EFFECT OF NEONATAL FASTING ON THE ASSIMILATION OF MATERNAL IMMUNITY (IGY), YOLK ABSORPTION AND INTESTINAL GENE EXPRESSION OF IL8, IL17 AND TNF-ALPHA

A Dissertation

by

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ABSTRACT

Protection of newly hatched chicks against disease depends greatly on innate immunity and maternal antibodies acquired in a passive manner through the yolk sac. The impact of fasting on yolk utilization in fasted vs fed chicks has been well studied; however, the impact of a neonatal fast on the assimilation of maternal antibodies is not well established. Several studies were conducted to examine the impact of a 4-day fast on yolk and IgY assimilation in broiler chicks. The hypothesis was that fasted chicks will catabolize IgY to supply nutrients at the expense of maternal immunity. All chicks hatching within a 2-hour period were immediately placed into one of three treatment groups (Fasted, Water only or Feed and water). Blood samples were collected from the jugular or brachial vein in the first hour post hatch and on day (d) four IgY titers were measured by sandwich ELISA. Blood volume was estimated by treatment using the Evans Blue technique and whole body IgY levels were estimated. Body weights were recorded on d 0, BW and yolk sac weight were measured on d 4. Day 0 BW was not significantly different among the treatments, d 4 BW of the fed group was significantly higher than that of water only and the fasted treatments. Compared to the fasted birds, absolute yolk weight d 4 was significantly lower for the water only and fed birds, relative yolk weight (% of BW) was significantly lower in fed birds compared to fasted or water only treatments. IgY titers did not differ on d 0. Day 4 IgY titers were significantly higher in fasted birds compared to those given water only, which in turn was significantly greater than fasted birds. Blood volume estimates indicated that the

blood volume of the fasted chicks was greatly reduced compared to those receiving feed. When total IgY per bird was estimated from blood volume measurements, there were no significant differences among the treatments. The level of the gene expression of the (IL-8, IL-17, and TNF) were measured and were less in the fasting group compared to the fed group.

These data indicate that compared to fed birds, fasted birds and those provided water only do not utilize yolk materials nor assimilate maternal antibodies at differing rates. Indicating that under these conditions, fasted or birds provided water only do not catabolize maternal antibodies at rates differing from fed birds. Gene expression analysis revealed that this will negatively affect the local immunity of intestine.

DEDICATION

To the soul of my father KHUDHAIR, may God have mercy on him for his support until the last moment of his life, and my mother for her support, love ,and prayer that helped me reach my dreams.

To my charming wife Helen and my wonderful kids Tuqa, Jana, Ali and Jude, thank you from the bottom of my heart, for your tolerance and unconditional love through this long journey.

To my big family, my brothers, and my sisters.

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Contributors

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NOMENCLATURE

υ	upsilon
µL	Microliter
Ab	antibody
Abs	Antibodies
BF	Bursa of Fabricius
C_H	Heavy chain constant domain
C_L	Light chain constant domain
DM	Dry matter
ELISA	Enzyme-linked immunosorbent assay
IACUC	Institutional Animal Care and Use Committee
IB	Infectious bronchitis
IBD	Infectious bursal disease
Ig	Immunoglobulin
IgA	Immunoglobulin type A
Igs	Immunoglobulins
IgY	Immunoglobulin type Y
Il-18	Interleukin 18
IL-7	Interleukin 7
KDa	Kilo Dalton
mL	Milliliter

ND	Newcastle Disease
PBS	Phosphate Buffered Saline
PV	Plasma Volume
PVCF	Plasma Volume Correction Factor
qPCR	quantitative polymerase chain reaction
TBV	Total Blood volume
TNF-a	Tumor Necrosis Factor alpha
TPV	True Plasma Volume
V_H	Heavy chain Variable Domain
V_L	Light chain variable variable domain
V_LD_L	Very Low-Density Lipoprotein
VTG	Vitellogenin

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CHAPTER I

INTRODUCTION

The role of maternally derived antibodies is important to the health of newly hatched chicks. The protection of newly hatched chicks against diseases depends on their innate immunity and maternal antibodies in the yolk sac, acquired in a passive manner from the hen (Tizard, 2009). It has been demonstrated that maternal antibodies of isotype IgY, are transferred from hens vaccinated against different diseases like infectious bursal disease (IBD) to their chicks. Maternal antibodies are capable of protecting chicks against the challenge of the disease for a period of up to 4 weeks (Darbyshire & Peters, 1985),(Chhabra et al., 2015). As shown in Figure 1, local immunity in the intestine works differently without the maternal immunity. If the IgY is available in the intestine, the immunoglobulins will protect the intestine. In the intestine that does not have IgY or maternal immunity, the intestinal immune activity will increase the T-dependent immunity or cellular immunity against commensals (Takiishi et al., 2017).

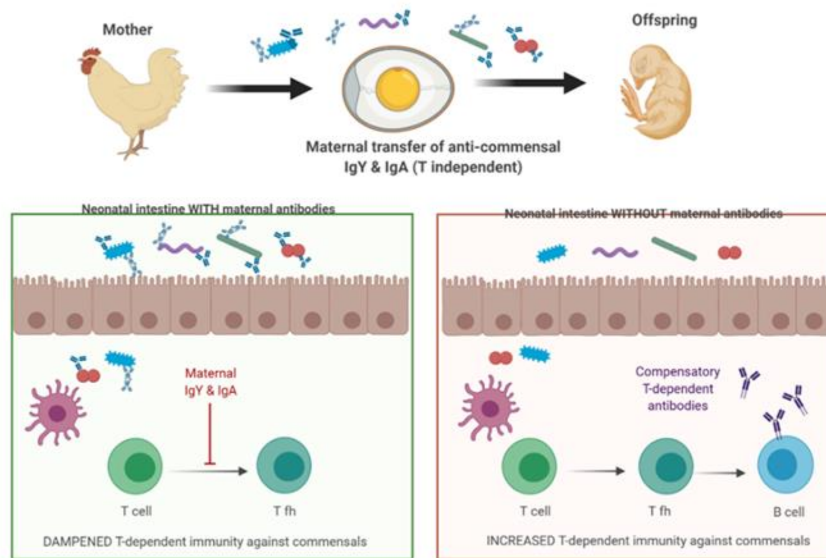

Figure 1 The difference between neonatal intestine with maternal antibody and the neonatal intestine without maternal antibody. Figure modified from Takishi et al (2017)

In modern poultry production, the separation of the hatchery from the production facility means that the hatchling will spend a period of time without the provision of feed or water. The time period between processing and placement is highly variable. It depends on the availability of transport equipment, distance to the placement facility, and hatchery practices. In some parts of the world, producers strive to place the neonates for 12-24 hr to allow them to mature and to initiate a vaccine response while the birds are under a low immunological challenge from other antigens. In other parts of the world, the practice is to hold the birds a period of a few hours. This reduces stress and

gets the birds to feed and water. Often the producer has no options, for example during shipment of birds over long distances. Rest and recovery from processing may have an additional benefit. Just before the hatching process begins, the bird internalizes what is left of the yolk sac. The residual yolk protein is the source of antibodies from the hen (Larsson et al., 1993). To be effective, maternal antibodies must not only move from the residual yolk into the bloodstream but must also diffuse to sites of vulnerability, in particular to the mucosal surfaces where organisms are most likely to enter the body. The problem is that birds are generally not fed in the hatchery (even when held overnight) - nor are they fed during transport. Producers may feel that feeding is not essential during this period because conventional wisdom says that the bird can survive on its residual yolk (Esteban et al., 1991). The survival of a hatchling may indeed depend, in the absence of other feed, upon its use of residual yolk as a nutrient source (Noble & Cocchi, 1990). This may be a valid point but does not completely represent the objectives of modern chick or poult production. Producers want the high weight of chicken in a short time, which intuitively would favor short holding times.

There is interest in intermittent fasting and fasting in general in support of health benefits in humans. Much research has been conducted on humans and few on animals The results have been associated with a wide array of potential health benefits, including improving blood sugar control, heart health, brain function and cancer prevention in general health (Collier, 2013). Information about the effect the fasting on maternal immunity or the immune system, in general, is lacking. Prolonged fasting may or may not negatively interfere with the absorption of immunoglobulins (Igs) because in order to

take care of their physiological needs, birds under a prolonged fasting period may or may not metabolize Igs of the yolk sac in order to obtain energy (Noble & Cocchi, 1990).

Literature Review

Structure of Ovarian Follicles and Yolk Formation.

In ovarian follicles of birds, several layers of supporting tissue surround the developing oocyte, also called the yolk. From the periphery inwards, these are: (1) the theca layer, consisting of the theca externa (a broad layer of stratified cells) and the narrower theca interna, separated by interstitial cells; (2) an acellular layer commonly designated the basement membrane; (3) an epithelial layer of granulosa cells; and (4) the perivitelline membrane.

Most of the yolk components and immunoglobulin are derived from blood plasma. The yolk precursors are mainly synthesized in the liver. The thecal layers of ovarian follicles are very well vascularized with permeable capillaries that allow the blood reach the surrounding tissues with yolk precursors and most yolk components including the immunoglobulins. Immunoglobulins and yolk precursors have to pass the basement membrane, which acts as a filter preventing the passage of larger plasma components. The immunoglobulins and yolk precursors are transported by receptor-mediated transcytosis using the chicken yolk sac IgY receptor (FcRY) and polarized epithelial cells. . Finally, the immunoglobulins and yolk precursors reach the oocyte plasma membrane, where ferritin-conjugated IgY binds to the oocyte plasma membrane at the coated pit (Murai, 2013).

The major yolk precursors are lipoproteins such as very low-density lipoprotein (VLDL) and protein rich-lipoproteins including vitellogenin (VTG). These are incorporated into yolks by receptor-mediated endocytosis. In the final 7-day period of rapid growth of the ovarian follicles, oocyte internalizes about 5 g of lipid and protein, reaching a diameter of nearly 35 mm until it is expelled from the ovarian follicle by ovulation (Figure 2).. The receptor that interacts with VLDL and VTG is called LR8. This receptor is a 95kDa molecule and is expressed in the granulosa cells (Bujo et al., 1994). It is hypothesized that the synthesized LR8 are embedded in the perivitelline and/or oocyte plasma membranes, which interacts with a specific region of apoprotein molecule and VTG. LR8 belongs to the LDL receptor gene family and is known to be a multi-binding receptor. It also interacts with riboflavin-binding protein (Mac Lachlan et al.,1994) and complement component C3 (Recheis et al., 2005). Uptake of yolk precursors into egg yolk is a dramatic example of receptor-mediated endocytosis pathway. This process plays a crucial role in egg formation (Murai, 2013).

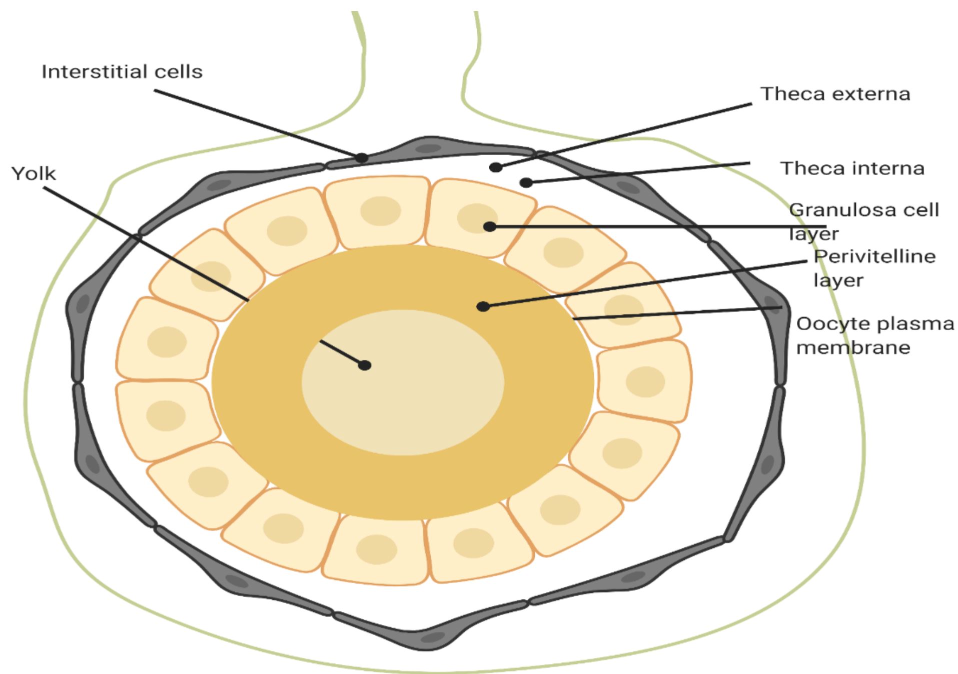

Figure 2 Cross-section diagram of an ovarian follicle of birds. Yolk, designated as oocyte cytoplasm, is concentrically surrounded by follicular tissue layers. Yolk components are incorporated from capillaries embedded in theca layer into oocyte cytoplasm by passing through gaps between the follicular tissues. Figure modifie from Murai (2013)

Egg Components and How They are Absorbed by the Chick's Embryo

A fertile egg contains all the essential nutrients a chick embryo needs (Vleck & Hoyt, 1991). All of these nutrients are very important and utilized to build new tissue, maintain existing tissue and muscular activity, and sustain development through hatching (Vleck, 1990). The egg's nutrients are furnished in the albumen and yolk, which comprise protein 48.5%, 45% lipids, and 3.7% carbohydrates on a dry matter (DM) basis, with exact composition varying depending on factors such as egg weight,

genetic strain, and hen age. The process of yolk fat utilization has been extensively studied. Sahan et al., (2014) observed a higher ratio of fat concentration in fresh yolk derived from old flock eggs (50 wk) compared to that of young flock eggs (30 wk). These results indicated that hens from an older flock deposited more yolk and fat in their eggs compared with eggs from a younger flock. This is associated with changes in yolk size and energy content of the yolk (Şahan et al., 2014).

The yolk represents approximately 70.6% of the total egg dry mass, accounting for 99% of the lipids and 47% of the protein present in an egg. The egg yolk is a primary nutrient source for the growth and energy production of embryos (Wallace, 1985). From the early stages of embryo formation until hatching, the embryos utilize egg materials for development (Wallace, 1985). Through the yolk sac membrane and the surrounding vascular system all the yolk nutrients are transported (Noble & Cocchi, 1990). The chick embryo uses nutrients and fats from the yolk sac to initiate body growth and development of the small intestine and other organs. This process increases after 12 d of incubation, at which time a rapid transfer of lipids from the yolk to the embryo begins (Speake et al., 1998). The yolk sac is drawn into the body cavity on d 19 of incubation, and the entire yolk residue is located within the abdomen at the end of 20 d of incubation. At hatch, about 30% of the original nutrients in the yolk are still present in the yolk residue constituting approximately 15% of the chick's body weight (BW) The main function of these nutrients is to serve as a source of energy for the hatchling during the first few days of life (Noy & Sklan, 2001).

The albumen is a relatively diluted source of nutrients, as it contains approximately 90% water (Freeman & Vince, 1974). The DM of albumen is 90% protein, and carbohydrates represent much of the remaining DM of albumen (7.8%). There is a small (0.26%) amount of fat in the albumen DM.

The eggshell of a chicken is covered with as many as 17,000 tiny pores. Eggshell is made almost entirely of calcium carbonate ($CaCO_3$) crystals. It is a semipermeable membrane, which means that air and moisture can pass through its pores. The shell also has a thin outermost coating called the bloom or cuticle that helps keep out bacteria and dust.

Contrasts Between Avian Igy and Mammalian Igg Immunoglobulins

In birds, there are three classes of immunoglobulins which are IgY, IgM and IgA. Functionally, IgY is dominant and generated mainly in secondary antibody responses working and behaving like mammalian IgG despite some differing biochemical properties (Davalos-Pantoja et al., 2000). IgY in serum exists as a monomeric form comparable to mammalian IgG. IgY has two heavy (H) and two light (L) chains joined by inter chain disulfide bonds (Figure 3), about 115 amino acid form the heavy and light chains. The heavy and light amino terminal domains are highly variable (V) and the pairing of V_H and V_L domains creates the antigen-binding site known as the Fab region, which confers antibody specificity. Each IgY molecule has two V_H–V_L pairs, therefore two antigen-binding sites. The other domains are referred to as the constant (C) region domains C_H and $C_{L;}$ this also refers to the FC region, confers biological properties for membrane transportation including half-life and secondary effector properties (Davison

et al., 2008). The heavy chains or the upsilon (υ) of the IgY possess one Vυ region, four C region domains (Cυ1 to Cυ4) and no genetic hinge. In the other side IgG_{γ} chains possess 1 V_{γ} region, 3 C domains ($C_{\gamma}1$ to $C_{\gamma}3$) and a genetic hinge between $C_{\gamma}1$ and $C_{\gamma}2$ (Figure 3), (Shimizu et al., 1992). The IgY Cυ3 and Cυ4 are homologues of the IgG $C_{\gamma}2$ and $C_{\gamma}3$, while the hinge region of IgG corresponds to the Cυ2 domain of IgY (Shimizu et al., 1992). The molecular weight of IgY is 180 kDa, it is heavier than IgG which is 150 kDa. The heavy chain of IgY has an additional constant domain instead of the hinge region of mammalian IgG. IgY is similar in this respect to mammalian IgE composed of four constant domains per heavy chain. Molecular analysis shows that IgY is the evolutionary precursor of both mammalian IgG and IgE (Warr et al., 1995). Comparison of the constant region sequences of avian IgY and mammalian IgG shows that Cυ3 and Cυ4 domains of avian IgY are most closely related to Cγ2 and Cγ3 domains of mammalian IgG (Parvari et al., 1988).

Additional structural differences between avian IgY and mammalian IgG include N-glycosylation patterns. Avian IgY contains two potential N-glycosylation sites located on the Cυ2 and Cυ3 domains. The one located on the Cυ2 domain (N308) is absent in mammalian IgG. The site located in the Cυ3 domain (N407) corresponds to the Cγ2 domain of mammalian IgG (N297). Spectrophotometer test indicates that the chicken IgY glycopeptides found in chicken Cυ3 domain contained only high-mannose-type oligosaccharides, whereas chicken Cυ2 domain contained only complex type N-glycan's (Suzuki and Lee, 2004). Mass spectrometry indicated that the N-glycosylation pattern of

avian IgY is more analogous to IgE and it appears to be slightly different than IgG (Murai, 2013).

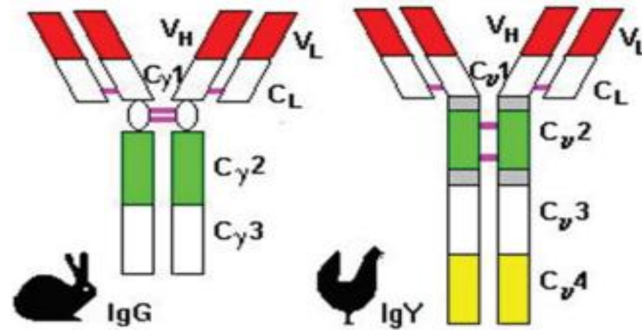

Figure 3 Structural differences between mammalian IgG and chicken IgY. Figure modified from Szabo et al (1998)

Each molecule has two heavy (H) and two light (L) chains joined by inter-chain disulphide bonds. The H chains of IgG are known as gamma (γ) chains, the H chains of IgY, as upsilon (u) chains. In both molecules the antigen-binding site is formed by the variable (v) region of the H and L chains. Remaining domains are known as the constant (C) region domains . In the rabbit, the gamma chains possess on v γ region , three C domains (C γ1 to C γ3) and a genetic hinge between C γ1 and C γ2. In the chicken , the upsilon chains possess one vu region, four C regain domains (Cu1to Cu4) and no genetic hinge. The chicken Cu3 and Cu4 correspond to the mammalian C γ2 and C γ3; the Cu2 domains of chicken IgY correspond to the hinge region of mammalian IgG. Figure modified from Szabo et al (1998).

Concentration of Igy In Egg Yolks and Newly Hatched Chicks.

Determination of IgY concentration in yolk and blood provides us insight into the existence of selective IgY transport mechanisms in ovarian follicles of birds. The concentration of IgY in egg yolks of chickens has been measured by many investigators with reported IgY concentration varying 1 - 25mg/g yolk (Hamal et al., 2006). It seems likely that the variability in yolk IgY concentration is caused by multiple reasons including differences in strains of chickens (Martin et al., 1990) and daily fluctuation (David et al., 2001). But, a major part of IgYs are probably used as a nutrient source in the development of the chick embryo. The reason for this thought is because of the big difference of the concentration of IgY in the yolk compared to the total amount of IgY in the hatched chick circulation (Carlander, 2002). Research has established IgY as the predominant Ig isotype transferred to the egg as well as to the newly hatched broiler chick. The amount of IgY deposited in the egg and the levels transferred to the offspring in 2 genetic meat lines of chickens were directly related to the circulating levels of IgY in the dam (Kitaguchi et al., 2008). The transfer expressed as the percentage of the dam's plasma IgY levels of 3-day-old chicks (approximately 30%) was similar in the 2 lines of chickens. This observation suggests that the amount of maternal antibody present in chicks is primarily influenced by the levels in the dam and argues against line differences in transport of IgY to the egg or from the egg to the offspring's circulation (Hamal et al., 2006). There is an alternative route of IgY transfer directly to the chick

embryo via the yolk stalk, which is through the highly vascularized yolk sac membrane (Noble & Cocchi, 1990). The yolk sac membrane is divided into two structural areas, an outer mesodermal layer of flattened cells, which performs a supportive role, and an inner endodermal layer of cylindrical epithelial cells, which is responsible for yolk absorption (Speake et al., 1998). The increase in post-hatch growth rate and concurrently the increase in metabolic rate of broiler chickens as a consequence of genetic selection during the last five decades also increased embryonic metabolism. The higher metabolic rate during incubation would imply a lower residual yolk weight and possibly lower energy reserve for the hatchling. This might affect post hatch development and performance. The residual yolk weight at hatch, and yolk utilization were studied by Van der Wagt et al., (2020). The researcher aimed to investigate whether residual yolk weight and composition has changed during the last eight decades, and which abiotic factors affect yolk utilization in poultry during incubation and the early post hatch period. They conclude that the residual yolk weight and the total solid amount of the residual yolk at hatch seem to have decreased in the recent decades. It cannot be concluded whether the differences between old and modern strains are due to genetic selection, changed management and incubation conditions, or moment of sampling. It is remarkable how genetic progress, improved management and incubation conditions over the last eight decades have impacted yolk utilization efficiency and embryonic growth. The factors especially affecting residual yolk weight at hatch include egg size and incubation temperature, whereas breeder age has more influence on nutrient composition of the residual yolk and yolk weight (van der Wagt et al., 2020).

Ontogeny of Immune System

The immune system development of embryonic and neonatal chickens is a dynamic process involving the migration of lymphocyte stem cells to primary immune glands. There is a rapid and extensive proliferation of lymphocytes in the absence of antigen within the bursa and thymus. Concurrent differentiation of bursal and thymus lymphocytes is also present in the process as well as the eventual seeding of differentiated lymphocytes to the peripheral lymphoid organs such as the spleen and cecal tonsils. Stem cell migration to the chick thymus occurs in three waves at 6.5, 12, and 18 days of embryogenesis, and in the bursa between 7.5 and 14 days of embryogenesis. Both lymphocyte sub-populations undergo somatic mutation during embryonic development. T-cells rearrange the T-cell receptor genes once and express the receptor proteins over days 12–14 of embryonic development. B-stem cells undergo rearrangements of the variable region of the immunoglobulin (the protein family containing antibodies and B-cell receptors) light and heavy chain genes and express surface IgM, the immunoglobin class composed of μ heavy chains that is the first to appear on B-cells and is the first to be secreted, as early as day 12 of embryonic development. Also, gene conversion is a typical avian source of antibody diversity which is extremely important. IgY, the immunoglobulin class composed of γ heavy chains that is the most abundant class of immunoglobulin in the plasma, is expressed by day 14 of embryonic development. The secondary immune organs, such as the spleen, cecal tonsils, and Meckel’s diverticulum, are incomplete at hatch. Although B-cells are

detected in cecal tonsils at hatch, only IgM is expressed. There are T-cells in the lamina propria and epithelium of the gut and other secondary immune organs, but they only develop cytotoxic ability sometime after hatch (Owen, 1972). In the early stages, the induction of a specialized differentiation of the Ig- producing cell system (plasma cells) is a function of the bursa of Fabricius, which develops as a hindgut lymph epithelial organ at day 5 of embryonic development. Plasma cell differentiation begins with the migration to stem cells from the yolk sac to the bursa, which provides the essential environment for maturation by days 12 to 13 of embryonic life. Within one or two days, stem cells differentiate into bursa lymphocytes, which synthesize and express IgM on their cell surface (Cooper et al., 1972) Bursa cells, however, do not secrete IgM, which is attributed to a lacking galactose attachment to the H chains. This detachment is a prerequisite for Ig secretion. In the absence of exposure to antigens, this cell population multiplies rapidly within the bursa, and after approximately 20 generations, IgY-producing cells arise from the IgM cloning of cells. IgA-forming cells are derived from cells formerly making IgY (Leslie & Martin, 1973). The bursa cells which were originally producing IgM may express residual IgM on their surface for a few generations following conversion to cells genetically programmed for IgG synthesis by antigenic stimulation (Cooper et al., 1972). This can be taken as evidence for a sequential IgM -> IgY -> IgA differentiation of bursa cells, although an alternative pathway of IgM -> IgA without passing through the IgY phase may occur (Bienenstock et al., 1973). The second stage of plasma cell differentiation is considered to begin when bursal lymphocytes migrate to the peripheral lymphoid tissue. Corresponding to the

sequence of differentiation within the bursa, IgM bearing bursa cells are the first to enter the circulation. The idea that all cells committed to synthesis of IgY and IgA originally came from IgM-synthesizing precursors may be useful in understanding the pathway of immunological deficiencies in chickens.

Baby Chick's Immune System or Immunology

Immunity in birds develops through their lymphoid system as it does in mammals. The immune system organs are classified as primary and secondary lymphoid organs. The bursa of Fabricius and thymus are primary lymphoid organs in which lymphocyte precursors develop into immune-competent native lymphocytes. These lymphocytes are a class of white blood cells that bear variable cell surface receptors for antigens and are responsible for adaptive immune responses. The two main types, B-lymphocytes (B-cells) and T lymphocytes (T-cells), function in the humeral immunity component of the adaptive immune system by secreting antibodies. B-cells mature in the bursa fabricius, and T lymphocytes, which mediate cell immunity, mature in the thymus gland.

In antigen recognition, a lymphocyte enlarges to form a lymphoblast and then proliferates and differentiates into an antigen-specific effector cell (Cooper et al., 1966). The secondary lymphoid tissues are the spleen, Harderian gland, bone marrow, pineal gland, and organized lymphoid tissues associated with mucosal surfaces, including bronchial-associated lymphoid tissues, gut-associated lymphoid tissues, conjunctival-associated lymphoid tissues, and other less well-organized groups of lymphoid cells in

various organs. These lymphoid tissues are located at strategic sites where foreign antigens entering the body from either the skin or a mucosal surface can be trapped and concentrated (Warner et al., 1969). Professional antigen-presenting cells (APCs) are highly specialized cells that can process antigens and display their peptide fragments on the cell surface in the context of MHC-II, together with co-stimulatory proteins required for activating naïve T helper cells. The main APCs for naïve T helper cells are dendritic cells. Other regulatory cells (T helper and T cytotoxic) are also located in these anatomically defined tissues and are thought to be organized into structures that optimize cellular interactions that support the efficient removal of unwanted pathogens.

Fate of the Immunoglobulins

Under normal circumstances, most proteins that have a large size (> around 60 kDa) are removed by pinocytosis, a process that occurs in all nucleated cells as they obtain their essential nutrients from plasma. This process explains the half-life or the fate of immunoglobulins IgA and IgM, but (IgY) has a concentration-dependent half-life of around 21 days due to recycling by FcRn receptors in case of mammalian, and FcRy in the case of birds (Kim et al., 2007). In mammals, transport of maternal IgG by the neonatal Fc receptor (FcRn) occurs *in utero* or after birth through uptake of IgG in ingested milk (Roopenian & Akilesh, 2007). FcRn receptors are found in most nucleated cells, even renal podocytes, which may account for the presence of IgG in the urine at high serum concentration (Akilesh et al., 2008). In this way, this receptor and the IgG immunoglobulin are transported from the pregnant mother to the fetus in the last trimester of pregnancy. However, non-mammalian species like birds and some reptiles

transfer maternal IgY to offspring via the egg yolk which is then transported across the yolk sac membrane into the embryonic bloodstream during late embryonic development (Kowalczyk et al., 1985). The yolk sac membrane of chicks expresses an IgY binding receptor (FcRY) with functional characteristics similar to FcRn (Tressler & Roth, 1987). Affinity purification of the IgY binding protein from chicken yolk sac and molecular cloning of its gene, revealed it to be a new class of Fc receptor (West Jr et al., 2004). FcRY lacks sequence and architectural similarity to FcRn or the Ig superfamily Fc receptors that recognize mammalian IgG, IgA, IgE (Nimmerjahn & Ravetch, 2006) or avian IgY (Viertlboeck et al., 2007). Instead, FcRY is the avian homolog of the mammalian secretory phospholipase A2 receptor (PLA2R), a member of the mannose receptor (Sunder et al.) family (Sunder et al.) (West Jr et al., 2004). FcRY is the only member of the MR family known to function as an Ig receptor, although other members of this receptor family participate in immune recognition. For example, MR binds pathogens via recognition of carbohydrates rarely found in mammalian glycoproteins, and the dendritic cell receptor DEC-205, another MR family member, regulates antigen presentation (Llorca, 2008). In common with PLA2R and other MR family members, FcRY is a type I membrane glycoprotein with a large ectodomain including 10 domains of known structure: an N-terminal cysteine rich (CysR) domain, a fibronectin type II (FNII) repeat, and eight C-type lectin-like domains (East & Isacke, 2002). A recombinant form of the FcRY ectodomain was shown to bind IgY and the FcY fragment of IgY with high affinity at acidic, but not basic, pH (West Jr et al., 2004). Full-length FcRY expressed in polarized mammalian epithelial cells functioned in

endocytosis, bidirectional transcytosis, and recycling of chicken FcY/IgY (West Jr et al., 2004) analogous to the functions of FcRn in epithelial and endothelial cells (Roopenian & Akilesh, 2007).

The Development of GI Tract In Chicks and the Nature of the Immune System of GI in Chickens

The gastrointestinal tract has a surface that is more exposed than any other part of the body and is always in contact with many different substances that could be harmful. The GI tract is made up of physical, chemical, immunological, and microbiological parts that work to protect the bird's tissues from its luminal environment. These parts have a delicate balance that can be negatively impacted by many factors related to diet and disease which could result in the decline of the bird's health and productivity. The weight of the embryonic small intestine increases at a much higher rate than body weight during incubation processes. The ratio of small intestine to body weight in the last three days of incubation increases from about 1% on day 17 of incubation to 3.5% at hatch (Uni et al., 2003). After hatching, the mass of the small intestine continues to grow faster than the rest of the body mass. Increases in the weight and length of the duodenum, jejunum, and ileum are similar (Noy & Sklan, 2001). Development of the intestine's enzymatic and absorptive activities is also very rapid after hatch (Uni et al., 1999). The newly hatched chick's small intestine is immature and undergoes the most dramatic changes during the first 24 hours and continues morphological, biochemical, and molecular changes for two weeks after hatching. The maturing process of the chicken small intestine is very similar to that of neonatal

mammals (Geyra et al., 2001). The residual yolk sac is internalized into the abdominal cavity toward the end of incubation. Much of the nutrition for the embryo is provided by the yolk directly through the circulation during incubation. The yolk material may directly reach the GI tract close to hatch and thereafter (Noy & Sklan, 1998). The yolk contributes to the development and maintenance of the small intestine during the first 48 hours post-hatch. At this time, the chick must transition to utilizing energy in the form of exogenous carbohydrate-rich feed rather than utilization of lipid supplied by the yolk (Noy & Sklan, 1999). The rapid development of the GI tract and associated organs is supported by the consumption of exogenous feed. After hatch, the timing and form of available nutrients is critical for intestinal development. The growth and development of the intestinal tract is stimulated and the post-hatch uptake of yolk by the small intestine is enhanced by early access to feed (Uni et al., 1998). It has also been demonstrated that the development of the intestinal tract may be enhanced by *in ovo* feeding to supply nutrients to the growing embryo. The intestinal development of chicks at 17 to 18 d of incubation is enhanced by increasing the intestinal capacity to digest disaccharides and by increasing the size of the villi when exogenous nutrients are administered into the amniotic fluid (Llorca, 2008). These observations indicate that the small intestines of *in ovo*fed chicks and conventionally fed 2-day-old chicks were functionally equivalent. Body weight of controls was less than that of in ovo fed chicks throughout the 10-day experiment (Tako et al., 2004). Injection of arginine and β-hydroxy-β-methyl-butyrate into the amnion of turkeys at 23 d of embryonic development resulted in improved

intestinal digestive and absorptive capacity compared to conventional poults (Foye et al., 2007).

When access to feed is delayed, birds show depressed performance and slower intestinal development (David et al., 2001). The transition time between hatching and placing chicks on feed and water often takes 24 to 72 hours (Uni et al., 1999). The depression in intestinal function and bird performance that results from the lack of access to feed at this time may not be overcome at a later stage in life (Geyra et al., 2001).

Genetic, physiological, nutritional, and environmental factors directly influence the overall organization and mechanisms of immunity in birds (Qureshi et al., 1998). To produce a protective immune response, several cells and soluble factors that compose the complex immune system of birds must work together. The intensive rearing conditions that most commercial flocks are raised under cause a properly functioning immune system to be of special importance to poultry. The rapid spread of infectious agents and disease outbreaks are a concern to flocks under such conditions (Sharma et al., 2003). The gut lining is the important interface between the bird and foreign material such as feed and microflora. These materials are capable of stimulating an immune response, although most of them are not pathogenic (Hughes, 2004). The immunological responses in chickens can be affected by the form of antigen (solid or dissolved), its rate of degradation in the gut, rate of distribution along the intestinal tract, and the rate of absorption (Phillips et al., 2000). Immune responses to GI antigenic stimulation are

energetically expensive, could have negative impacts on feed efficiency, and divert nutrients away from production(Santos & Ferket, 2006).

Rapid development of the GALT (gut-associated lymphoid tissue) occurs concomitant with the development of digestive structures and functions. The GALT is a component of the mucosal associated lymphoid tissue (MALT) as well as bronchial, salivary, nasopharyngeal, and genitourinary lymphoid tissues. The first line of defense on mucosal surfaces is MALT (Friedman et al., 2003). The intestinal arm of the immune system is represented by lymphoid structures that are distributed throughout the intestinal tract in a way that is not uniform across different segments of the intestine (Qureshi et al., 1998). The chicken's hindgut has numerous lymphoid follicles present, especially in the ceca, but the foregut has a relatively poor number of follicles (Friedman et al., 2003). Functioning of GALT is delayed as a result of delayed onset of feeding (Shira et al., 2005). The effects of short-term feed withholding (for the first 72 hours after hatch) on GALT development in newly hatched broiler chicks was investigated by Shira et al., (Borst & Conover). The GALT activity was determined by antibody production (systemic and locally in the gut), distribution of B and T lymphocytes in the gut, expression of lymphocyte-specific genes, and distribution of B and T lymphocytes in the bursa. GALT activity in the hindgut was significantly delayed during the first 2 weeks of life, although development of GALT in the foregut (duodenum, jejunum, and ileum) was only slightly and temporarily impeded by feed withholding.

The bursa of Fabricius plays a major role in antibody production in chickens. The development of the bursa and other GALT such as cecal tonsils and Peyer's patches

begins during late embryogenesis, (Kajiwara et al., 2003). Simultaneously, the bursal duct opens and transport of environmental antigens into the bursal lumen and further into the lymphoid follicles begins at the time of hatching. The earlier the feed passes through the GI tract, the sooner the proliferating.

The Role Of IL-8 & IL-17 on the Maturation of the Gut Immune System and Immune Response

There is a lack of natural flora providers in commercial poultry production because chickens are hatched in the relatively clean environment of the hatchery. Immediately after hatching, the interaction between the immune system of the gut and commensal microbiota begins and leads to a low level of inflammation characterized by increased interleukin-8 (IL-8) expression (Bar-Shira & Friedman, 2006). Chicken cecum was protected by increased expression of chicken β-defensins for the first 3 d of life, which dropped after 4 d (Amit-Romach et al., 2004). Physiological inflammation and maturation of the gut immune system is indicated by a transient increase in interleukin-8 (IL-8) and IL-17 expression, which could be observed in chicken cecum on day 4 of life. This results in the normalization of the gut immune system and infiltration of heterophils and lymphocytes into the lamina propria or the gut epithelium (Van Immerseel et al., 2002). Depending on the composition of gut flora, infiltrating lymphocytes develop further either in terms of changes in αβ T-cell receptor repertoires (Mwangi et al., 2010) or in terms of a decreasing ratio of αβ to γδ T lymphocytes in the lamina propria or the gut epithelium (Lillehoj & Chung, 1992). Gut microflora has been reported to induce the Th1 and Th17 arms of the immune response in mice, but not in chickens, with IL-17

playing an important role in the maturation of the murine gut immune system (Ivanov et al., 2008). In mice, IL-17 is induced after *S. enterica* serovar Typhimurium infection (Raffatellu et al., 2009), and interestingly, IL-17 has been shown to be important for defense against bacterial and fungal pathogens (Curtis & Way, 2009). This means that the patterns of immune response to pathogens and commensals may overlap, the latter being a subset of the former. Especially in the early days of life, a detailed understanding of these responses may allow active modification of gut microbiota composition and potentiation of the immune response against particular bacterial or fungal pathogens.

Effect of Nutrients on Immune System Function

Immune response of an animal can be impacted by specific nutrients. Depending on the level of other nutrients in the diet, energy restriction of birds has a varying effect on immune function. Feeding a calorie-deficient and amino acid-sufficient diet resulting in over-consumption of amino acids has been associated with decreased antibody responses (Cook, 1991). The specific (Fritsche et al., 1991) and the inflammatory (Korver et al., 1998) aspects of the immune response can be significantly affected by the fatty acid composition of the diet. These actions are mediated largely through the activity of eicosanoids, which are metabolites of 20-carbon PUFA. When certain eicosanoids are derived from n-6 PUFA (prostaglandin E2 and leukotriene B4) they have much higher potencies as pro-inflammatory mediators than do the corresponding eicosanoids derived from n-3 PUFA (Prostaglandin E3 and leukotriene B5). The release of pro-inflammatory cytokines from effector cells such as macrophages as well as the effect of those cytokines at the level of the target tissues can both be affected by the

eicosanoids. The n-6 PUFA tend to have a pro-inflammatory effect while the n-3 PUFA tend to have an anti-inflammatory effect. Antibody response, mitogen-induced blastogenesis and mixed-lymphocyte reactions in mice can be decreased by copper deficiency and primary antibody response increases with addition of copper to poultry diets (Cook, 1991). Immune functions in mammals and poultry are suppressed by zinc deficiency (Cook, 1991). The requirement of methionine for maximum antibody titers has been shown to be greater than that for growth (Tsiagbe et al., 1987). Many nutrients are very important in early development of the immune system. For example, vitamin A is necessary to maximize immune competence for optimum growth and feed efficiency (Sklan et al., 1994). Additionally, there are several nutrients, which can affect early immune development such as linoleic acid, iron, selenium and specific B vitamins (Korver et al., 1998). Some vitamins have regulatory roles in the immune system such as vitamins A, D and E (Cook, 1991). The immune response complex requires vast communication with immune cells and immune molecules. Eventually, through the nutrients ingested by the host, all these mediators are derived. Some of the mediators are derived from dietary fatty acids that can have altered potency based on the precursor molecule (Korver et al., 1998). The early nutrient supplement of polyherbal organisms (*Phyllanthaus emblica, Terminalia chebula*) to broiler chicks enhanced the immune system. The adaptogenic immune modulation and immunopotentiating properties increase their potency by using these herbs (Kadam et al., 2009).

Effect of Feed and Water Status on Body, Intestine, and Muscle Development in Chickens

The development of the gastrointestinal system plays an essential role in the early stages of the chick's growth (Nitsan et al., 1991). In practice, the feeding of chicks is delayed 10 to 60 h through hatching and transportation procedure (Noy & Sklan, 1996) During the fasting period, the nutrients in the residual yolk supply the lack of food (Bigot et al., 2003). However, these nutrients represent an insufficient contribution to the nutritional requirements for both maintenance and growth in today's broilers chicks (Bigot et al., 2003). In the first few days of life, delayed feeding causes a decrease in the final body weight (Noy & Sklan, 1999) as well as adverse effects on the immunological capacities (Dibner et al., 1998). Early feeding is recommended in order to reduce these effects. The rate of higher initial growth in delay-fed chicks, induced by feed intake after two days of fasting, remained insufficient to overcome the consequences of the delay on body weight at 6 d of age. It is likely that this occurs in the longer term as well (Noy & Sklan, 1999). The relative body weight gain reached similar levels after 4 d of age in both early-fed and delay-fed chicks. The authors state that the growth mechanism released post-fast was probably not compensatory growth. Part of the body weight variation in delay-fed chicks immediately after feeding was not actual body weight but rather larger amounts of food stored in the crop. This was greater in delay-fed chicks than in early-fed chicks due to hunger, which developed during fasting. The rate of maximal growth is obviously attained when chicks are fed (Murakami et al., 1992).

Intestinal growth during the fasting period and the weight of intestinal tract increased during the fast period but remained lower than early fed chicks even after access to feed (Bigot et al., 2003). Post-hatching fasting delayed pectoral muscle weight gain and weight increase occurred only after chicks had access to feed. Intestinal growth during post-hatch fasting showed that this growth may be preferential in early development in chicks compared to muscle growth (Noy & Sklan, 1999). Chicks that had access to feed in the initial post-hatch period had increased yolk sac absorption through the intestine by greater intestinal development, mainly because of increased peristaltic activity of the intestine (Noy et al., 1996). The researchers observed no difference in yolk sac resorption between early-fed and delay-fed chicks despite differences in intestinal tract growth between the two groups (Bigot et al., 2003). Noy et al. (1996) observed variations in the amounts of yolk utilized between the studies. They found that fast duration, water availability, or both might explain these. The yolk sac nutrients might play a complementary role rather than exacerbate the lack of food and nutrients and represent an insufficient contribution to the nutritional requirements for both maintenance and growth of chicks. It also plays the role as a main source of maternal antibodies of passive natural humoral protection until they have a mature immune response (Bigot et al., 2001).

Effect of feed and water status on energy utilization in chicks

During embryonic development, energy is derived from the yolk (Romanoff, 1960). Twenty percent of a newly hatched chick's body weight consists of the yolk, which provides immediate post-hatch energy for the chick (Romanoff, 1960). The yolk

is used for maintenance and exogenous nutritional energy is utilized for growth (Anthony et al., 1989). Chicks must undergo metabolic adaptations while moving from embryonic yolk dependence to utilization of exogenous feed. Pancreatic and brush border enzymes must be available in sufficient quantities for digestion and furthermore the uptake processes must transfer the required quantities of nutrients. The presence of pancreatic enzymes in the intestine have been observed during late embryonic development (Marchaim & Kulka, 1967). Secretion of pancreatic enzymes per gram of feed intake vary little at 4 d of age (Uni et al., 1996). Digestion of starch, protein, and fat was 85%, 78%, and 87%, respectively (Noy & Sklan, 1995). Chicks with delayed access to feed fall behind in body weight by 7.8% in the 48 h post-hatch period, which was equivalent to 5.3 kcal/45 g chick/d. However, during this period small intestine weight and protein content increased by 80% or more (Noy et al., 1996) . Decreases in yolk fat and protein could account for most of the changes in body composition in the feed-deprived chicks. In contrast, fed chicks grew by 5 g and used 4.5 kcal/d for maintenance. During this period small intestines increased in weight by 110 % (Jin et al., 1998). Noy and Sklan, (1996) examined nutrient absorption from hatch through 4 d by administration of a bolus of labeled glucose, methionine, or oleic acid, together with a no absorbable reference substance. The absorption rate of fatty acid was more than 80% at hatch and was higher than that of glucose and methionine, which appeared to be low especially when the bolus was administered as a solution. Absorption of all the tested nutrients increased with age and were more than 80% at 4 day. In hatching chicks, duodenal in situ uptake of oleic acid from yolk and saline solutions was high compared

to glucose and methionine solutions. The chicks exhibited low uptake from yolk but higher uptake from saline solutions (Noy & Sklan, 1996).

Effect of feed and water status on immune function

In commercial poultry operations, hatch is distributed over a 2-day period and the chicks are transferred from the hatcher only when the majority of them clear the shell. Other practices such as sexing, vaccination and packaging are carried out following removal from the hatcher but only before they are boxed for transportation. Potential for poor viability and retarded growth occurs in practice because some chicks may spend 36-48 h without initial access to feed or water. A critical period in the development of hatchling poultry is the time from hatching to the onset of receiving nutrition. The response of birds to a vaccination can be enhanced by short-term feed restriction (12-24 h) relative to fasted or *ad lib* fed birds (Cook, 1991). Deleterious effects on the immune response associated with increasing levels of corticosterone can result from longer periods of restriction or fasting (up to 48-72 h). Early feeding can help increase immune response through antibody production. Early feeding allows antigens to enter the chick's digestive system thus leading to production of antibodies. Most development of immune tissue occurs at the late incubation period, and in the early part of life. Therefore, maternal immunity and early nutrition play important roles in immune system modulation. The antibody titer against Newcastle disease after vaccination (when chickens are at 21 days of age) was higher in the broiler chicks fed in earlier life than chicks fed after 48 hours post-hatch (Sunder et al., 2007).

From the beginnings of the chick embryo, the avian immune system continues growing for many weeks after hatching. Several different factors influence the early development of an immune system in a newly hatched chick, and one of the most important factors is initial feed intake (Dibner et al., 1998). The transition from use of yolk sac-derived nutrients to those present in exogenous feeds provides the correct platform for rapid functional and structural development of the gut (Geyra et al., 2001). Feed provides nutrient substrates, which is essential for growth and development of both primary and secondary lymphoid organs (Dibner et al., 1998). The immune system of the hatchling, particularly the mucosal immune system, also requires oral feed intake for rapid development (Shira et al., 2005).

Early feed restrictions were reported to produce metabolic changes leading to impaired systemic immune competence most likely due to depleted lymphoid organ growth (Dibner et al., 1998; Panda et al., 2010). The thymus, is very sensitive to the time of feed deprivation, and causes a rapid decline in its cellularity and weight gain (Klasing, 2007). Feed deprivation beyond 48 hours in chicks impaired the activity of gut associated lymphoid tissue in the hind gut and delayed the development of lymphocyte populations in the bursa during the first two weeks of life (Shira et al., 2005).

The avian immune system begins during embryogenesis, but is not developed until a few weeks of age post-hatch, and can be undersized due to the unavailability of nutrients if hatchlings are deprived of food immediately after hatch. Early feeding is not only connected with immune organ development, but also with the functioning of the immune system in broiler hatchlings. With the continued rise in the economic

importance of broiler chickens, comprehension of the development and function of the immune system in these birds and their capacity to respond effectively to divergent antigens is necessary (Panda et al., 2010).

CHAPTER II

EFFECT OF NEONATAL FASTING ON THE ASSIMILATION OF MATERNAL IMMUNITY (IGY) AND YOLK ABSORPTION

Introduction

The role of maternally derived antibodies is important to the health of newly hatched chicks. The protection of newly hatched chicks against diseases depends on their innate immunity and maternal antibodies in the yolk sac, acquired in a passive manner from the hen (Tizard, 2009). It has been demonstrated that maternal antibodies of isotope IgY, are transferred from hens vaccinated against different diseases, like multiple virus vaccines to their chicks. The maternal antibodies are capable of protecting chicks against the challenge of the disease for a period of up to 4 weeks (Darbyshire & Peters, 1985), (Chhabra et al., 2015).

In modern poultry production, the separation of the hatchery from the production facility means that the hatchling will spend a period without the provision of feed or water. The time period between processing and placement is highly variable. It depends on the availability of transport equipment, distance to the placement facility, and hatchery practices. In some parts of the world, producers strive to place the neonates for 12-24 hr. to allow them to mature and to initiate a vaccine response while the birds are under a low immunological challenge from other antigens. In other parts of the world, the practice is to hold the birds a period of a few hours. This reduces stress and gives the birds access to feed and water. Often the producer has no options, for example during

shipment of birds over long distances. Rest and recovery from processing may have an additional benefit.

Just before the hatching process begins, the bird internalizes what is left of the yolk sac. The residual yolk protein is the source of antibodies from the hen (Larsson et al., 1993). To be effective, maternal antibodies must not only move from the residual yolk into the bloodstream but must also diffuse to sites of vulnerability, in particular to the mucosal surfaces where organisms are most likely to enter the body. Thus, a delay in placement can leave the hatchlings better able to respond to the environment once they are placed. The attendant problem is that birds are generally not fed in the hatchery (even when held overnight), nor are they fed during transport. Producers may feel that feeding is not essential during this period because conventional wisdom says that the bird can survive on its residual yolk (Esteban et al., 1991). The hatchling may indeed depend, in the absence of other feed, upon its use of residual yolk as a nutrient source (Noble & Cocchi, 1990). However, access to feed between 8-24 h post-hatch supported faster utilization of residual yolk compared to those chicks that remained unfed for 40-48 h (Bhanja et al., 2009).This may be a valid point but does not completely represent the objectives of modern chick or poultry production. Producers want the high weight of chicken in a short time, which intuitively would favor short holding times and early feeding.

There is interest in intermittent fasting and fasting in general in support of health benefits in humans. Much research has been conducted on human and few onanimals. The results have been associated with a wide array of potential health benefits, including

improving blood sugar control, heart health, brain function and cancer prevention in general health (Collier, 2013). Information about the effect the fasting on maternal immunity or the immune system, in general, is lacking. Prolonged fasting may negatively interfere in the absorption of immunoglobulins (Igs) because in order to take care of their physiological needs, birds under a prolonged fasting period may metabolize Igs of the yolk sac in order to obtain energy.

This research was conducted in order to characterize the maternal antibodies and the yolk sac absorption after a period of fasting. The hypothesis is that the level of plasma antibodies in chicks fasted after hatching decrease sharply after four days without food because the chicks will catabolize the immunoglobulins as a source of nutrients.

Hypotheses

This research will be conducted in order to characterize the maternal antibodies and the yolk sac absorption after a period of fasting and the intestinal expression of interleukins that are responsible for the immune system maturation of the intestinal tract. The hypothesis is that the level of antibodies in chicks fasted after hatching will decrease sharply after four days without food because the chicks will catabolize the immunoglobulins as a source of energy. In addition, the yolk sac of the chicks not fed will have less weight and volume because the chicks will absorb the egg yolk faster than the chicks with access to feed and water.

Material and methods

Eight trials were conducted, each lasting 4 days. There were three treatments: fasting, feed and water, and water only. From each treatment, 7 chicks were utilized for body weight, yolk sac, and serum IgY measurement. In the last 3 experiments, gene expression for IL-8, IL-17 and TNF- alpha were measured in the cecal wall . RNA extraction and gene expression will be described in detail in the next chapter. From experiment 4 to experiment 8 an additional 7 birds per treatment were utilized for blood volume estimation.

Ninety fertile eggs (Ross 708) at d 18 of incubation were obtained from a local hatchery (Sanderson Farms, Bryan TX). The eggs were incubated at Texas A&M Poultry Science center. Incubation continued at a temperature 98-100F, and 75 to 85 percent relative humidity. Hatching was monitored closely and hatchlings were removed every 2 hours. The chicks utilized for the trials hatched within the same 2-hour time period near the peak of the hatching period. At hatch, chicks were weighed, wing banded, and randomly assigned to treatments. The research was conducted under an approved animal use protocol from the Texas A&M IACUC.

On day 0, three chicks were taken randomly from each group and euthanized, to measure their yolk sac weight. The concentration of maternal antibodies (IgY) in the yolk was measured to establish IgY levels at hatch. Intestine samples were taken and saved in RNAlater™ (Invitrogen, Carlsbad, CA) to process them for qPCR.

Blood samples were collected from jugular vein in the first hour of hatching from all groups and held in tubes without heparin or EDTA. Alcohol 70% was used to

disinfect the region, and 1-ml disposable syringes were used to collect the blood, which was stored in a sterile 3ml tube. After clot formation samples were centrifuged samples at 2,000-x g for 10 minutes, serum was collected, and stored at -20 ° C until further analysis.

Chicks were housed in separate adjacent floor pens (4 square feet for 14 birds) by treatment. The room was thermostatically controlled at a temperature of 29.4 -31.18 degrees Celsius , lighting was provided for 24 hr. Feed was provided as a crumble starter feed during all experiments. Body weights were recorded on d 0 and 4. Feed and water consumption were measured.

On day 4, 7 birds of each group were bled (via jugular vein) for ELISA test to measure the IgY concentration in the circulation.

In an attempt to determine the relative amount of IgY in the total circulatory system of chicks, the product of serum volume estimates, and serum IgY concentrations were calculated. Three hundred thirty-six blood samples for 3 groups (fasting, feed and water, and only water) were collected over 8 experiments. Sandwich ELISA plates pre-coated with anti- IgY chicken monoclonal antibody (Abcam, Cambridge, MA) was utilized. Abcam's IgY Chicken ELISA kit is an in vitro enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of IgY in Chicken serum and plasma samples. In this assay the IgY present in samples reacts with the anti-IgY antibodies which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-IgY antibodies conjugated with horseradish peroxidase (HRP) are added. These enzyme-labeled antibodies form

complexes with the previously bound IgY. Following another washing step, the amount of enzyme bound in complex is measured by the addition of a chromogenic substrate tetramethylbenzidine (TMB). The quantity of bound enzyme varies proprotionately with the concentration of IgY in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of IgY in the test sample. The quantity of IgY in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample (Abcam 2019) dilution .The assays were conducted with reagents, according to the manufacturer's standard protocol. Five µl were transferred to 495 µL of 1X normal saline diluent (for a 1/100 dilution). The samples were further diluted by transferring 2 µL to 998 µL of 1X diluent for a final dilution of serum 1/100. Each sample was run in triplicate. The concentration levels were obtained from an ELISA reader (BioTek, Winooski, VT) at 405 nm. Concentration was calculated by interpolating the blank control subtracted absorbance values against the standard curve provided by the manufacturer.

On day 0, and day 4, residual yolk IgY concentration was measured. After measuring the volume of the yolk sac, the egg yolks were collected from the residual yolk sac, then 2 volumes of cold (4°C) 1X Cell Extraction Buffer PTR to 1 volume of the egg yolk were added and homogenized for 1 minute using a blender at high speed (BIOSPED PRODUCTS, Bartlesville, Oklahoma). Samples were centrifuged at 16,000 x g for 10 minutes at 4°C. Supernatant was collected for assay. The egg yolk suspension was pre-diluted to 1:100,000 or 1: 1,000,000 into 1X Cell Extraction Buffer PTR (Abcam, Cambridge, MA). Un-diluted egg yolk suspensionwas stored at -20ºC. The

samples were thawed and diluted 1:100,000 by sample diluent normal saline. Total yolk sac IgY was estimated by sandwich ELISA test as previously described for the serum.

On d 4, the blood volume of 7 chicks per treatment was estimated using the Evan's blue dye method (Ertl et al., 2007). Briefly, blood was drawn from the left wing vein for the undyed sample. Next, 0.3 mg of dye (1mg/ml) per kilogram of body weight were injected i.v. an . After allowing 10 min of thorough mixing of the dye, 300 µl of blood was drawn from the right wing vein. Two hundred µl were diluted with 800 µl of 0.9 % saline. The remaining 100 µl was used to measure hematocrit. The optical density was read at 620 nm and blood volume estimated using the following formulas.

PV (plasma volume) = M*K/D.

In which M = mg of dye injected, K = Constant, whose value is determined by dividing the density of different concentrations of dye in plasma by the concentrations used, and

D = Density reading of the dyed sample from the spectrophotometer.

PVCF =%PV/8+(PV*2) which is Plasma Volume Correction Factor

In which % PV is the percent plasma volume from the hematocrit reading. The value thus obtained is multiplied by the PV of the previous formula in order to determine the True Plasma Volume (TPV): TPV = PV*PVCF. The Total Blood Volume (TBV) was calculated from the formula:

TBV= TPV/P

In which P is the corrected volume of plasma per 100 ml of blood calculated as the follows: P= [Volume of cells] * 100/Volume of cells and plasma.

On d 4, serum percentage in blood volume of each chick in each group was estimated. After blood samples were collected from jugular or brachial veins on day 4 post- hatch from all groups to evaluate the concentration of IgY in the serum. The samples were collected in tubes without heparin or EDTA. After clot formation, the samples were centrifuged at 2,000-X g for 10 minutes and serum collected. The serum volume was measured of each sample by using the micropipette, then percentage of the serum of each sample was recorded. For instance, the blood volume was 300-μL blood, and the serum was 150 μL, the percentage of the serum will be 50%. After that the total blood volume was multiplied X serum percentage = serum volume of each sample. Then, the concentration of IgY in 1mL X serum percentage in all blood volume was multiplied to get the total IgY in circulation for each group.

Results and discussion

Table 1 shows the impact of the treatments on body weights at 0 and 4 d. No differences in body weight were evident at d 0 but birds receiving feed and water were significantly heavier at d 4 than those receiving water only or fasting.

Table 1 The Body weights at day 0 and 4– Individual1 ("Identification of a differentiation stall in epithelial mesenchymal transition in histone H3–mutant diffuse midline glioma,")

Treatment	*Day 0*	*Day 4*
Fasted	45.0	34.5 b
Water only	44.9	36.0 b
Fed	44.9	80.5 a
SEM2	0.50	0.90
P	0.9831	<0.0001

[a–b] Means within a column lacking a common superscript differ significantly ($P < 0.05$).
[1] Each value is the average of 8 trials each with 7 chicks per treatment (n=56).
[2]SEM=standard error mean.

Table 2 shows feed and water intake, the chicks at day 4 consumed an average of 81.5 g of feed per chick Chicks in the feed and water groups consumed more water (151 ml) per chick compared to the water only group (52 ml) per chick. These results were consistent with Osborne and Mendel, (1915) and Palvink and Hurwiz, (1985). Their results show that severe feed restriction for broiler chicks during the first few days of age will have negative effects on body weight until market age. These results were also consistent with Henderson et al., (2008); their results show that early feeding can have significant effects on early growth, and the dependence of chicks on residual yolk sac during the first few day's post hatch limits the growth potential of

modern broilers. Body weight, feed and water consumption of the fed birds are within expected values for birds under these treatments.

Table 2 Feed and water consumption[1]

Day 4	*Feed Consumption*	*Water Consumption*
Fast	n/a	n/a
Water	n/a	52 ml
Feed	81.5 g	151 ml

[1] Each value is the average of 8 trials each with 7 chicks per treatment (n=56).

Table 3 shows absolute yolk sac and relative yolk sac weight at d 4. Fasted birds had significantly heavier yolk sacs than any other treatment. There was no difference in yolk sac weight between birds fasting and those receiving only water. The yolk sac expressed as a percentage of body weight was significantly greater for the fasted birds compared to the birds receiving water only. Relative yolk sac weight of the birds receiving water was significantly greater than that of the fed birds. This is a combination of the effects of the treatments on body weight and the residual yolk sac weight, further indicating that the fasted birds were not utilizing yolk sac material at the same rate as the fed birds.

Table 3 The residual yolk weight at day 4 and the relative yolk weight with percentage of body weight[1].

Treatment	*Absolute (g)*	*% of BW*
Fasted	0.596 a	0.017 a
Water	0.517 a	0.013 b
Fed	0.369 b	0.005 c
SEM2	0.045	0.001
P	<0.0019	<0.0001

[a–c] Means within a column lacking a common superscript differ significantly ($P < 0.05$).
[1] Each value is the average of 8 trials each with 7 chicks per treatment (n=56).
[2]SEM=standard error mean.

These data indicatethat without ingestion of feed or water, the utilization of yolk sac material is significantly reduced. Noy, et al., (1996) noted that the utilization of yolk by the newly hatched chick was dependent on peristaltic activity of the intestine. It is apparent that the fasted birds did not initiate this peristaltic action in the absence of feed and water. Because this movement (peristaltic) was absent, the amount of yolk that would enter the intestine was less than the amount of yolk that would enter with peristaltic movement. Therefore, the relation is inverse between the yolk weight and the state of fasting. The more fasting there is, the less yolk will be absorbed and the weight of the yolk sac would be higher. This result is similar to previous results by (Noy et al., 1996) and (Noy & Sklan, 2001). They reported that yolk utilization was more rapid in

fed than in fasted chicks suggesting that the transport of yolk material to the intestine could be increased by the greater intestinal activity found in fed chicks.

Serum IgY titers on 0 and 4 d are shown in Table 4. Titers did not differ among the treatments on d 0. On d 4, titers in the fasted birds were significantly higher compared to those receiving only water, which were in turn significantly greater than the titers of the fed birds. It is without question that the hydration status of the fasted birds is drastically different from the fed birds on d 4. These results are supported by those of Aarcon et al., (2011) who stated that the increase in the titer of antibodies of the fasting birds during fasting was probably due to a reduction in the plasmatic volume as a consequence of the chronic deprivation of water.

Table 4 The IgY concentration of serum[1].

Treatment	*Day 0 (mg/ml)*	*Day 4 (mg/ml)*
Fasted	3.13	8.194 a
Water	2.72	7.423 b
Fed	2.99	2.969 c
SEM2	0.148	0.465
P	0.1662	<0.0001

[a–c] Means within a column lacking a common superscript differ significantly ($P < 0.05$).
[1] Each value is the average of 8 trials each with 7 chicks per treatment (n=56).
[2]SEM=standard error mean.

In trials 5-8, the Evan's Blue dye method was utilized to estimate the blood volume on d 4 as described previously (Ertl, et al., 2007). The fasted birds had an estimated blood volume of 3.07 ml per bird (Table 5), the fed birds had an estimated blood volume of 9.32 ml per bird and the birds receiving water only had an estimated blood volume of 3.57 ml per bird. While these estimates may have some confounding factors, they clearly indicate that the hydration status, and therefore blood and serum volume of the fasted birds were considerably reduced compared to the fed birds. These results ae supported by previous research (Kotula and Helbacka, 1968) that stated that the normal blood volume is approximately equal to nine percent of the chick's body weight. The results of the present studies are consistent with Kotula and Helbacka (1968), the percentage of each treatment was close to 9% of total body weight.

The percentage of serum was significantly different among the groups (Table 5). The fed group serum volume was higher compared with the water group serum volume. The water group serum volume was higher compared with the fasting group serum volume. These results look logical, depending on the blood volume. This indicates that the percentage of serum in the blood is proportional to the volume of the blood. However, there were no significant differences among the treatments in the total IgY in circulation (Table 5). These results compare favorably with those of (Aarcon et al., 2011), they also reported that a period of up to 72 hours of post hatch fasting did not alter the decay of maternal antibodies and did not reduce the IgY titer; this again indicates that the antibodies were not utilized as a nutrient source.

Table 5 Total IgY in circulation and the percentage of the serum volume[1].

Treatment	*Blood volume*	*% serum*	*Total IgY(mg)*
Fasting	3.07 ml c	46.6 c	14.4
Water	3.57 ml b	56.6b	14.4
Feed	9.32 ml a	68.9a	13.7
SEM2	0.077	0.18	0.75
P	<.0001	<0.0001	0.7412

[a–c] Means within a column lacking a common superscript differ significantly ($P < 0.05$).
[1] Each value is the average of 8 trials each with 7 chicks per treatment (n=56).
[2]SEM=standard error mean.

Conclusion

The fasting chicks did not use the immunoglobulins as nutrients at a rate different from fed birds as measured by total circulating IgY levels. The total circulating IgY levels were not different among the treatments.. In the fasted birds the IgY was not reduced after fasting.

The reasons for the IgY concentration in the fasting group was higher (per mL) than the feed, and water group, because the blood volume of this group was less than the others. Therefore, when the concentration was measured (per mL), it was logical that the concentration was higher. Nevertheless, the total IgY was measured in all the serum of blood volume, the total IgY was not different.

The fasted birds were not utilizing the yolk sac material at the same rate as the fed birds. This indicates that without the ingestion of feed, the utilization of yolk sac material is significantly reduced.

The utilization of yolk by the newly hatched chick was dependent on peristaltic activity of the intestine. It is apparent that the fasted birds did not initiate this peristaltic action in the absence of using this movement (peristaltic) was absent; the amount of yolk that would enter the intestine was less than the amount of yolk that would enter with peristaltic movement.

Therefore, the relation is inverse between the yolk weight and the feed and water. The more fasting there is, the less yolk will be absorbed and the weight of the yolk sac would increase.

However, through the highly vascularized yolk sac membrane, there is another way to transfer IgY directly to the chick embryo, rather than yolk stalk.

The average yolk sac weight on day 0 was 2.73 grams but the fasting group's yolk sac weight was less on day 4 even without peristaltic movement. However, the weight of the fasting group's yolk sac weight was heavier than the other two groups (feed & water, and water only). This reveals how the fasting chicks are impacted by the highly vascularized yolk sac membrane.

There are two different structural areas that make up the yolk sac membrane, an inner endodermal layer of cylindrical epithelial cells, which is responsible for yolk absorption, and an outer mesodermal layer of flattened cells, which performs a

supportive role. This will help us understand why the weight of the yolk sac decreased on day four, compared with yolk sacs of all groups at day zero.

CHAPTER III

THE GENE EXPRESSION FOR IL-17, IL-8 AND TNF-ALPHA

Introduction

In modern poultry production, the separation of the hatchery from the production facility means that the hatchling will spend a period without the provision of feed or water. The time between processing and placement is highly variable. It depends on the availability of transport equipment, distance to the placement facility, and hatchery practices. In some parts of the world, producers strive to place the neonates for 12-24 hr to allow them to mature and to initiate a vaccine response while the birds under a low immunological challenge from other antigens. In other parts of the world, the practice is to hold the birds a period of a few hours. This reduces stress and gets the birds to feed and water. Often the producer has no options, for example during shipment of birds over long distances. Rest and recovery from processing may have an additional benefit. Just before the hatching process begins, the bird internalizes what is left of the yolk sac. The residual yolk protein is the source of antibodies from the hen (Larsson et al., 1993).

After all this time, there will be a significant lack of natural flora providers in commercial chicks, because the chicks are hatched in the relatively clean environment of a hatchery. Physiological inflammation and maturation of the gut immune system is indicated by a transient increase in interleukin-8 (IL-8) and IL-17 expression, which could be observed in chicken cecum on the fourth day of life. This results in the

normalization of the gut immune system and infiltration of heterophils and lymphocytes into the lamina propria or the gut epithelium (Van Immerseel et al., 2002).

Gut microflora has been reported to induce the Th1 and Th17 arms of the immune response in mice, but not in chickens, with IL-17 and IL-8 playing an important role in the maturation of the murine gut immune system (Ivanov et al., 2008). This means that the patterns of immune response to pathogens and commensals may overlap, especially in the early days of life.

Therefore, it is prudent to study the effect of fasting on the immune system, in the intestine of the early hatching chicks. Especially, the expression of the genes (IL-8, IL-17, and TNF-alpha), because of their importance in the maturity and inflammation in the intestine of the baby chicks.

Hypotheses

The level of the IL-8, IL-17 and TNF expression will be lower in chicks that have not been fed compared to those receiving feed and water.

Materials and Methods

Three trials were conducted, each lasting 4 days. There were three treatments: (Fasting, Feed and Water, and Water only). From each treatment, 7 chicks were utilized for body weight, yolk sac, serum IgY measurement, and gene expression of IL-8, IL-17 and TNF in cecal wall.

Ninety fertile eggs (Ross 708) at d 18 chick of incubation were obtained from a local hatchery (Sanderson Farms, Bryan TX). The eggs were incubated at Texas A&M Poultry Science center. Incubation continued at a temperature 98-100F and 75 to 85 percent relative humidity. Hatching was monitored closely, and hatchlings were removed every 2 hours. The chicks utilized for the trials hatched within the same 2-hour time period near the peak of the hatching period. The research was conducted under an approved animal use protocol from the Texas A&M IACUC.

On day 0, three chicks were taken randomly from each group and euthanized. Intestine samples were taken and saved in RNAlater^{TM} (Invitrogen, Carlsbad, CA) to process them for qPCR to establish the gene expression level at hatch.

Chicks were housed in separate adjacent floor pens (4 square feet for 14 birds) by treatment. The room was thermostatically controlled at a temperature of 85-88 degrees Fahrenheit; lighting was provided for 24 hr. Feed was provided as a crumble starter feed during all experiments. On day 4 all groups were euthanized. Intestinal samples from three chicks from the fed and fasted groups were saved in the RNA later ^{TM} (Invitrogen, Carlsbad, CA) to process them for qPCR.

The total RNA extraction and cDNA synthesis were measured. The cecum samples were collected, and were directly added to 1ml Trizol reagent (Ambion, life technologies) in homogenizing tubes. The tissue was homogenized in a bead beater at 1500 rpm for 60 seconds. The RNA extraction was performed as described in the manufacturer’s protocol, and the RNA pellet was dissolved in nuclease free water. The RNA was validated for its quality and quantity using Nanodrop. The total RNA (500ng)

was used to synthesize cDNA using Superscript* II Reverse Transcriptase (Invitrogen) as per manufacturer instructions. cDNA synthesis was performed immediately following RNA extraction using the High Capacity cDNA Reverse Transcription Kit (SuperScript ™ II RT, London, United Kingdom). The manufacturer protocols were followed using 500 ng of each respective RNA sample. cDNA was stored at −20◦C until analysis.

RT-qPCR was measured. To analyze the cytokine expression during fasting and feeding, the expression of cytokines *i.e.* Interleukin-8 (IL-8), Interleukin-17(IL-17), and Tumor Necrosis factor alpha (TNF) was performed. qPCR analysis used the SYBR® Green master mix (Applied Bio systems, Foster City, CA) with the 7900HT Fast Real-Time PCR System (Applied Bio systems, Foster City, CA). The PCR reactions were carried out in seven replicates for 3 trials in 10µl volumes using 5µl of SYBR® Green master mix, 1µl of 1:10 diluted cDNA, 0.2 ul of 10mM of gene-specific primers for cytokines (IL-8, IL-17, TNF) per reaction (table 6). Absolute quantification was performed for each gene by preparing three 10-fold serial dilutions to ensure if cDNA synthesis reagents did not impair PCR efficiency. Thermal cycling conditions for the PCR reactions comprised initial denaturation of 95°C for the 10 minutes followed by 40 cycles of 95°C of 5s and 60°C for the 60s. Melting curve analysis was performed at the dissociation step in the thermocycler conditions to validate the specificity and consistency of the amplified product. RT-qPCR efficiency was measured for each gene with the slope of the linear regression model and standard curves. Amplification efficiency and correlation coefficients for each primer pair were calculated. In expression studies, the relative expression of genes in biological samples was estimated

using the ΔΔCt method normalized with *actin* as a reference gene and compared with feeding control. The primers were designed depending on oligo analyzer program from IDT (Sanders et al., 2020) website as a table 6.

Table 6 Primers design

Gene name	*Primer Sequence*
GG_IL-8_F	ATGGAAGAGAGGTGTGCTTG
GG_IL-8_R	CACAGTGGTGCATCAGAATTG
GG_IL-17_F	GGCCGTAACGATTAGCAAGA
GG_IL-17_R	AAGCTTTCCCACCACTCTTC
GG_Actin_F	TGTCTGGATTGGAGGCTCT
GG_Actin_R	TTAGAAGCATTTGCGGTGGA
GG_TNF_F	GAAGAAAGCCCACGTGTTTG
GG_TNF_R	TGCCAGCAGTATAGCAAGAA

Results and Discussion

To study the cytokine expression in fasting chickens, the IL-8, IL-17, TNF was evaluated in the birds fasting for 4 days as compared to fed birds. There was a significant reduction in mRNA levels in fasted birds (93.7%, 75.2%, and 62.82% of IL-8, IL-17,

TNF, respectively) compared to the fed control (Figures 4, 5 and 6, respectively). Even in basal samples (0 day intestine samples), a significant decrease in mRNA expression levels to 95.6% , 77.8% and 72.8% of IL-8, IL-17 and TNF respectively was observed compared to fed birds (Figures 7, 8 and 9). In addition, the expression of cytokines also was altered in response to feeding or fasting. For instance, in fasted birds it has been observed that there was a significant reduction in mRNA levels of IL-8 and TNF-alpha (Faris et al., 2012) and viceversa with consumption of a high fat diet (Borst & Conover, 2005). During feeding, the gut is exposed to numerous environmental stimulators, which results in physiological inflammation and maturation of the gut immune system (Pont et al., 2019). This inflammation in the gut is normally initiated by various cytokines such as IL-8 and IL-1 (Maresca et al., 2008). Similarly, our studies indicate that the expression of cytokines was significantly reduced, which illustrates the low integrity of gut epithelial cells while fasting. According to Hirata et al, (2008), TNF alpha and IL-17 both induce IL-8 secretion, which is sometimes triggered by microbiome development in the ceca (Crahnova et al. 2011). In addition, these cytokines are mostly expressed during interaction between the gut immune system and commensal microbiota at early stages after hatching in chickens (Crahnova et al. 2011). IL-17 is mostly secreted by T-helper cells, which enhances the IL-8 secretion (Hirata et al 2008). IL-8 is a chemokine secreted by macrophages and neutrophils (Hirata et 2003). Generally, its secretion triggers the JAK3 pathway, which leads to proinflammation (Henkels et al., 2011). These factors play the utmost role in immune maturation and cytokine secretion during feeding.

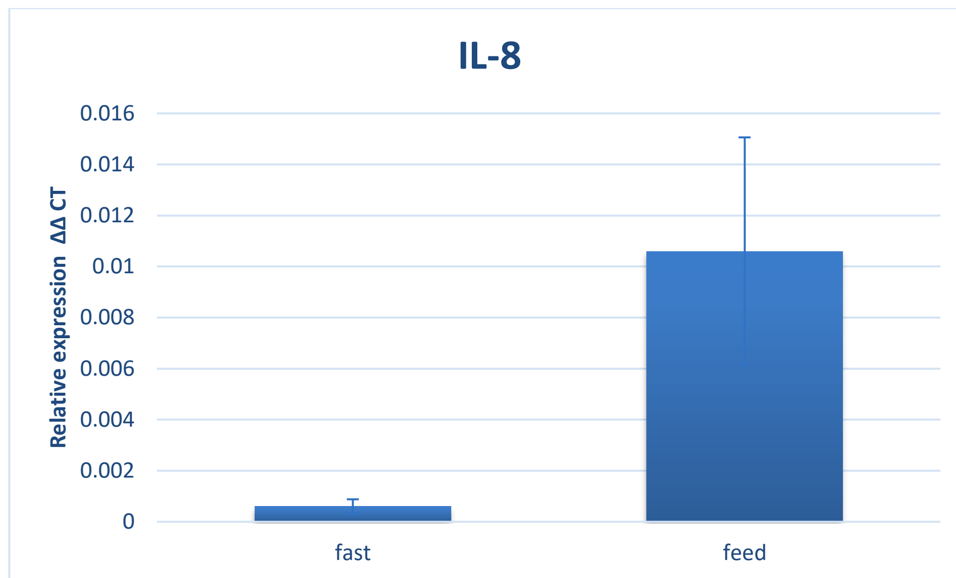

Figure 4 IL-8 relative expression in fasting compared to fed chickens
Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the relative quantification method (2 -ΔΔCt) to calculate the relative level of mRNA expression. The error bars represent the standard deviation for 21 replicates and represents significant differences in mRNA levels compared to that in control fed birds ($P \leq 0.05$, Student's t-test).

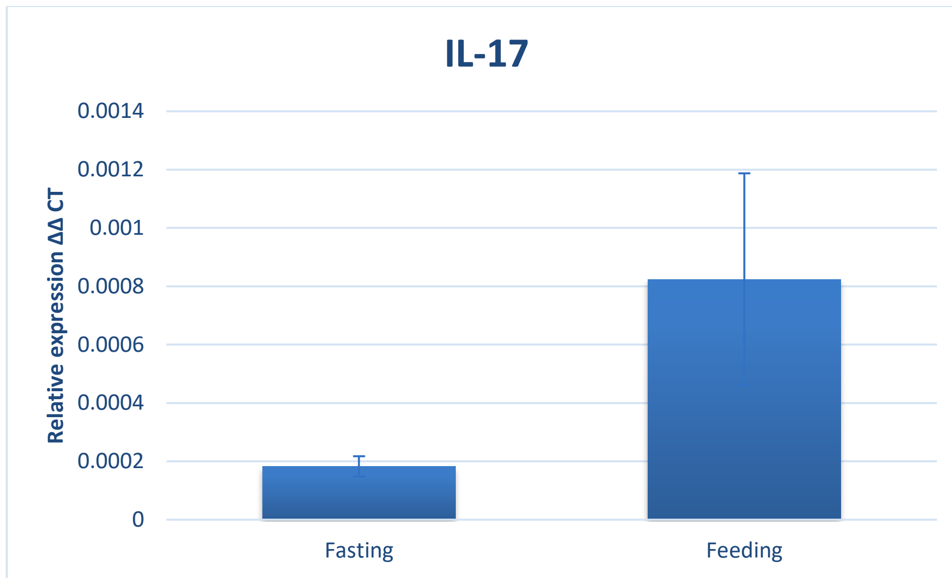

Figure 5 IL-17 relative expression in fasting chickens comparative to feeding chickens.

Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the relative quantification method (2 -ΔΔCt) to calculate the relative level of mRNA expression. The error bars represent the standard deviation for 21 replicates and represents significant differences in mRNA levels compared to that in control—fed ($P \leq 0.05$, Student's t-test).

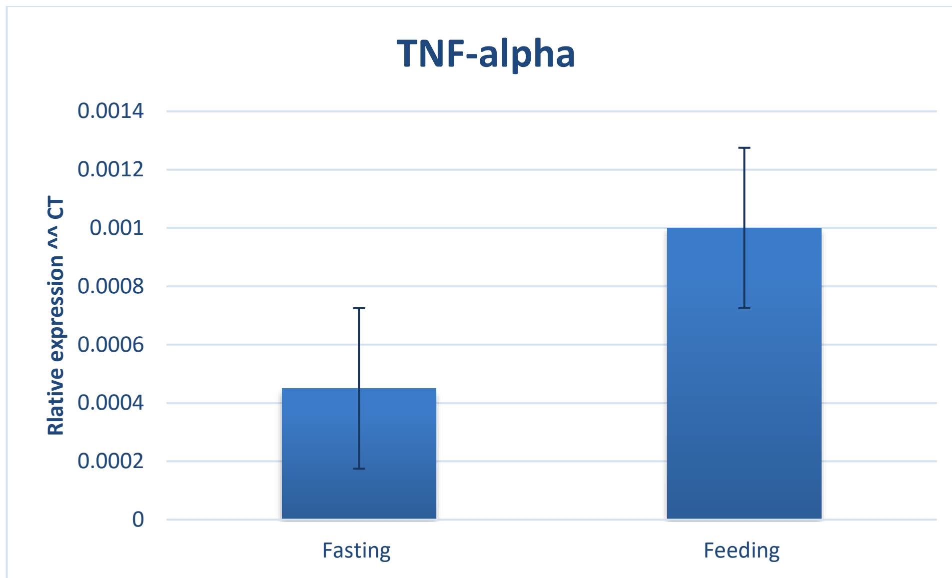

Figure 6 TNF relative expression in fasting chickens comparative to feeding chickens.

Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$) to calculate the relative level of mRNA expression. The error bars represent the standard deviation for 21 replicates and represents significant differences in mRNA levels compared to that in control—fed ($P \leq 0.05$, Student's t-test).

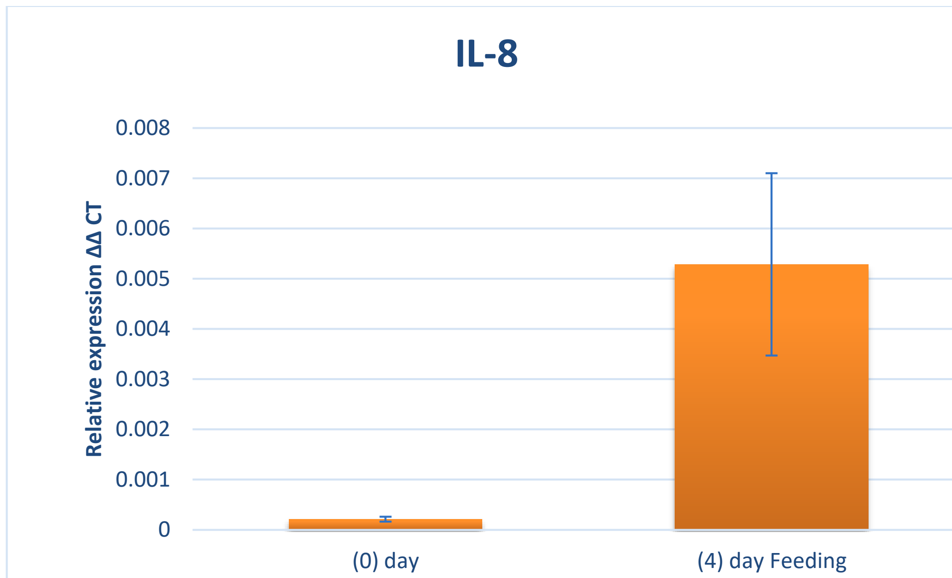

Figure 7 IL-8 relative expression in basal chickens comparative to feeding chickens. Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$) to calculate the relative level of mRNA expression. The error bars represent the standard deviation for 9 replicates for basal, 21 replicates for control—fed , and represents significant differences in mRNA levels compared to that in control—fed ($P \leq 0.05$, Student's t-test).

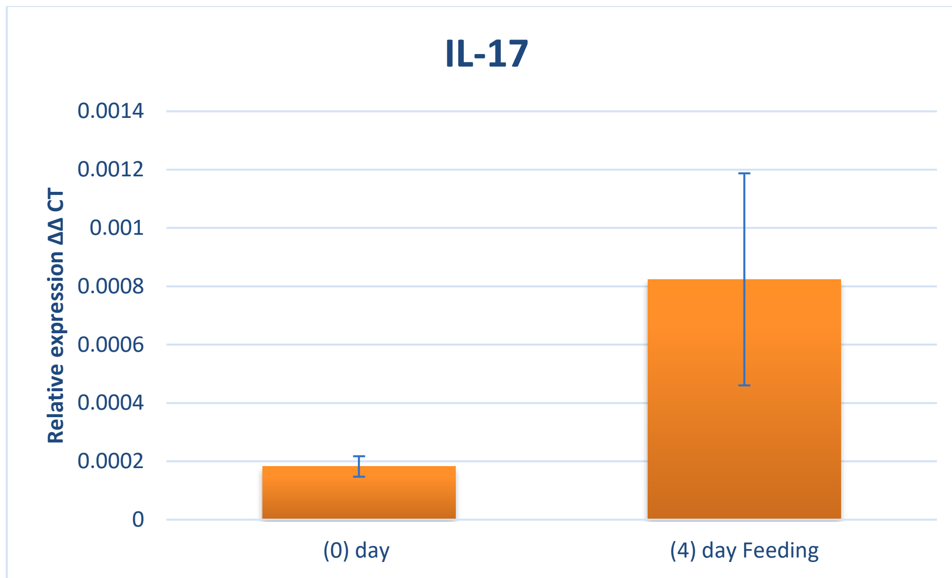

Figure 8 IL-17 relative expression in basal chickens comparative to feeding chickens.

Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$) to calculate the relative level of mRNA expression. The error bars represent the standard deviation for 9 replicates for basal, 21 replicates for control—fed , and represents significant differences in mRNA levels compared to that in control—fed ($P \leq 0.05$, Student's t-test).

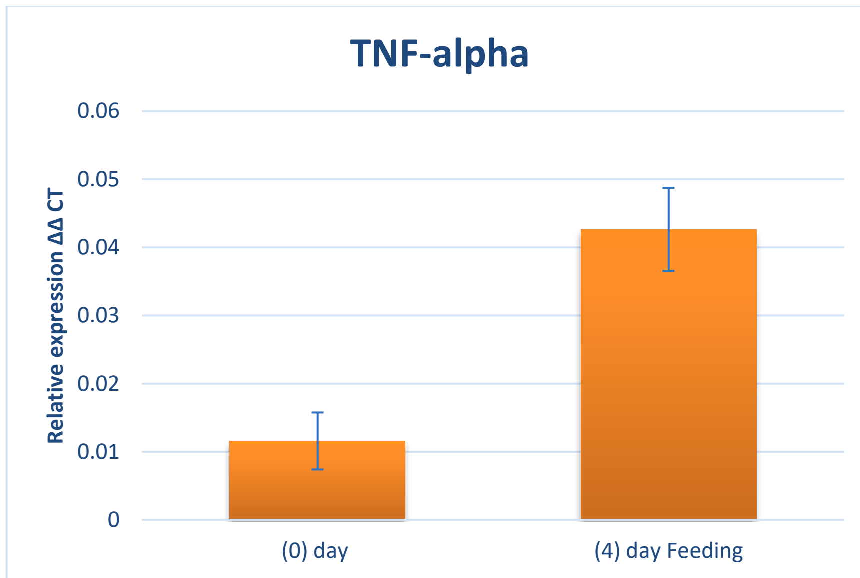

Figure 9 TNF relative expression in basal chickens comparative to feeding chickens Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$) to calculate the relative level of mRNA expression. The error bars represent the standard deviation for 9 replicates for basal, 21 replicates for control—fed , and represents significant differences in mRNA levels compared to that in control—fed ($P \leq 0.05$, Student's t-test).

Conclusion

The level of the gene expression of the (IL-8, IL-17, and TNF) were lower in the fasting group compared to the fed group. That will affect negatively the maturity of the immune system in the intestine and will negatively affect the inflammation process in the intestine of the baby chicks.

CHAPTER IV

INVESTIGATION OF THE RELATIONSHIP BETWEEN HUMORAL AND EGG YOLK ANTIBODIES IN LAYING HENS

Introduction

The role of maternally derived antibodies is important to the health of newly hatched chicks. Common vaccination programs attempt to increase the titer of these antibodies in fertile eggs by robust immunization programs for breeder hens (Murai, 2013). There are no recent studies that provide information regarding the relationship between hen humoral antibody titers and egg antibody titers.

The transfer of antibodies especially IgY from the hen to her offspring takes place in a 2-step process. In the first step, the antibody is taken up from the hen's blood into the egg yolk by antibody receptors on the ovarian follicle. The antibody is transferred from egg yolk to the offspring via the embryonic circulation in the second step (Hamal et al., 2006). This study focused on the evaluation of the relationship between hen humoral antibody titers and egg yolk antibody titers. The impact of time on these titer levels was also examined.

Materials and Methods

The study utilized ten mid-production (40-50 weeks of age) hens previously subjected to multiple vaccinations for NDV. Blood samples were collected from jugular vein and held in EDTA tubes until centrifuged and sera were stored at -20C until further

analysis. Eggs were collected the next day, broken and the contents gently transferred to a petri dish. From the intact yolk 1 ml of yolk was collected and diluted in 9 ml of PBS, mixed well and centrifuged at 2000 X g for 20 minutes (Figure 4). From the supernatant 2 ml was collected and kept at -20C until subjected to ELISA analysis. A total of 70 blood samples and 70 egg samples were collected over a 6-week period.

ELISA procedures. Commercially available plates (ProFLOK®, Parsippany, New Jersey) pre-coated with Newcastle disease virus were utilized. The assays were conducted with ProFLOK® reagents, according to the manufacturer's standard protocol. Each serum or yolk sample was run in duplicate. The titer levels were obtained from an ELISA reader (BioTek, Winooski, VT) at 410 nm. Titers were calculated using the formula provided by the manufacturer. The research was conducted under an approved animal use protocol from the Texas A&M IACUC.

Statistical analysis. The data were analyzed using the CORR procedure in SAS.

Hypothesis

The levels of NDV titers in yolk will be correlated to NDV titer levels in the hen's serum.

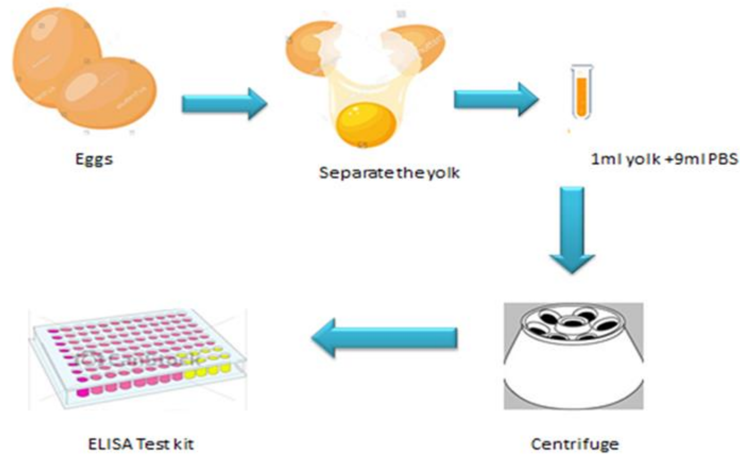

Figure 10 Yolk antibody extraction procedure.

Results and discussion

Figure 10 shows the results of mean antibody titers in the sera and yolk samples over the six-week collection period. There was an unexplained rise in serum antibodies in week 2, however the general trend is for a slow decay in titer levels over the collection period. This is what would be expected in the absence of a field challenge or other exposure to NDV. In the yolk samples, the titer levels also slowly decayed over the collection period. This is also expected in the absence of exposure to NDV. The difference between serum and yolk titers week to week remained consistent, excepting week 2.

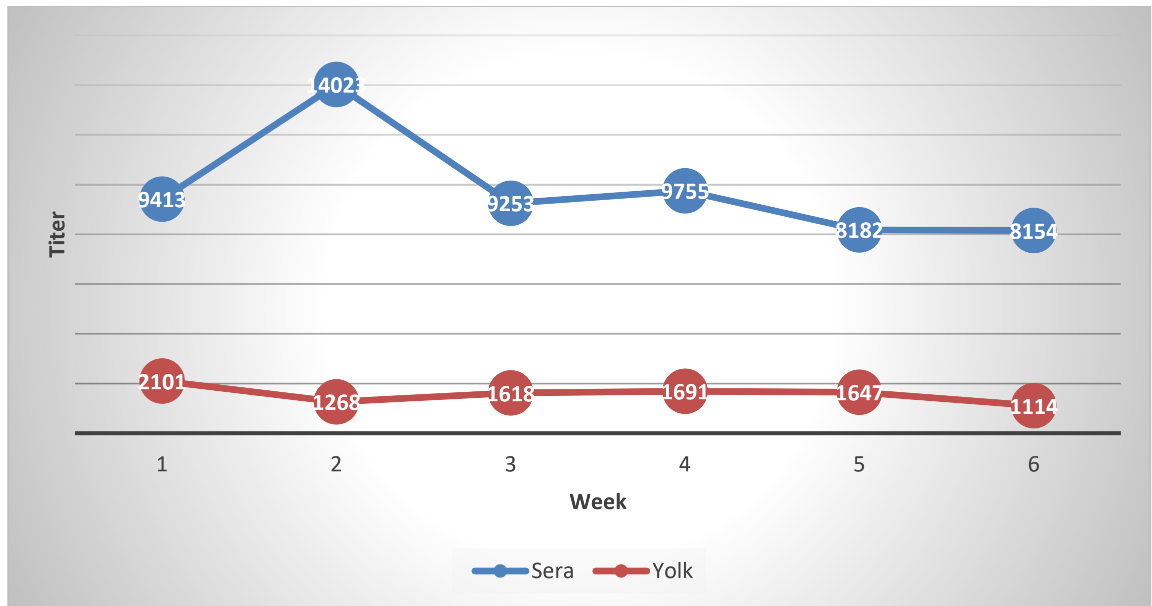

Figure 11. Weekly means of sera and yolk NDV titers.

Table 7 shows the mean concentration of maternal antibodies in serum and yolk samples. There was significant difference between serum and yolk antibody **titers. A** portion of this difference could be due to the dilution steps in yolk analysis.

Table 7 The concentration of the maternal antibodies in the hens and their eggs.

	ND Titer	*Standard deviation*
Week 1-7		
Serum	8383	2484
Yolk	10908	2095

Each value is the average of 3 trials each with 10 broiler breed hens per treatment (n=210).

Table 8 shows the correlation of maternal antibodies in the sera and yolk samples. The correlation was both strongly positive (R^2=0.648) and highly significant (P< 0.0001). In general, the use of egg yolk as source of antibody has been described by (Mohammed et al. 1986).

Table 8 The correlation of maternal antibodies in serum and yolk samples

Correlation of Serum – Egg Titers	
R2 =	0.648
P =	< 0.0001

There was a significant and positive correlation (p<0.0001) between the two variables (serum and yolk) maternal antibodies, R^2 =0.648 among 10 hens over 6 weeks

Conclusion

The humoral(Bigot et al., 2003) NDV antibody titers of the hens decayed slightly over the course of this study. The hens produced eggs with a slight decay in titer levels over time in this trial. There is a high correlation between the hens(Bigot et al., 2003) NDV antibody titers and the egg (yolk) NDV antibody (maternal) titers. The hypotheses are accepted.

CHAPTER V

CONCLUSIONS

Poultry immunity, vaccines, and the control of diseases are several factors that challenge the future growth of the poultry industry.

One important source of immunity, the maternal immunity, is the basis of innate immunity and acquired immunity. The protection of newly hatched chicks against diseases depends on their innate immunity and maternal antibodies in the yolk sac, acquired in a passive manner from the hen.

Therefore, this study tried to track the maternal immunity from the mothers to their yolk and then to the baby chicks.

The results showed there is a high correlation between the hens (Bigot et al., 2003) NDV antibody titers and the egg (yolk) NDV antibody (maternal) titers. The humoral (Bigot et al.) NDV antibody titers of the hens decayed slightly over the course of this study. As a consequence, the hens produced eggs with a slight decay in titer levels over time in this trial.

In modern production, the separation of the hatchery from the production facility means that the hatchling will spend a period without the provision of feed or water. This leadsto many questions: is this beneficial for the chick's immunity or will fasting help the chicks to absorb the yolk faster than the group getting feed early causing their immunity to be stronger than the others? Also, are the chicks in the fasting group going

to use the Immunoglobulin (IgY) of maternal immunity as a source of protein for their living needs?

The results showed that the fasting chicks did not use the immunoglobulins for nutrients at a rate different from fed birds. Especially in the maternal immunity, which is represented by IgY, as a source of protein for daily needs. The antibodies for the maternal immunity remained at the same level without having effect, comparative with regular feed- water group, and only water group. In addition, the fate of the antibodies was not degradation after fasting.

The IgY concentration in the fasting group was higher (per mL) than the feed and water group, because the blood volume of this group was less than the others. Therefore, when the concentration was measured (per mL), it was logical that the concentration was higher. Nevertheless, the total IgY was measured in all the serum of blood volume, but the total IgY was not significantly different.

The fasting birds were not utilizing the yolk sac material at the same rate as the fed birds. This indicates that without the ingestion of feed, the utilization of yolk sac material is significantly reduced.

The utilization of yolk by the newly hatched chick was dependent on peristaltic activity of the intestine. It is apparent that the fasting birds did not initiate this peristaltic action in the absence of using this movement (peristaltic); the amount of yolk that would enter the intestine was less than the amount of yolk that would enter with peristaltic movement.

Therefore, the relation is inverse between the yolk weight and the feed and water. The more fasting there is, the less yolk will be absorbed, and the weight of the yolk sac would increase.

However, there is another way to transfer IgY directly to the chick embryo, rather than yolk stalk. That happens through the highly vascularized yolk sac membrane. On day zero, their average weight was 2.73 grams (as a baseline), but the fasting group's yolk sac weight was less on day 4 even without peristaltic movement. However, the weight of the fasting group's yolk sac weight was higher than the other two groups (feed & water, and water only). This reveals how the fasting chicks are impacted by the highly vascularized yolk sac membrane.

There are two different structural areas that make up the yolk sac membrane, an inner endodermal layer of cylindrical epithelial cells, which is responsible for yolk absorption, and an outer mesodermal layer of flattened cells, which performs a supportive role. This helps explain why the weight of the yolk sac decreased on day four, compared with the yolk sacs of all groups at day zero.

Also, the local immunity of the intestine was checked by taking samples of the ceca and extract the RNA and measure the gene expression of interleukins (IL-8, IL-17, and TNF) which is responsible to mature the immunity and the inflammation of the intestine. The level of gene expression of the (IL-8, IL-17, and TNF) was lower in the fasting group compared with feed group. That has a negative effect on the maturity of the immune system in the intestine and also on the inflammation process in the intestine of the baby chicks.

From all of this, the recommendation for the companies and the owners is to make the feed open for chicks. This will help to get more absorption of yolk and at the same time make the local immunity of the intestine mature and more active as soon as possible. This will also facilitate reaching your goals of getting healthy chicks with high weight in a short time.

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