mid	Control		41.19	160.41	43.71
Mid	L-carnitine		0.00	147.84	40.09
Late	Control		5.17	118.84	30.20
Late	L-carnitine		5.58	118.52	28.82
SEM			0.28	13.51	5.00
Hen Age * Chick Age					
Early		Day 1	25.02[b]	.	.
Early		Day 4	60.83[a]	112.08[c]	36.44[b]
Mid		Day 1	1.61[d]	153.13[a]	36.43[b]
Mid		Day 4	12.84[c]	155.12[a]	47.37[a]
Late		Day1	0.26[d]	130.39[b]	28.56[c]
Late		Day 4	3.03[d]	106.97[c]	30.47[c]
SEM			0.28	14.62	5.41
Treatment * Chick Age					
	Control	Day 1	6.26	.	.
	Control	Day 4	25.43	121.96	38.48
	L-carnitine	Day 1	9.52	.	.
	L-carnitine	Day 4	25.71	127.49	37.71
SEM			0.22	12.52	4.63
Hen Age * Treatment * Chick Age					
Early	Control	Day 1	23.84	.	.
Early	Control	Day 4	65.47	99.52	35.53
Early	L-carnitine	Day 1	26.20	.	.
Early	L-carnitine	Day 4	56.19	124.64	37.34
Mid	Control	Day 1	0.00	162.75	39.77
Mid	Control	Day 4	11.29	158.07	47.65
Mid	L-carnitine	Day 1	0.00	143.52	33.08
Mid	L-carnitine	Day 4	14.38	152.17	47.09
Late	Control	Day 1	0.00	129.40	28.16
Late	Control	Day 4	0.00	108.28	32.25
Late	L-carnitine	Day 1	4.62	131.38	28.96
Late	L-carnitine	Day 4	6.55	105.67	28.69

SEM	0.39	20.62	7.63
P-Values			
Hen Age	<0.0001	<0.0001	<0.0001
Treatment	0.4961	.	.
Chick Age	<0.0001	.	.
Hen Age * Treatment	0.2492	0.0620	0.6296
Hen Age * Chick Age	0.0009	0.0097	0.0129
Treatment * Chick Age	0.5395	.	.
Hen Age * Treatment * Chick Age	0.4283	0.3550	0.1449

[abcd] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 12 per treatment

[4] Data log transformed to meet ANOVA assumptions, but presented as %

[5] Data for D1 chicks from Early hens lost

[6] Phagocytic capacity is an indication of the number of *E. coli* bioparticles phagocytized by each cell.

[7] Phagocyte activation is an indication of the number of cells which contain at least one *E. coli* bioparticles

[8] Fluorescence of treated cells used to estimate phagocytic capacity and activation in comparison to fluorescence of untreated cells.

Table 4.7. Effect of hen age and maternal L-carnitine on oxidative burst response in chicks

			Oxidative Burst	
			Mean Fluorescence Unit[5]	
			Chick Age	
Hen Age[1,3]	Treatment[2,3]	OB Time	Day 1	Day 4
Early			2.59[b]	2.50
Mid			5.57[a]	2.38
Late			2.17[b]	2.28
SEM			1.05	0.52
	Control		3.25	2.60
	L-carnitine		3.64	2.18
SEM			0.86	0.42
		5	1.72[b]	1.62[b]
		10	2.72[b]	1.94[b]
		15	4.19[a]	2.63[a]
		20	5.15[a]	3.37[a]
SEM			1.22	0.60
Interactions				
Hen Age * Treatment				
Early	Control		2.07[bc]	3.48[a]
Early	L-carnitine		3.12[b]	1.52[d]
Mid	Control		6.17[a]	2.67[bc]
Mid	L-carnitine		4.97[a]	2.10[cd]
Late	Control		1.52[c]	1.65[d]
Late	L-carnitine		2.81[bc]	2.92[ab]
SEM			1.49	0.73
Hen Age * OB Time				
Early		5	1.97[cd]	1.69
Early		10	1.92[cd]	1.78
Early		15	2.86[bcd]	2.67
Early		20	3.62[bc]	3.87
Mid		5	1.92[cd]	1.88
Mid		10	4.45[b]	2.14
Mid		15	7.02[a]	2.71
Mid		20	8.89[a]	2.80
Late		5	1.26[d]	1.29
Late		10	1.78[cd]	1.91
Late		15	2.69[bcd]	2.52
Late		20	2.95[bcd]	3.42
SEM			2.11	1.03
Treatment * OB Time				
	Control	5	1.43	1.70
	Control	10	2.51	2.26
	Control	15	3.97	3.01
	Control	20	5.11	3.43
	L-carnitine	5	2.01	1.54
	L-carnitine	10	2.92	1.62
	L-carnitine	15	4.41	2.26
	L-carnitine	20	5.20	3.30
SEM			1.72	0.84
Hen Age * Treatment * OB Time[4]				
Early	Control	5	1.41	2.33
Early	Control	10	1.65	2.48
Early	Control	15	2.25	4.02

Early	Control	20	2.98	5.11
Early	L-carnitine	5	2.53	1.05
Early	L-carnitine	10	2.20	1.07
Early	L-carnitine	15	3.48	1.32
Early	L-carnitine	20	4.26	2.64
Mid	Control	5	1.77	1.62
Mid	Control	10	4.64	2.65
Mid	Control	15	7.78	3.30
Mid	Control	20	10.49	3.11
Mid	L-carnitine	5	2.08	2.13
Mid	L-carnitine	10	4.27	1.63
Mid	L-carnitine	15	6.26	2.13
Mid	L-carnitine	20	7.29	2.50
Late	Control	5	1.10	1.15
Late	Control	10	1.26	1.66
Late	Control	15	1.89	1.71
Late	Control	20	1.85	2.07
Late	L-carnitine	5	1.42	1.44
Late	L-carnitine	10	2.29	2.16
Late	L-carnitine	15	3.50	3.33
Late	L-carnitine	20	4.04	4.76
SEM			2.98	1.46
P-Values				
Hen Age			<0.0001	0.7086
Treatment			0.3853	0.0502
OB Time			<0.0001	<0.0001
Hen Age * Treatment			0.0352	<0.0001
Hen Age * OB Time			0.0051	0.4887
Treatment * OB Time			0.9822	0.6378
Hen Age * Treatment * OB Time			0.6676	0.0905

[abcdefg] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] Values reported are average OB over length of assay

[4] n = 12 per treatment

[5] Oxidative products quantified via fluorescence intensity

Table 4.8. Effect of hen age and maternal L-carnitine on weekly chick body weight and gain

		Body Weight				Average Daily Gain		
		g				g/d/chick		
		D0	D7	D14	D21	Week 1	Week 2	Week3
Hen Age[1]	Treatment[2]							
Early		41.51[c]	124.48[c]	340.30	724.04	11.85[c]	27.97	54.90
Mid		46.41[b]	139.80[b]	389.46	789.25	13.42[b]	35.92	56.34
Late		48.77[a]	153.98[a]	389.13	824.36	15.03[a]	33.27	62.93
SEM		1.11	11.47	46.59	107.57	1.56	7.47	8.51
	Control	45.27	136.21	366.37	754.92	13.04	32.29	56.94
	L-carnitine	45.86	142.63	379.55	803.50	13.82	32.48	59.18
SEM		0.90	9.37	38.04	87.83	1.27	6.10	6.95
Interactions								
Hen Age * Treatment[3]								
Early	Control	40.75	118.98	323.09	696.23	11.18	27.64	53.54
Mid	Control	45.94	140.08	398.10	771.12	13.61	36.71	57.29
Late	Control	49.11	149.56	377.90	797.41	14.35	32.54	59.98
Early	L-carnitine	42.28	129.99	357.50	751.84	12.53	28.30	56.26
Mid	L-carnitine	46.88	139.51	380.81	807.37	13.23	35.14	55.40
Late	L-carnitine	48.43	158.39	400.35	851.31	15.71	34.00	65.88
SEM		1.56	16.23	65.89	152.13	2.20	10.56	12.03
P-Values								
Hen Age		<0.0001	0.0006	0.0638	0.1606	0.0033	0.9486	0.1313
Treatment		0.1746	0.1595	0.4616	0.2489	0.2038	0.1006	0.4917
Hen Age * Treatment		0.1201	0.5189	0.4667	0.9763	0.4010	0.8995	0.6121

[ab] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 10 per treatment per chick age

Table 4.9. Effect of hen age and maternal L-carnitine on weekly chick liver weight

		Liver Weight			
		g			
		D0	D7	D14	D21
Hen Age[1]	Treatment[2]				
Early		1.034[b]	.	12.60[b]	22.18
Mid		1.25[a]	5.82	14.49[a]	24.04
Late		1.22[a]	5.85	14.12[a]	21.90
SEM		0.077	0.73	1.31	2.16
	Control	1.14	5.78	13.00[b]	23.00
	L-carnitine	1.20	5.89	14.47[a]	22.41
SEM		0.063	0.73	1.07	1.76
Interaction					
Hen Age * Treatment[3]					
Early	Control	1.07[cd]	.	11.66	21.57[b]
Mid	Control	1.16[bc]	5.81	13.73	26.44[a]
Late	Control	1.18[b]	5.74	13.61	21.01[b]
Early	L-carnitine	1.00[d]		13.53	22.80[b]
Mid	L-carnitine	1.33[a]	5.82	15.26	21.64[b]
Late	L-carnitine	1.26[ab]	5.97	14.63	22.80[b]
SEM		0.11	1.03	1.85	3.05
P-Values					
Hen Age		<0.0001	0.9152	0.0157	0.1011
Treatment		0.0626	0.7425	0.0079	0.5021
Hen Age * Treatment		0.0109	0.7635	0.8063	0.0048

[abcd] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1] Hen ages are defined as production stages. Early = 31 to 33 wks; Mid = 45 to 47 wks; and Late = 57 to 59 wks

[2] Control diet contains no supplemental L-carnitine, L-carnitine diet supplemented with 50 mg/kg diet

[3] n = 10 per treatment per chick age

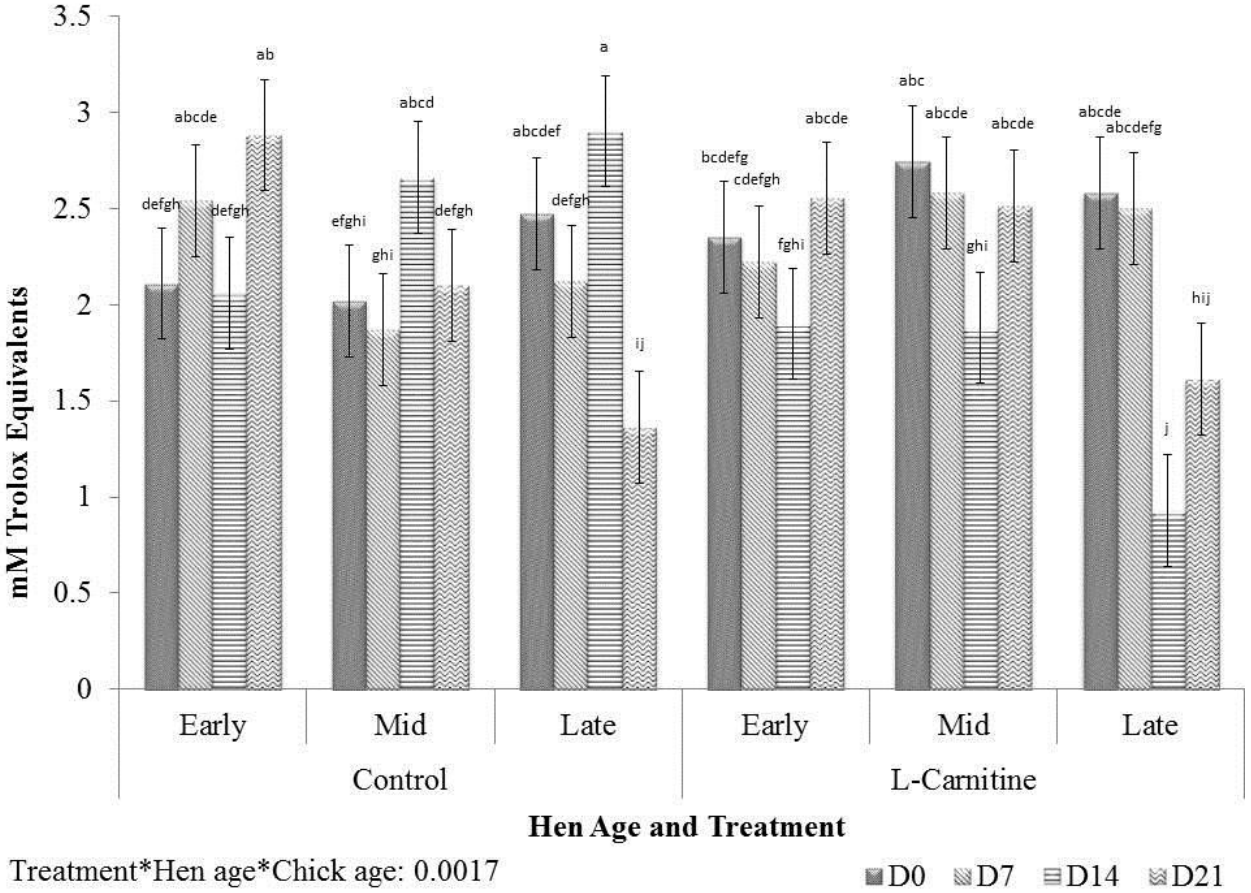

Figure 4.1. Effect of maternal L-carnitine[1], hen age[2] and chick age on chick total plasma antioxidant capacity

[abcdefghij] Indicate differences ($p \leq 0.05$) among bars.

[1] Hens supplemented with either 0 or 50 ppm L-carnitine.

[2] Early: 33 wks; Mid: 47 wks; Late: 59 wks

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5. THE EFFECT OF HEN AGE AND INCUBATION TEMPERATURE ON CHICK POST-HATCH INNATE IMMUNE FUNCTION INDICES

5.1 INTRODUCTION

It has long been thought that chicks from older hens are of higher quality than chicks from younger hens because they possess greater growth rates and therefore reach greater weights at market age. In actuality the effect of hen age on chick growth potential is inconsistent. There are data supporting that chicks from older hens reach greater market weights (Ulmer-Franco et al., 2010; Peebles et al., 1999), but there are also data reporting that chick market weight is unaffected by hen age (Tuft and Jensen, 1991; Hulet et al., 2007). Although there are inconsistencies in chick growth rate associated with hen age, chick first week mortality may be associated with hen age. Yassin et al., (2009) reported that first week mortality was much lower among chicks from 38 to 44 wk hens (1.02%) compared to chicks from 26 wk (1.82%) or 60 wk (1.20%) hens. Reasons for this increase in mortality have not yet been investigated, but possibilities include reduced availability of yolk fatty acids to chicks from young hens (30 weeks) resulting in fewer post-hatch resources (Yadgary et al., 2010) and that chicks from young (26 weeks) and old (64 weeks) hens do not utilize yolk fatty acids as efficiently as chicks from middle aged (51 weeks) hens (Latour et al., 1998). It is also possible that first week mortality is associated with the development of the immune function.

The immune system is not fully mature until several weeks post-hatch (Crhanova et al., 2011). For the first week of life, the chick's acquired immune system is minimally functional (Bar-Shira et al., 2003). However, the innate immune system is sufficiently mature to afford some protection against pathogens to the chick (Crhanova et al., 2011), thus post-hatch chicks rely on the innate immune response. We had previously hypothesized that chicks from middle aged hens (45 to 47 weeks) would have a greater innate immune response because of the lower first week mortality reported by Yassin et al., (2009). What was actually observed was that chicks from young hens (31 to 33 weeks) had greater innate immune responses as measured by *Escherichia coli* (***E. coli***) bactericidal capacity than chicks from middle aged (45 to 47 weeks) or old hens (57 to 59 weeks; Chapters 2, 3 and 4). This could have been the result of increased egg size as hens aged and the relationship between egg size and incubation temperature.

The relationship between hen age and egg size (and therefore embryo size by association) is well established; as hens age they lay larger eggs (Anderson et al., 1978; Gibson et al., 2008; Tůmová and Gous, 2012). Furthermore, larger eggs contain larger embryos which produce greater amounts of metabolic heat (Lourens et al., 2006); and chicks incubated at temperatures promoting an eggshell temperature greater than 37.8 °C had lower post-hatch development (Molenaar et al., 2011; Joseph et al., 2006) and greater mortality than chicks from eggs incubated at 37.8°C (Molenaar et al., 2011). To obtain an eggshell temperature of 37.8°C for larger eggs, which are usually from older hens, the incubator temperature has to be adjusted downward (Lourens et al., 2006). Further

complicating matters, the development of the avian innate immune response is affected by incubation temperature. When the incubation temperature was lowered by 6°C, tree swallow (*Tachycineta bicolor*) nestlings had lower abilities to kill *E. coli ex vivo* than nestlings not exposed to a reduced incubation temperature (Ardia et al., 2010). Conversely, when the incubation temperature was increased to 38.8°C from 37.8°C, the development of the bursa and thymus of broilers were impaired (Oznurlu et al., 2010).

Previously, we incubated eggs under the same incubation conditions (37.5°C, 56% relative humidity; **RH**) regardless of egg size (or hen age) or the subsequent differences in expected metabolic heat produced by embryos of different sizes. Given the relationship between immune function development and incubation temperature, and incubation temperature and egg size, it is possible that an incubator temperature effect on chick post-hatch innate immune function was observed rather than a hen age effect. The objective of the current research was to determine the effects of hen age and incubation temperature on chick post-hatch innate immune function indices. It was hypothesized that chick innate immune function would increase with increasing hen age and decreasing incubation temperature, and furthermore that reducing the incubation temperature from 37.5°C to 36.5°C would reduce differences in innate immune function indices observed between chicks from Young (26 to 34 weeks) and Old (46 to 54 weeks) hens.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Treatments

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with the Canadian Council of Animal Care (1993) guide. Hatching eggs were obtained from commercial flocks of Ross 708 broiler breeder hens of three different age groups. The flock ages were Young (Y): 26 to 34 wk, Mid (M): 35 to 45 wk, and Old (O): 46 to 54 wks. The following procedure was repeated eight times for two replications of each experimental temperature. The data collected were pooled resulting in n = 12 eggs for each hen age at each experimental temperature. Eggs (n=18) within 0.5 g of the average for each flock age group were collected prior to each temperature replication and stored for 2 to 4 days. They were then incubated for 14 days at 37.5ºC and 56% RH in a Jamesway AVN incubator. At 15 days of incubation, 6 fertile eggs from each of the three flock ages were individually incubated in one of 24 metabolic chambers (for metabolic measurements taken for a concurrent study) placed inside the incubator until hatch. The incubator was set at one of the four incubation temperature treatments: 36.0, 36.5 37.0, or 37.5ºC (Control) until 21.5 days of incubation.

5.2.2 Innate Immune Function Indices

Whole blood collected from the chicks on day of hatch was used to determine *E. coli* bactericidal activity (**EBC**), to assess the number of cells containing at least one *E. coli* (phagocyte activation) and the average number of

E. coli/cell (phagocytic capacity) and oxidative burst response (**OB**) *ex vivo*. Assay conditions were as described in Chapter 2, Experiment 1.

5.2.3 Statistical Analysis

Data were analyzed as a two-way ANOVA using the Mixed procedure of SAS (SAS Institute Inc., 2002-2010), with hen age and incubator temperature as the main effects, initial egg weight was used as a covariate. Differences of least square means were considered significant when $p \leq 0.05$ (Steel and Torrie, 1960).

5.3 RESULTS AND DISCUSSION

Ex vivo E. coli bactericidal capacity was greatest among chicks from Young hens in agreement with our previous findings (Chapters 2, 3, and 4; Table 5.1); but unlike previous observations the EBC of chicks from Mid and Old hens did not differ from each other. Incubating eggs at 36.5°C resulted in the greatest EBC (72.1%) while an incubator temperature of 36.0°C resulted in the lowest EBC (23.0%). Incubating eggs at 36.5°C or 37.0°C resulted in intermediate EBC (~37%; Table 5.1). Phagocytic capacity was unaffected by hen age, but was greatest when eggs were incubated at 36.0 or 37.5°C and lowest when eggs were incubated at 36.5°C (Table 5.1). Phagocytic activation was unaffected by either hen age or incubator temperature (Table 5.1). There were no interactions.

The effect of hen age on innate immune function as measured by EBC has consistently been observed (see Chapters 2, 3 and 4). The decreased EBC of chicks as hens age could be related to maternal stress. Chronic stress is associated with immuno-suppression; however acute stress, such as that associated with handling or being moved to a new environment, may have a temporary immuno-

enhancing effect (Dhabhar and Viswanathan, 2005). Young hens, which have been recently transferred to the lay house from the pullet barn, are stressed (Voslarova et al., 2011; Singh et al., 2009) and adapting to a new environment. It has been demonstrated that perceived stress, such as handling, temporarily upregulates the innate immune response of birds. For example, in 3 week old turkey poults exposure to handling stress increased the oxidative burst of heterophils, which is part of the innate immune response (Huff et al., 2010). Furthermore, maternal stress may affect the innate immune function of offspring; this was demonstrated by exposing late gestation sows to handling stress. The piglets of stressed sows had increased levels of pro-inflammatory cytokines in response to lipopolysacharide (**LPS**) challenge (a physiological stress) compared to piglets of sows which were not stressed late in gestation (Collier et al., 2011). A common measure of stress in chickens is the heterophil:lymphocyte ratio. A stressed chicken has increased numbers of heterophils (cells of the innate immune response) and decreased numbers of lymphocytes (cells of the acquired immune response) (Gross and Siegel, 1983). The stress level (heterophil:lymphocyte ratio and egg corticosterone level) of caged laying hens continued to decrease as hens aged from 22 to 35 to 45 weeks of age (Singh et al., 2009), suggesting that the Young hens in the current study may also have had greater stress than Mid and Old hens. Therefore, the Young hens could have experienced greater levels of perceived (handling) stress which prepared their chicks for physiological stress (*E. coli* challenge) and promoted the development of the innate immune response compared to chicks from older hens.

The effect of incubation temperature on EBC was highly significant and may be related to heat stress experienced by the embryos incubated at 37.0 and 37.5°C during incubation. Eggshell temperature (**EST**) data collected concurrently during the incubation of the eggs used in the current study (Hamidu et al., unpublished data), demonstrated that when eggs were incubated at 36.5°C the EST of those eggs were close to 37.8°C. Eggshell temperatures above 37.8°C have been associated with reduced post-hatch growth (Joseph et al., 2006; Molenaar et al., 2011) and increased post-hatch mortality (Molenaar et al., 2011). It is possible that at the investigated incubation temperatures above and below 36.5°C, embryos experienced heat or cold stress respectively which impaired their immune function development. Lowering the incubation temperature by 6°C reduced the EBC in tree swallow nestlings (*Tachycineta bicolor*) (Ardia et al., 2010). Conversely, high incubation temperatures (40.1°C) impaired development of the thymus and bursa, which are immune tissues, in broiler chicks (Oznurlu et al., 2010). The current results showed that reducing the incubation temperature from 37.5°C or 37.0°C to 36.5°C increased the innate immune function as measured by EBC. However, the temperature range in the current study was 1.5°C, and the optimal temperature for innate immune development was 1°C lower than the industry standard incubation temperature of 37.5°C (which maximizes hatchability; French, 1997). The current data infer that commercial incubation conditions may result in chicks with an underdeveloped, or even impaired, immune function. This could contribute to the five percent mortality that is expected in industry (Heier et al., 2002; Heier et al., 2001). The current data

support the hypothesis that incubation temperature affects the development of the avian immune system *in ovo*, and furthermore suggests that the incubation temperature range which is optimal for the development of the immune system may be quite narrow.

It was interesting that although the innate immune function as measured by EBC was greatest when the incubation temperature was 36.5°C, the phagocytic capacity was lowest at that temperature and greatest at 36.0 and 37.5°C. The reason for this is unknown. Quinteiro-Filho et al., (2010) reported that exposing broilers to heat stress (31°C or 36°C for 10 hrs per day) reduced phagocytic cell activity in 35 to 42 day old broilers; although this appeared not to be the case with the current study. It has also been reported in six to eight week old broilers, that heterophils are capable of recognizing pathogenic vs. non-pathogenic strains of *Staphylococcus aureus* and are more responsive to the pathogenic strains than the non-pathogenic strains (Andreasen et al., 1993). In the current research a pathogenic strain of *E. coli* was used to determine the EBC, while a non-pathogenic strain of *E. coli* was used to determine phagocytic capacity and phagocytic activation. Considering that stress upregulated the innate immune response in turkey poults (Huff et al., 2010), it is possible that the chicks from eggs incubated at 36.5°C, which were apparently not stressed and had the greatest immune development, possessed phagocytes that were able to differentiate between pathogenic and non-pathogenic *E. coli*. Conversely, the phagocytes of the chicks from eggs incubated at 36.0°C, 37.0°C or 37.5°C, which may have been thermostressed and have comparatively underdeveloped immune function, may

not have been able to differentiate between the pathogenic and non-pathogenic strains of *E. coli* used. This may explain the differences in phagocyte capacity at the various incubation temperatures investigated, but why EBC was greatest at 36.5°C while phagocytic capacity was lowest is still unknown. The incongruence of EBC and phagocytic capacity effectiveness at 36.5°C incubation temperature suggest that mechanisms other than phagocytosis, such as heterophil extracellular traps (Chuammitri et al., 2009), and degranulation or antimicrobial peptides (Segal et al., 1980) should be considered in this type of research. Crhanova et al. (2011) have demonstrated that during the first four days of a chick's life the cecum was protected by chicken β-defensins, the gallinacins 1, 2, 4 and 6, and that thereafter the immune response changed to a more cell mediated response.

5.4 CONCLUSIONS

It was determined that chicks from young hens have a greater innate immune response as measured by EBC than chicks from middle aged or old hens; and incubating eggs at 36.5°C resulted in the greatest EBC, regardless of hen age. The results of the current research suggest that the innate immune response mechanisms of post-hatch chicks needs investigation to further understand which components are responsible for conferring protection to the chick in the first week of life. The current data support the existing literature that incubation temperature affects the development of the avian immune system *in ovo*, and furthermore suggests that the incubation temperature range which is optimal for the development of the immune system may be quite narrow, and lower than optimal for hatchability.

Table 5.1. Effect of hen age and incubator temperature on chick innate immune function

		E. coli Bactericidal Capacity[4]	Phagocytic Capacity[5]	Phagocytic Activation[6]
		%	Mean Fluorescence Unit[7]	
Hen Age[1]	Incubation Temperature			
Young		60.78[a]	483.26	44.69
Mid		35.36[b]	388.21	44.70
Old		30.91[b]	215.78	40.20
SEM		19.32	205.67	6.90
	36.0	22.97[b]	449.32[a]	40.15
	36.5	72.08[a]	177.31[b]	45.09
	37.0	37.49[b]	319.32[ab]	44.34
	37.5	36.86[b]	503.72[a]	43.20
SEM		15.47	205.59	5.52
Interaction				
Hen Age * Incubator Temperature (°C)[2]				
Young	36.0	42.35	526.39	42.04
Young	36.5	85.40	310.41	48.87
Young	37.0	47.73	526.55	45.53
Young	37.5	67.65	569.68	42.31
Mid	36.0	15.20	437.38	44.94
Mid	36.5	73.57	193.19	44.99
Mid	37.0	27.86	286.09	44.49
Mid	37.5	24.83	636.19	44.36
Old	36.0	11.37	384.20	33.45
Old	36.5	57.29	28.33	41.41
Old	37.0	36.88	145.32	43.01
Old	37.5	18.10	305.29	42.92
SEM		28.66	354.18	10.23
P-Values				
Hen Age		0.0128	0.1021	0.2994
Incubation Temperature		<0.0001	0.0144	0.3101
Hen Age * Incubation Temperature		0.3813	0.7796	0.6451
Egg Weight[3]		0.0125	0.0076	0.0337

[ab] Means within column with differing letters are different ($p \leq 0.05$) within effect or interaction

[1]Hen ages are defined as production stages. Young = 26 to 34 wks; Mid = 35 to 45 wks; and Old = 46 to 54 wks

[2] n = 12 eggs per hen age, n = 2 for incubation temperature

[3] Egg Weight used as covariate to accurately assess effect of hen age. Coefficient: 3.46

[4] Data log transformed to meet ANOVA assumptions, but presented as %

[5]Phagocytic capacity is an indication of the number of *E. coli* bioparticles phagocytized by each cell.

[6] Phagocyte activation is an indication of the number of cells which contain at least one *E. coli* bioparticle

[7] Fluorescence of treated cells used to estimate phagocytic capacity and activation in comparison to fluorescence of untreated cells.

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6. SYNTHESIS

The post-hatch chick particularly relies on its innate immune system, the development of which may be affected by hen age and nutrition. Antioxidant ingredients may play an important role in the innate immune response because of the production of reactive oxygen species as a bactericidal mechanism (Yamamoto and Johnston, 1984). There are no published data investigating the effect of hen age on chick immune function, but there is reason to investigate whether such a relationship exists. Chick first week mortality is reportedly greatest among chicks from young hens (26 weeks; Yassin et al., 2009). However the inflammatory response to lipopolysaccharide (**LPS**) challenge was lower in poults from older hens (55 weeks) than in poults from younger hens (33 weeks; Schaefer et al., 2006). Although there are many potential causes for first week mortality, it is logical to make a connection between mortality and the immune function. This research investigated the effects of hen age and three antioxidant ingredients reported to possess immune-modulatory properties on chick post-hatch innate immune response which were the carotenoid pigment canthaxanthin (Koutsos et al., 2006), vitamin E (Han et al., 2006) and L-carnitine (Izgüt-Uysal et al., 2003) .

Supplementing hens with 6 ppm canthaxanthin increased the innate immune function as measured by *Escherichia coli* (***E. coli***) bactericidal capacity, but did not affect the specific mechanisms measured which were phagocytosis and oxidative burst. Although its incorporation into the egg and tissues of the chick increased with increasing level of maternal supplementation, 12 ppm consistently

did not affect the indices of innate immune function differently from no supplementation. Hen supplementation of canthaxanthin also affected the plasma total antioxidant capacity of chicks, but in an inconsistent manner which was affected by hen age.

Although natural and synthetic forms of vitamin E were differentially incorporated into the egg, this was not consistently reflected on indices of chick innate immune function. However, natural vitamin E supplementation nearly increased *E. coli* bactericidal capacity and increased oxidative burst of innate immune cells at day four. As observed with maternal canthaxanthin supplementation, the form of maternally vitamin E affected chick plasma total antioxidant capacity, but inconsistently as chicks aged and was affected by hen age. It was intended to supplement hens with industry standard levels of vitamin E (40 IU/ kg) which are fairly high relative to the levels recommended by NRC (1994), but the actual level of vitamin E available to the hens was around 80 IU/kg which was substantially greater than intended. The high levels of supplementation may have masked effects of natural vitamin E vs. synthetic vitamin E.

Hen dietary supplementation of L-carnitine did not affect *E. coli* bactericidal capacity but may have had a stabilizing effect on phagocytosis and increased the oxidative burst response of day four chicks. As with the previous two antioxidant ingredients, maternal L-carnitine supplementation affected chick plasma total antioxidant capacity in a three way interaction with hen age and chick age, but the effect of L-carnitine on chick plasma total antioxidant capacity

was inconsistent. The effect of L-carnitine on chick oxidative burst suggests that it possesses some immuno-modulatory properties in chicks.

Consistently in all three of the antioxidant ingredient studies, chick innate immune function, as measured by *E. coli* bactericidal capacity, decreased as hens aged. Given the effect of incubator temperature on immune function development and the effect of egg size on incubator temperature, which was unaccounted for in the three previous experiments it was unclear whether the hen age effect was confounded by an incubator temperature effect on innate immune function was observed. To address this question a follow up study investigating the effects of hen age and incubator temperature on chick innate immune function was conducted. Although chick innate immune function was determined to be greatest in chicks from young hens and in chicks incubated at 36.5°C, there was no interaction between hen age and incubator temperature. The data from this research supports published data that incubation temperature affects the development of the chick's immune system (Oznurlu et al., 2010), but also strongly suggests that hen age affects the development of the chick's immune system.

One challenge encountered in this research was the development of a reliable method to measure the content of vitamin E in the matrices intended to be sampled in this research: feed, eggs, plasma (hen and chick) and chick liver. The difficulty in developing a reliable method which produced reproducible results in eggs and feed translated into a lack of time to analyze all of the samples. Analysis of the liver and plasma contents of vitamin E may have provided valuable insight

into the results of this study, especially with regards to chick plasma total antioxidant capacity. As the vitamin E analysis took close to 12 months to develop, the analysis of the samples taken for the study of L-carnitine was not able to be commenced, nor were plasma levels of canthaxanthin able to be analyzed. Another challenge was interpreting the three-way interactions between hen treatment and age and chick age on chick plasma total antioxidant capacity.

The complexity of chick plasma antioxidant capacity results was not anticipated. There are published data demonstrating that the antioxidant enzyme activities in chicks are highly variable during embryonic development (Surai, 1999), this could also be the case in the post-hatch chick. In hindsight, including other measures of antioxidant capacity such as antioxidant enzyme activities as chicks aged, oxidation production products and other antioxidant molecules present in the blood such as ceruloplasmin (Wiggens et al., 2006) may have provided a more complete description of chick plasma antioxidant capacity and resulted in greater understanding.

In hindsight, making this research more focused and contributing much more information about a single antioxidant ingredient in chickens would have been preferable to studying three antioxidant ingredients in the broader context. In addition to studying only one antioxidant ingredient, it would have been beneficial to focus on either the innate immune function during the first week as affected by maternal nutrition or to focus on the effect of antioxidant supplementation to chicks on their innate immune function as they age. By focusing on one or the other methods of nutrient supplementation a greater array

of innate immune function mechanisms such as the expression of antimicrobial peptides, cytokine profiles, immune cell populations and heterophil extracellular traps could have been measured.

Going forward with this research it would be interesting to investigate the immune function of hens as they age. In addition a comparison of innate and acquired immune responses should be measured in both hens and chicks to determine if there is a relationship between the two in hens that is affecting the chicks' immune response. It would also be interesting to study the effect of the antioxidant ingredients investigated on antioxidant status throughout the day given reports that antioxidant status follows a diurnal fluctuation (Albarrán et al., 2001). Finally, much is yet unknown about the transport mechanisms of carotenoids into various tissues in chickens. It would be beneficial to understand how canthaxanthin, with respect to this research, is transported and deposited into eggs and from there into chicks.

It has been suggested that the dietary requirements of certain nutrients, such as vitamin E, for optimizing immune function in chicks may actually be greater than required for optimal growth and performance (Haq et al., 1996). With the possibility that prophylactic use of antibiotics in animal agriculture may be banned, as is the case in the EU, it is prudent to determine what levels of nutrients optimize not only the immune response, but also do not negatively affect growth and performance which are probably the most important parameters for the poultry industry. The goal of dietary modulation of the immune system is to increase its efficiency and reduce its energetic cost to the bird by providing

nutrients that prime the various components (Klasing, 1998). However, before dietary levels of ingredients to optimize immune response can be recommended, the use of these ingredients in the immune function must be determined. This research has provided direction for future research on the effects of the antioxidant and immuno-modulatory ingredients canthaxanthin, vitamin E and L-carnitine on chick immune response development.

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