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Effects of the In Ovo Injection of Inovocox Em1 Vaccine on the Embryogenesis, Posthatch Performance, and Gut Pathology of Ross Ross 708 Broilers

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Effects of the in ovo injection of Inovocox EM1 vaccine on the embryogenesis, posthatch performance, and gut pathology of Ross × Ross 708 broilers

By

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Effects of the in ovo injection of Inovocox EM1 vaccine (**EM1 vaccine**) suspended in commercial diluent on developing broiler embryos were investigated in 3 trials. Effects of the EM1vaccine administered by in ovo injection on broiler embryogenesis and posthatch performance was determined by evaluating site of injection (**SOI**), embryo staging (**ES**), hatchability, and chick quality parameters. Oocyst output, microscopic lesion scores, and grow-out performance were further examined through day 35 posthatch. In these studies, it was shown that oocyst output began at day 3 posthatch (6 days post-injection), and peaked at day 7 posthatch (10 days post-injection). The EM1 vaccine had no effects on hatchability, various and chick quality parameters that were examined in the study. Similarly, grow-out performance through day 35 posthatch was not affected by the EM1 vaccine. SOI and ES provided information on the accuracy of in ovo vaccine delivery to the embryos, and were found to be significantly influenced by embryo age.

In conclusion, in ovo injection of the EM1 vaccine has no detrimental effect on broiler embryogenesis, hatching chick quality, or the performance characteristics of Ross × Ross 708 broilers.

DEDICATION

I dedicate this dissertation first to God, to my lovely and supportive wife, Remi Sokale, and to our precious adorable son, Oluwatobi Sokale.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AC	air cell
AL	allantois
BW	body weight
BWG	body weight gain
CPM	cumulative percent mortality
d, D	day
doi	day of incubation
ED	embryonic day
EM	embryo body
ES	embryo staging
EM1	Inovocox EM1
FCR	feed conversion ratio
FI	feed intake
h	hour
HBW	hatchling body weight
HI	hatchability of injected eggs
IW	intestine weight
LW	liver weight
m	meter

N number of observations

PIM post injection embryonic mortality

Poh post hatch

SEM standard error of the mean

SOI site of injection

YFBW yolk free body weight

YFBM yolk-free body mass

YS yolk sac

YSW yolk sac weight

CHAPTER I

INTRODUCTION

The broiler chicken industry has grown exponentially over the past two decades. This growth can be attributed to the ability of the commercial poultry industry to incorporate advanced scientific methods into their production of fast-growing broiler chickens that are raised under intensive management systems. In spite of the numerous advantages associated with increased poultry production, which essentially provides food security in the world, the intensive system of poultry production provides opportunities for the development and transmission of numerous poultry diseases that limit the ability of the industry to meet the increasing demands for poultry products. Enteric diseases are of greatest global concern to broiler chicken producers because they cause production losses associated with increased morbidity, mortality, and risks associated with the contamination of poultry products that make them unsafe for human consumption.

The infectious disease, coccidiosis, which is caused by a protozoan parasite of the genus *Eimeria*, is still considered the greatest disease challenge to the worldwide poultry industry (Tyzzer, 1932; Chapman et al., 2002). Coccidia parasites multiply in the intestinal tract, and cause damage to intestinal tissues, resulting in reductions in feed intake, and body weight gain that are accompanied by diarrhea, morbidity, mortality, and an increased susceptibility to other diseases (Williams 1999a; Mc Dougald, 2003). The control of coccidiosis by the use of anticoccidial drugs dates back to the early twentieth

century. However, coccidia have developed various degrees of resistance to all anticoccidial drugs that have been introduced to date (Chapman, 1997a; Chapman, 2014). Although several poultry integrators have adopted rotational or combinational usages of various anticoccidials in order to overcome the problem of drug resistance. The deposition of drug residues in poultry products has become a bigger concern among consumers. This has led to the withdrawal of several in-feed coccidial drugs from broiler feed (McEoy, 2001; Young and Craig, 2001, Farrant, 2001). Therefore, poultry producers are shifting from the chemotherapeutic control of coccidiosis to the use of vaccines. Coccidiosis vaccines consist of a low dose mixture of live *Eimeria* oocysts that can be administered to embryos or day-old chicks. This has been shown to produce adequate immune responses against coccidial challenges that may occur later in the chicken's life (Chapman, 2014). Since the inception of the Coccivac vaccine in 1952, several commercial coccidiosis vaccines have been made available. Inovocox EM1 vaccine is a non-attenuated vaccine that is administered to 18 to 19 day-old embryos using a commercial in ovo injection machine. This method of coccidiosis control ensures the delivery of a precise vaccinal dose of *Eimeria* oocysts to each embryo in order to achieve an early onset of immunity in the hatchling. Several studies have been conducted to evaluate the effects of coccidiosis vaccines on various performance variables in broiler chickens. For example, an improvement in posthatch performance of broilers and a reduction in the number of shed oocysts have been reported following day-of-age spray application of Coccivac B, in the presence of mixed coccidia infection (Schering-Plough Animal Health, 2007; Mathis, 1999). However, there is very limited information regarding the effects of the EM1 vaccine on the physiological characteristics of broiler

embryos and hatched chicks, as well as the posthatch performance characteristics of broiler chickens during grow-out. Therefore, the objective of this current study was to investigate effects of the Inovocox EM1 vaccine suspended in commercial diluent, when injected in ovo at various embryonic age and dosages on embryogenesis, posthatch performance, and gut pathology of broilers.

CHAPTER II

REVIEW OF LITERATURE

Embryogenesis and incubation

The development of the chicken (*Gallus gallus domesticus*) embryo from fertilization to hatching is a rapidly changing process that lasts approximately 21 days. This process of development is influenced by a combination of several endogenous and exogenous factors. More specifically, the process is optimized by environmental factors that are provided during incubation, namely temperature, turning, humidity, and ventilation (Romanoff, 1929; Molenaar et al., 2008; Willemsen et al., 2008). These environmental factors influence the developmental and metabolic requirements of the chicken embryo, and in addition can affect posthatch chick development (Molenaar et al., 2010). Optimum incubational dry and wet bulb temperatures are approximately 37.5 ± 0.5 ^{o}C and 28.9 ± 0.5^{o}C, respectively. During embryonic development, the embryo produces internal heat as a result of its metabolic processes. The external temperature need of the embryo therefore decreases as the embryo matures towards hatch. A lower set temperature is necessary on the machine beginning from at d 15 of incubation in order to coincide with the heat production of the embryo, especially in large size embryos (Lourens et al., 2006). The impact on temperature on embryos and chick performance has been studied extensively. Sozcu and Ipek (2014) showed that chronic high eggshell temperature in late-stage embryos can affect their hatchability, performance, and the

incidence of ascites. In addition to temperature, relative humidity is an important component of incubation. A relative humidity of 50 to 65% is further required for optimal incubation (Peebles and Brake, 1987; Pulikanti et al., 2012). Humidity occurs in consonance with temperature, in that as temperature increases, relative humidity decreases during incubation (Townsley, 1931). During the process of incubation, the embryonated egg loses some amount of moisture (Rahn, 1977). The amount of water loss can affect hatchability (Ar and Rahn, 1980). Hatchability is not affected when moisture loss is between 12 to 14 % of the fresh egg weight at d 18 of incubation. However increased embryonic mortality can occur with moisture loss that is lower than 9 % or greater that 18.5 % (Buhr, 1995; Davis and Ackerman, 1987). Humidity is essential for control of the loss of moisture from the incubated egg (Landauer, 1948; Buhr, 1995). During incubation when temperature is high, a decrease in relative humidity will allow for an increase in moisture loss. However, during late-stage incubation as temperature is decreased as embryo approach hatch, the relative humidity is increased (Hamdy et al., 1991). Oxygen is a key nutrient required by the growing embryo, and is influenced by the incubator ventilation. Adequate ventilation not only ensures that a uniform temperature is maintained, but also that an adequate supply of oxygen and the removal of carbon dioxide is maintained within the incubator as the embryo develops (Atwood and Weakley, 1915; French, 1997). Further, efficient air circulation within the incubation system ensures that incubation temperature, oxygen, and carbon dioxide are maintained at levels which are acceptable to the growing embryo. In addition to incubation ventilation, egg pore quality impact gaseous exchange between the embryo and its external environment. A decrease in the number of pores on the egg surface can lead to

reduced oxygen availability to the growing embryo (Wineland, 2014). Oxygen demand by the embryo increases as the embryo increases in size. This need for oxygen increases for embryo growth and development especially in the first 14 days of incubation. A plateau phase of oxygen utilization occurs at d 14 of incubation due to maximum saturation of the chorio-allontoic membrane, and egg pores with oxygen. Oxygen demand increases again when the embryo begins pipping at d 19 of incubation. During the plateau phase, the embryo reverts back to alternative energy sources that are anaerobic such as glycogen or protein (Wineland, 2014). A gentle turning frequency of at least 3 times and no more than 96 times every 24 h at an angle of 45^{o} is essential for the incubation process, especially during the first 14 days of incubation (Olsen and Byerly, 1936; Funk and Forward, 1960; Elibol and Brake, 2006). Embryonic development begins with fertilization of the ovum by spermatozoa, which occurs in the infundibulum and, results in fusion of the two gametes to produce the zygote (Romanoff, 1960). Following formation of the zygote prior to oviposition, cell division (cleavage) begins in the germinal disc. The process of cleavage begins with a cluster of cells in a single layer called the blastoderm. Continuation of the process of cleavage produces the blastodermal stages (Patten, 1920; Fig. 2.1). Following oviposition, when eggs are collected and stored under cold conditions (at physiological zero), embryonic development is arrested. Appropriate incubation conditions are necessary to reinitiate cell divisions in the blastoderm (Edwards, 1902). As cell differentiation continues, the primitive streak arises from the blastoderm (epiblastic portion of the blastoderm) resulting in formation of the germinal epithelia, which consists of the ectoderm, endoderm and mesoderm (Patten, 1920). The ectoderm differentiates into the integuments, nervous system, and sense

organs. The endoderm develops into the epithelial lining of the respiratory, digestive and secretory organs, while the mesoderm differentiates into the circulatory, lymphatic, and reproductive systems (Patten, 1920)**.** The development of embryos during incubation can generally be categorized into three phases: early, mid, and late. The early phase of development occurs during embryonic days (ED) 1 to 7, the mid phase extends from ED 8 to 14, and the late-phase extends from ED 15 to 21 (Hamburger and Hamilton,1992). The early- and mid- phases are characterized by formation of the organ and systems, while growth and maturation of those systems occurs during the late-phase. The daily developmental stages of the chicken embryo as they relate to the days of incubation, are presented in Table 2.1. In addition, Hamburger and Hamilton (1992) described and classified the stages of chicken embryonic development into 45 stages based on morphogenetic changes from cell division to hatching (Fig. 2.2). The early stages (1 to 6) correspond to d 1 of incubation and are characterized by the development of the primitive streak. The middle stages (7 to 14) are characterized by organ systems, and correspond to d 2 to 3 of incubation. The last stages (15 to 45) which corresponds to d 4 to 21 of incubation, are characterized by morphological changes leading up to the maturation of features that become more evident as hatch approaches. For example, stage 28 of the Hamburger and Hamilton classification scheme corresponds to d 6 of incubation and is characterized by rapid differentiation, with the formation of appendages and the egg tooth. In general, the initial stages of chick embryonic development are very rapid. For example, as early as 44 h into incubation, the vascular system is beginning to function, with full formation of the heart occurring by d 7 of incubation. By d 3 to 6 of incubation, the limb buds are visible, the auditory pits are established, the reproductive organs are

formed, and sex differentiation begins. By the mid-phase of incubation, the appendages (beak and toes) are fully formed, and can be used as points of reference for distinguishing the various phases of incubation. By end of the mid-phase (d 14 to 15 of incubation), the embryo begins to move into hatching position, with its head turned toward the large end of egg. During the late-phase, the developing embryo undergoes several maturation changes in preparation for hatching. By d 17 of incubation, the chick's beak is turned toward the air cell. By d 19 of incubation, the yolk sac begins to enter the body cavity, and internal pipping begins. By d 20 to 21 of incubation, the yolk sac is completely drawn into the body cavity and the hatching process begins.

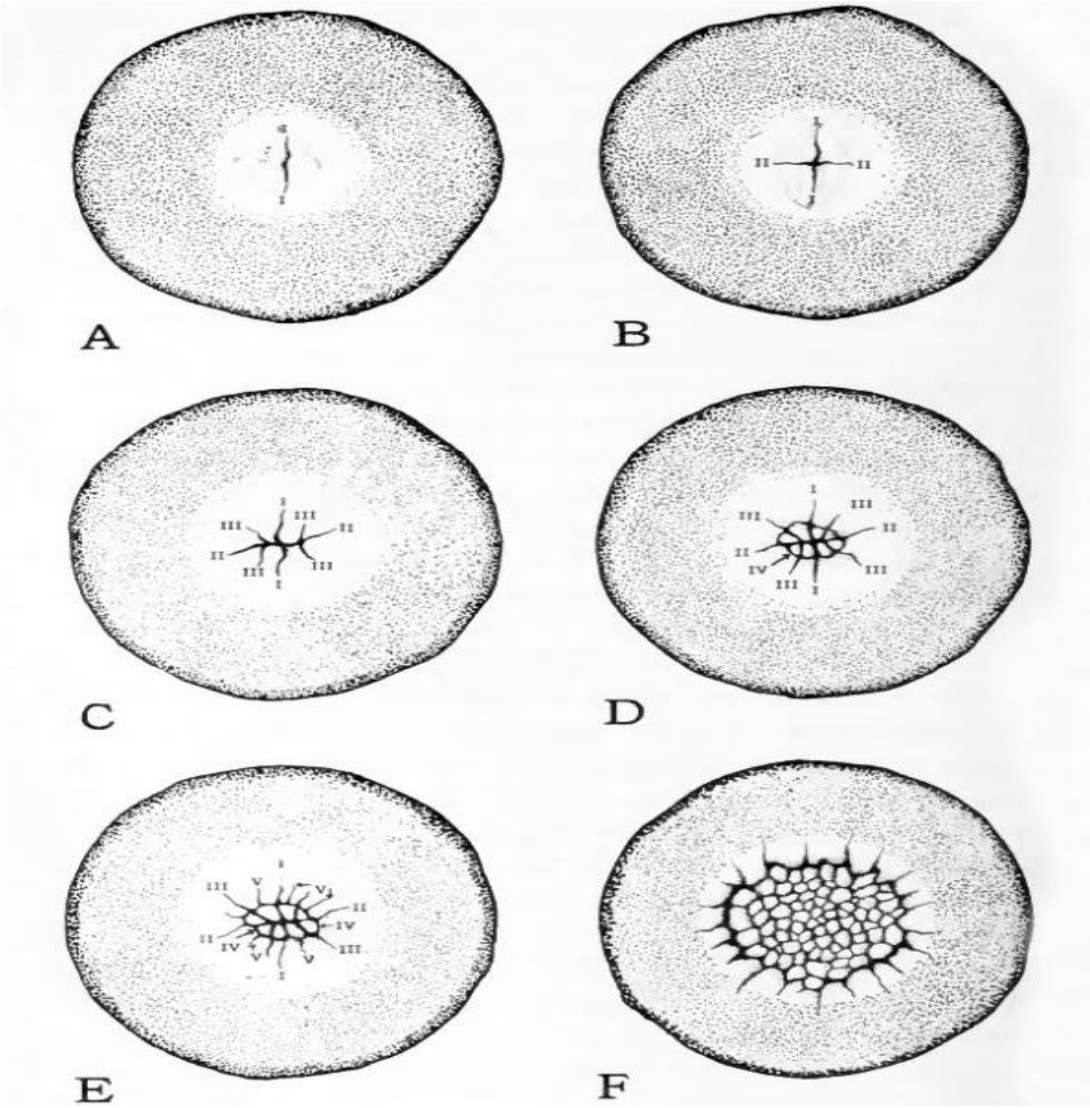


Figure 2.1 The process of cleavage leading to the formation of blastodermal stages. Drawing taken from Patten (1920)

Table 2.1 Stages of embryonic development during of incubation

Days	Embryonic Developmental Event
1	Formation of the blastoderm. Area pellucida and opeca on blastoderm. Primitive streak and somite appears
2	Heart is beat, vascular systems are linked.
3	Circulatory system continues to develop, limb buds for wings and legs are visible, auditory pit is established
4	Eye pigment present, leg and wing buds present, vasculature is present
5	Formation of reproductive organs, sex differentiation
6	Complete embryo differentiation, embryo has bird-like appearance, beak and egg tooth formed, embryo voluntary movement evident
7	Feather tracts becoming prominent. heart is completely enclosed in the thoracic cavity
8	Eyes becoming prominent
9	Allantois completely covers the embryo, amnion, and yolk
10	Beak and toes hardens and are fully formed
11	Comb serration, and down feathers begin to appear
12	Eye lids closed
13	Overlapping scales on legs, down feathers covers the body of embryo
14	Head turns toward the large end of egg, embryo begins turning to hatching position.
15	Rapid-eye-movement, intestines enters the abdomen, embryo reaches a growth phase prior to hatching
16	Feather cover are present, yolk is the primary source of nutrient, albumen regressed
17	Amniotic fluid begins to regress and embryo begins to prepare for hatch with head between legs.
18	Regression of amniotic fluid, beginning of yolk sac absorption into the embryo's body cavity. Head under right wing
19	Complete regression of the amniotic fluid , yolk sac half drawn into body cavity, beak pips through air-sac (internal pipping), and embryo starts to breath with the lungs
20	Yolk sac completely drawn into the body, external pipping and vocalization, allantoic respiration and circulation changes to pulmonary respiration
21	Pipping completed, normal hatching.

(Mauldin and Buhr, 1990; Parkhurst and Mountney, 1988)

Functional structure of the embryonated chicken egg

The functional compartments of the chicken embryo during incubation are shown in Fig. 2.3. As the embryo differentiates from the blastoderm stage to a fully developed embryo, there are at least 5 important compartments that must encompass and support the

embryo in its development. These include: the eggshell, yolk, chorioallantois, allantois, and amnion. In addition, during embryonic development, the embryo utilizes the nutrients that are contained within the maternal egg. These nutrients are found specifically in the yolk, albumen, and shell.

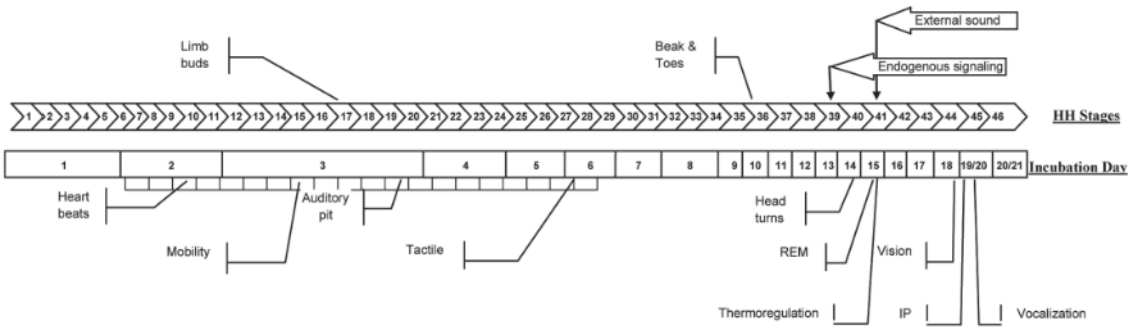


Figure 2.2 Comparisons of major embryonic developmental milestones between Hamburger and Hamilton stage classification and the entire embryonic incubational period.

Drawing taken from Hamburger and Hamilton (1992).

Yolk

The yolk is an important extra-embryonic structure, providing essential nutrients for the embryo throughout incubation (Romanoff, 1960) and during the first 3 to 4 days of the chick's posthatch life (Parkhurst and Mountney, 1988). The chicken embryo eventually develops from secondary oocyte containing a large amount of yolk that is ovulated by the hen. The yolk proteins are primarily formed in the liver and are transported to the ovary via the blood. The yolk is formed from one of many small cellular structures called oocytes located on the surface of the ovary. Once formed, a secondary oocyte is released (ovulated) from the ovary and is captured by the infundibulum of the oviduct, and after further maturation to an ovum is fertilized by a

sperm. The yolk makes up approximately 30 % of the total weight of an egg, and is composed of water (48 %), digestible proteins (17 %), lipids (31 %), vitamins, and minerals. The yolk lipids provide the energy needed for rapid embryonic development and growth of the embryonic tissues (Moran, 2007). The fat soluble vitamins A, D, E, and K, and the water soluble vitamins B2 (riboflavin) and B9 (folate), are distributed in the yolk. In addition, the egg yolk contains a sufficient amount of iron and phosphorus that support embryo growth and development. Other important function of the yolk include: (1) embryonic formation - the yolk houses the germ cell (where fertilization occurs) which is present at the upper pole of the yolk; (2) embryonic development - the yolk provides a readily available source of nutrients to the developing embryo; (3) immunological competence - the egg yolk contains yolk Immunoglobulins (IgY), which are synthesized in the hen's serum and are transported into the yolk to provide specific antibody in the embryonic chicken (Leslie and Clem, 1969).

Albumen

Albumen makes up approximately 60 % of the total egg. Of this, 87 % of the albumen is water, 11 % is protein, and 1 % is carbohydrate (Romanoff and Romanoff, 1949). The protein of the albumen maintains the viscosity and thickness of the albumen. Albumen proteins include ovalbumin, ovotransferrin, ovomucoid, ovoglobulin, lysozyme and ovomucin (Alleoni, 2006). Albumen also contains elements including potassium, sodium, phosphorus, calcium, and iron. The primary function of the albumen is to protect the embryo from adverse effects of microorganisms, as well as provide water, protein and minerals for the developing embryo. The albumen surrounds the yolk and is attached to the yolk by the chalaziferous layer. During embryonic development the albumen is

rapidly consumed by the embryo to supply amino-acids for protein synthesis within the body to enhance development. Albumen is essential for embryonic development compared to yolk and manipulating albumen can alter embryonic development during incubation (Romanoff, 1960). Al-Murrani (1982) showed that injection of albumen into late-stage embryo increased embryonic growth just prior to hatching. Similarly, when albumen was removed from the egg prior to incubation, whole-body protein synthesis was decreased (Muramatsu et al., 1990). These suggests that the albumen is plays a significant role in regulating protein synthesis of the whole body of the developing embryo during incubation.

Eggshell

As the egg travels down the oviduct, the eggshell is deposited around the egg in the shell gland (uterus) prior to being laid. It takes approximately 20 to 22 hours for complete egg shell calcification to occur in the uterus. The egg shell is made of calcite, which is a crystalline form of calcium carbonate. The egg shell is made up of approximately 98 % calcium carbonate, and 2 % organic shell matrix. The organic shell matrix is made up of layers of proteins and mucopolysaccharides on which calcification occur. Microscopically, the shell consists of inner and outer shell membranes that are made up of a mesh work of protein fibers that are semipermeable, thus permitting the passage of gases and water through them. These shell membranes stick together across the entire surface area of the egg, except at the large end of the egg where they separate to form the air cell. The egg shell consists of the mammillary layer, palisade layer, surface crystal layer, and the cuticle. The mammillary layer forms the bases of the palisade layer and is the site of initial calcium deposition on the shell membrane. The

palisade layer, which is composed of calcium carbonate in columns, forms over the mammillary layer. Outside of this, there is a vertical surface crystal layer that marks the actual beginning of the egg shell and is characterized by a dense crystalline structure. The cuticle is a thin, waxy coating made up of protein, polysaccharides, and lipid, which seals the pores that open on the surface of the egg shell, and protects the egg from moisture loss and microbial invasion. The major function of the eggshell is to protect and maintain the embryo within a controlled internal environment. The pigment of the eggshell lies beneath the cuticle (Parsons, 1982).

Amnion

The development of the amniotic membrane is evident by day 5 of incubation, and completely envelopes the embryo by day 9 of incubation. The amniotic membrane is formed by the differentiation of the ectoderm and mesoderm (Parkhurst and Mountney, 1988). The amniotic cavity becomes maximally filled with amniotic fluid at day 12 of incubation (Patten, 1920). The amniotic fluid hydrates the embryo (Romanoff, 1960), protects the embryo from physical and environmental changes during development, and provides the necessary environment needed by the developing embryo as it changes position during incubation (Patten, 1920). The embryo begins to ingest the amniotic fluid during late stage of incubation (approximately day 17 of incubation), with complete regression by day 19 to 20 of incubation. Jochemsen and Jeurissen (2002) showed that substances injected into the amnion of an embryo at day 18 of incubation are taken up through the mouth into the digestive and respiratory tract beginning 24 hours post injection. Similarly, Weber et al. (2001) found *Eimeria* oocysts within the intestine of the embryo within a few hours following the in ovo injection of *Eimeria tenella* life cycle

stages. Furthermore, Jochemsen and Jeurissen (2002) showed that when embryos were injected at day 16 of incubation, only 50 % of the injected substances were recovered from their organs. However by day 18 of incubation, all injected substances reached the embryonic organs. This showed that injection of substances into the amnion at day 18 of incubation is effective, due to the large size of the amnion at this time. In addition, late stage embryos, at day 18 of incubation, display strong rhythmic movement as they approach hatching, and are able to actively imbibe the amniotic fluid and its constituent substances, allowing these substances to be actively distributed throughout their organs and tissues as early as 24 hours post injection. Several authors have demonstrated great success in injecting in the amnion on day 18 of incubation (Sharma et al., 1984; Sharma, 1986; Jochemsen and Jeurissen, 2002; Weber et al., 2004; Williams and Hopkins, 2011; Zhai et. al., 2011a, b; Bello et al., 2013; Bello et al., 2014).

Chorio-allantois

The chorioallantoic membrane (CAM) of the chick embryo is formed by the partial fusion of the chorionic and allantoic membranes. The CAM completely surrounds the chicken embryo by day 10 of incubation, and along with other contents of the egg, becomes attached to the shell membrane, thus allowing the vascular system to be in direct contact with the eggshell membranes (Parkhurst and Mountney, 1988). Based on this arrangement, the CAM performs at least 2 important functions in the developing chick embryo. Firstly, the CAM is responsible for mobilizing shell calcium into the circulation of the embryo for bone ossification during embryonic development (Terepka et al., 1976). Secondly, the CAM functions as an embryonic lung by serving as the medium for gas exchange, as carbon dioxide is released and oxygen is taken in through the pores in

the eggshell. In addition, the CAM is responsible for the reabsorption of water and electrolytes from the allantois where urinary waste products are discharged, and it provides acid-base balance within the egg of the developing embryo (Gabrielli and Accili, 2010).

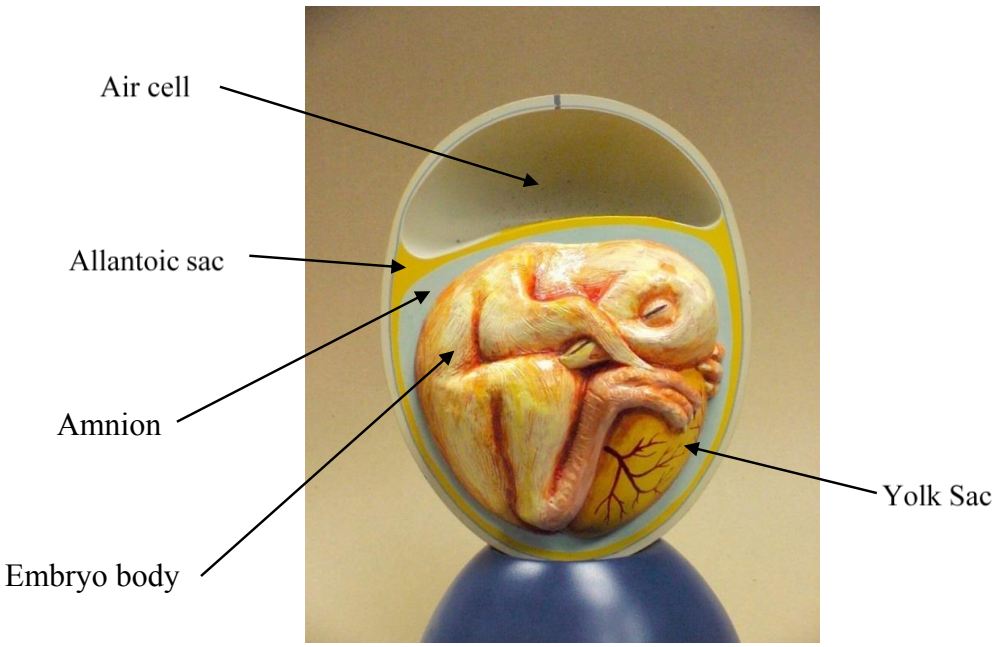


Figure 2.3 Compartments of the chicken embryonic egg on day 18 of incubation, accessible by *in ovo* injection

Image taken from Embryo Development 101; Zoetis Animal Health, Poultry Health Division.

Allantois

The allantoic membrane is a thin cell layer between the amnion and the chorion, and encloses the allantoic fluid (Gabrielli and Accili, 2010). The allantoic membrane is formed by the differentiation of the mesoderm layer at approximately day 5 of incubation (Parkhurst and Mountney, 1988). As the embryo continues to develop, the allantois stores metabolic wastes (uric acid) produced by the embryo. It also plays a role in the

excretion, respiration and metabolic processes of the developing embryo (Parkhurst and Mountney, 1988). During incubation, the volume of the allantoic fluid initially increases, reaching a maximum by day 13, which allows it to meet the functional needs of the embryo. It then declines rapidly until hatching. In addition, the allantoic membrane facilitates the reabsorption of water from the allantoic fluid, as well as facilitating the absorption of the remaining albumen (Parkhurst and Mountney, 1988).

Coccidiosis

Coccidiosis is an infectious disease caused by protozoa in the phylum Apicomplexa, and family Eimeriidae. Poultry coccidiosis is caused by species of the genus *Eimeria*, and there are at least 9 distinctly recognized species of *Eimeria* that infect chickens (Chapman, 2000; Dalloul and Lillehoj, 2005; Lillehoj and Lillehoj, 2000). *Eimeria* species are distinguishable by individual biological characteristics associated with each species. These biological characteristics include: oocyst size and shape, size of tissue stages produced during their life cycle, site of intestinal infection, pathogenicity, gross appearances of lesions, and immunogenicity (McDougald and Fitz-Coy, 2008). *Eimeria* species parasitizes and replicates in specific portions of the intestinal tract, resulting in various levels of pathogenicity (McDougald, 2003).

Coccidia life cycle

Development of *Eimeria* parasites within the host involves both endogenous and exogenous stages (Fig. 2.4). Within 48 h at optimal temperature (30 ^{o}C), oxygen concentration, and relative humidity levels, unsporulated oocysts present within the poultry environment become viable infective sporulated oocysts (Reid, 1978). Infection

occurs when a susceptible chicken host ingests sporulated oocysts from its environment. After the sporulated oocysts are ingested, they are subjected to enzymatic and grinding actions in the gastrointestinal tract (**GIT**) and gizzard, respectively. This processes leads to the excystation and release of sporozoites (Reid, 1978). Sporulated oocysts contain 4 sporocysts, with each sporocyst containing two sporozoites (infective stage). The grinding action of the gizzard causes a release of the sporocysts from the oocysts, whereas actions of digestive enzymes results in the release of sporozoites from the sporocysts. The released sporozoites initially penetrate the host's intestinal wall, extending from the surface epithelium to the deep epithelium of the intestinal glands. Invasion of the intestinal wall occurs in various regions of the intestine where specific *Eimeria* species have a predilection (Reid and Long, 1979).

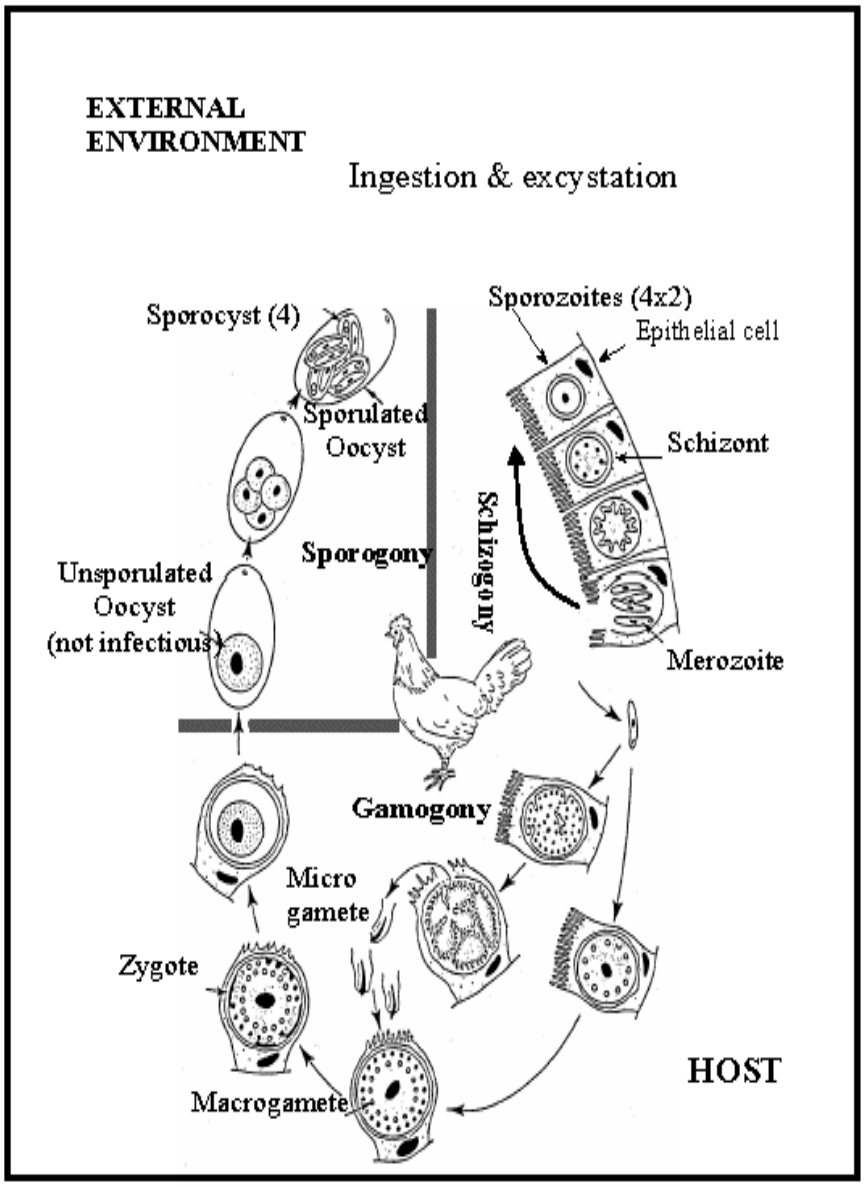


Figure 2.4 Stages of *Eimeria* life cycle occur in exogenous (external environment) and endogenous (within the host) phases

Drawing taken from Price (2012).

The sporozoites further develop into trophozoites (feeding stage), which absorb nutrients from the intestinal tissues. Trophozoites increase in size and undergo multiple asexual divisions in a process referred to as schizogony or merogony, which leads to the formation of schizonts. At maturity, schizonts rupture and release merozoites (small

parasitic stages within the schizont). The merozoites, which are released from the initial infected cells, infect new epithelial cells, and the process of development through the trophozoite and schizogony stages are repeated. At least 2 to 4 generations of merogony asexual development occurs depending on the species of *Eimeria* (Reid and Long, 1979). After completion of the last merogony cycle, the resulting merozoites penetrate new epithelial cells and initiate the sexual phase of the cycle. The sexual phase involves formation of male (microgametocyte) and female (macrogametocyte) gametocytes in a process referred to as gametogony (Fayer, 1980). During gametogony, the male gametocyte undergoes multiple divisions releasing numerous biflagellate male gametes (microgametes). The female gametocyte (macrogametocyte) matures to a single large macrogamete. A process of fertilization occurs between the microgamete and macrogamete within the host intestinal epithelium, leading to the formation of a zygote. The zygote forms a thickened wall and matures into an oocyst (Fig. 2.5). The mature oocyst, consisting of 4 sporocysts, each containing 2 sporozoites, is released from the host gut and passed out in the feces of the bird (Reid, 1978). The entire life cycle process may take up to 14 days, depending on the specie of *Eimeria* (Reid and Long 1979). The timelines encompassing the complete life cycle of the various species of *Eimeria* have been previously described (Reid and Long, 1979). These include the length of time for sporozoite excystation, which usually occurs within 2 h post ingestion of intact oocysts; the prepatent period (period required for complete endogenous development into a zygote i.e. from sporozoites excystation to gametogony stage), which can range from 4 to 7 days depending on the species of *Eimeria* (Reid and Long, 1979); and environmentally-

influenced exogenous phase, which can occur over a period of 2 to 3 days (Chapman et al., 2002).

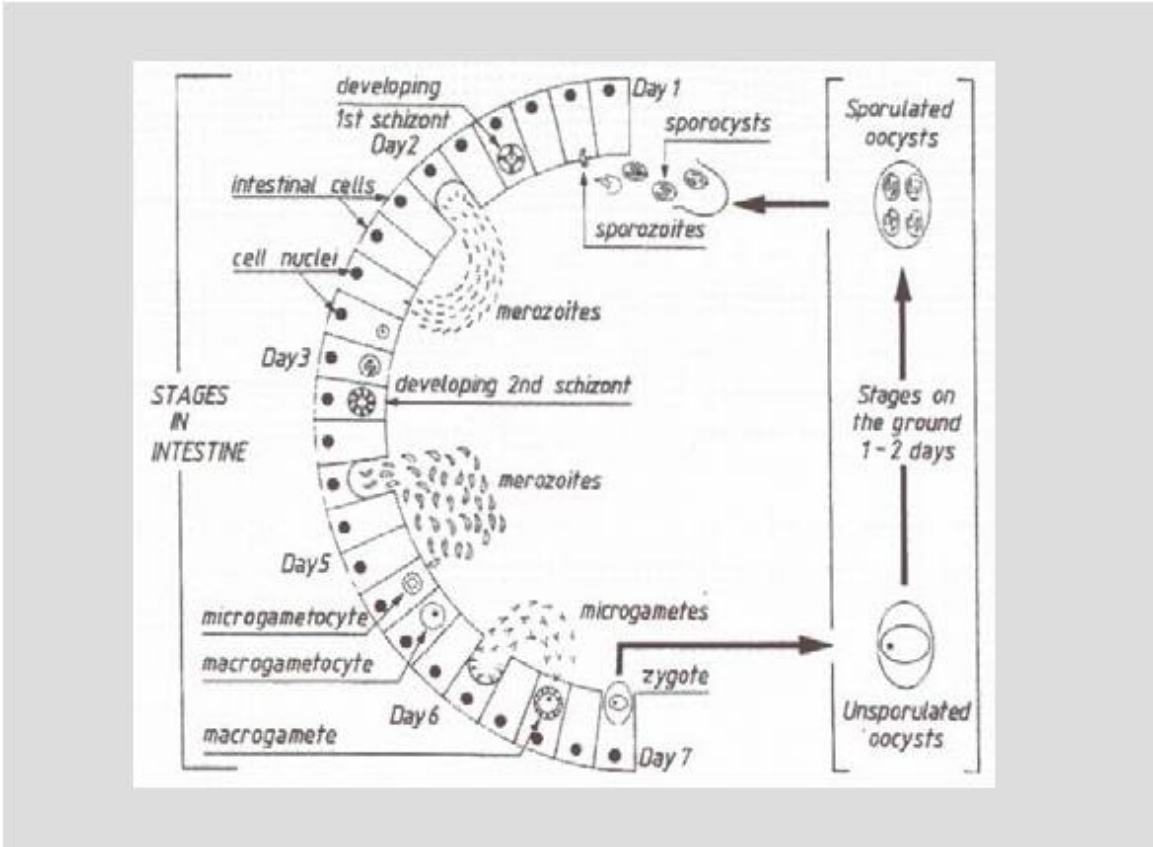


Figure 2.5 Overview of the 7-day life cycle of *Eimeria tenella*

Stages include 2 or more asexual and 1 sexual cycle during the 6 days following oocyst ingestion. Taken from McDougald and Fitz-Coy (2008).

Etiology, diagnosis and pathogenicity of coccidiosis in chickens

Coccidia oocysts can be shed in the feces of infected and recovered birds, contaminating feed, water, and litter. The coccidia oocyst is ubiquitous to the chicken house, especially under an intensive rearing system, providing an opportunity for increased fecal-oral transmission of viable oocysts among susceptible birds. Intact

unsporulated oocysts can remain in the chicken environment and remain non-infective for a long period of time. However, under appropriate optimal temperature, humidity, and oxygen concentration conditions, the oocysts sporulate and can become infective within 1 to 2 days (McDougald, 2003). The sporulated oocysts can survive in the environment for a long period of time, depending on environmental conditions. Although a total of 9 species of *Eimeria* affecting chickens have been described (*E. acervulina*, *E. brunetti*, *E. hagani*, *E. maxima*, *E. mitis*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella*), only 7 species commonly parasitize commercial chicken operations (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*). Of those, 3 are commonly reported in broiler chicken operations (*E. acervulina, E. maxima, and E. tenella*) (Chapman, 2000; Lillehoj and Lillehoj, 2000; McDougald, 2003). The development of *Eimeria* in specific locations of the chicken digestive tract (Fig. 2.6) has been used to differentiate the different types of *Eimeria* species (McDougald and Fitz-Coy, 2008). *Eimeria acervulina* and *E. praecox* parasitize the duodenum but can extend to the mid-intestine in heavy infections. *E. mitis*, *E. maxima*, and *E. necatrix* typically parasitize the mid-intestine, but can extend to the lower portion of the intestine in heavy infections. *Eimeria. tenella* parasitize the ceca, and *E. brunetti* parasitize the lower intestine and rectum (Levine, 1942; Joyner, 1978). The pathogenicity of coccidia infections range from moderate to severe, and may be influenced by the age, immune status and genetic background of the affected bird, its possession of concurrent diseases, the species of coccidia that infect it, and the number of coccidia oocysts that it ingests (Vermeulen et al., 2001). Infections by *E. acervulina* and *E. mitis* result in mild enteritis, which results in nutrient malabsorption, reduced growth rate, and poor feed efficiency. Infections by *E.*

brunetti and *E. maxima* result in inflammation and thickening of the small intestinal wall, with tiny petechial hemorrhages on the serosa and mucosa surfaces of the epithelium, and subsequent epithelial sloughing. Mild infections may go unnoticed, but may produce moderate mortality, reduced weight gain, and a poor feed conversion ratio. Infections by *E. necatrix* and *E. tenella*, which are the most pathogenic of chicken coccidia, result in weight loss, high morbidity and mortality, extensive hemorrhage, and bloody feces. Mortalities as a result of natural infections may be as high as 25 % in commercial flocks, and birds that survive the infection appear unhealthy, and suffer from cachexia and secondary infections. *Eimeria praecox* is generally considered to have little pathogenicity. However, heavy infections may cause intestinal contents to be watery, and may result in reduced weight gain, poor feed conversion, morbidity, and dehydration in birds.

Johnson and Reid (1970) developed a scoring system based on the visual examination of the different lesions produced by *Eimeria* species in different locations of the host's intestine. Lesion scoring was mainly initially used for experimental infections with known outcomes based on the species of *Eimeria*. However, the scoring system has become part of commercial operations, especially in the commercial broiler industry to continuously monitor the development of coccidiosis infections and the development of immunity within the broiler flock during grow-out. Lesion scoring is a technique which provides a numerical ranking based on the severity of gross lesions caused by different species of *Eimeria*. Scoring is based on the examination of 4 separate sections of the intestine. The upper intestine (duodenum) is examined and scored for lesions caused by *E. acervulina* and *E. mitis*. The mid-intestine (from the duodenum past the meckle's

diverticulum) is examined for lesions caused by *E. maxima, E. praecox, E. necatrix,* and *E. mitis*. The lower small intestine (from the yolk sac diverticulum to the cecal junctures) is examined for lesions caused by *E. maxima, E. brunetti, E. necatrix,* and *E. mitis*. The cecum is examined for lesions caused by *E. tenella,* only (Fig. 2.6). In the scoring technique developed by Johnson and Reid (1970), a score of 0 to 4 is assigned (0 = no gross lesions, and 4 = most severe gross lesion). In addition to gross lesion scoring, other diagnostic tools used to detect the severity of coccidiosis infections include microscopic lesion scoring, which is especially useful in detecting *Eimeria* species, such as *E. mitis* and *E. praecox*, that do not produce evident gross lesions (Idris et al., 1997), oocysts output (Idris et al., 1997), or histopathology (Idris et al., 1997; McDougald and Fitz-Coy, 2008). Several molecular methods that can differentiate between *Eimeria* species have been successfully developed. These methods include starch block electrophoresis, which differentiates between species based on isoenzyme patterns of the oocysts (Shirley 1975), and rRNA and rDNA probes which identify species by their characteristic restriction fragment patterns (Ellis and Bumstead, 1990). PCR techniques such as: conventional PCR and real-time PCR (Shirley, 1994); amplification of the internal transcribed spacer region 1 from genomic DNA (Shirley and Harvey, 1996); PCR RFLP techniques; and TaqMan probe-based qPCR (Woods et al., 2000), have all been used to detect and differentiate between *Eimeria* species.

Interaction between coccidiosis and gut health

Coccidia oocysts are ubiquitous in the environment because they are easily transmitted from one chicken house to another through the host (chickens), vermin, contaminated equipment, feed, litter, and humans (Williams, 2005). Once the coccidia

oocysts finds its way into the chicken house, it spreads via fecal-oral route, and birds may be exposed to coccidia throughout their lives. In practise, under the intensive management conditions, coccidiosis does not occur alone but rather occurs in consonance with other diseases that affect poultry, and management practices. In addition, the microflora of the gastrointestinal tract (GIT) consists predominantly of bacteria, and fungi and protozoa to a lesser degree. The interplay between these various organisms determines the microbial environment in the GI tract (Gabriel et al., 2006), and can influence the outcome of the coccidiosis infection. Because the GIT is a microbial community, several endogenous and exogenous factors can alter the microbiota balance within the GIT community thereby leading to presence of a disease state in the form of coccidiosis. These factors include infectious disease agents, nutrition, and management practices (Hughes, 2005). The interaction of coccidiosis with disease agents is reviewed in this section:

Coccidiosis and necrotic enteritis

Clostridium perfringens is part of the normal microflora of the chicken gut. However certain predisposing factors creates an imbalance of this bacteria within the gut, leading to clinical necrotic enteritis (NE). Damage to the GIT by bacteria such as *Clostridium perfringens* results in loss of performance e.g. reduction in BWG associated with coccidiosis. On the other hand it is thought that damage to the GIT by coccidiosis is a predisposing factor to NE, because coccidiosis often occur prior to or concurrently with NE outbreaks in the field (Williams, 2005; McDevitt et al., 2006). Therefore, evidence indicates that there is a relationship between NE and coccidiosis, so that the effects on performance in broiler may be synergistic in nature rather than coccidiosis alone. The

relationship between NE and coccidiosis has also been proven based on the methods of control (Williams, 2005).

a) Ionophores that are used in the control of coccidiosis also exert effect on NE.
b) The administration of coccidiosis vaccine in the absence of in-feed ionophores in leads to an increased risk of NE.
c) It is thought that mild coccidial infection produced by live-coccidial oocyst vaccine can predispose to NE. this potential risk associated with coccidial vaccine prevents its use by certain commercial producers.

Several studies have shown that vaccination with non-attenuated or attenuated anticoccidial vaccine, or medication with ionophores and antimicrobial growth promoter (**AGP**) can occasionally lead to the development of NE and coccidiosis in broilers (Williams, 2002a). Disturbances in balance of microbiota within the chicken gut is the single most important factor that may affect health status and production performance of birds in commercial poultry operations.

Coccidiosis and Immunosuppressive diseases

Immunosuppresive disease such as infectious bursal disease, chick infectious anemia virus and Marek's disease, and stressors may exacerbate or complicate *Eimeria* infection, producing severe coccidiosis (Rice and Reid, 1973; McDougald et al., 1979). In addition, gangrenous dermatitis (GD), caused mainly by *Clostridium perfringens* and *Clostridium septicum* in immunosuppressed birds, may predispose birds to coccidiosis (Li et al., 2010). The enteric and non-enteric viruses such as adenoviruses, reoviruses, enteroviruses, and rotaviruses, can complicate coccidiosis, leading to depression in

weight gain, impaired feed utilization, high morbidity, and poor flock uniformity (Reynolds, 2003; McNulty and Guy, 2003).

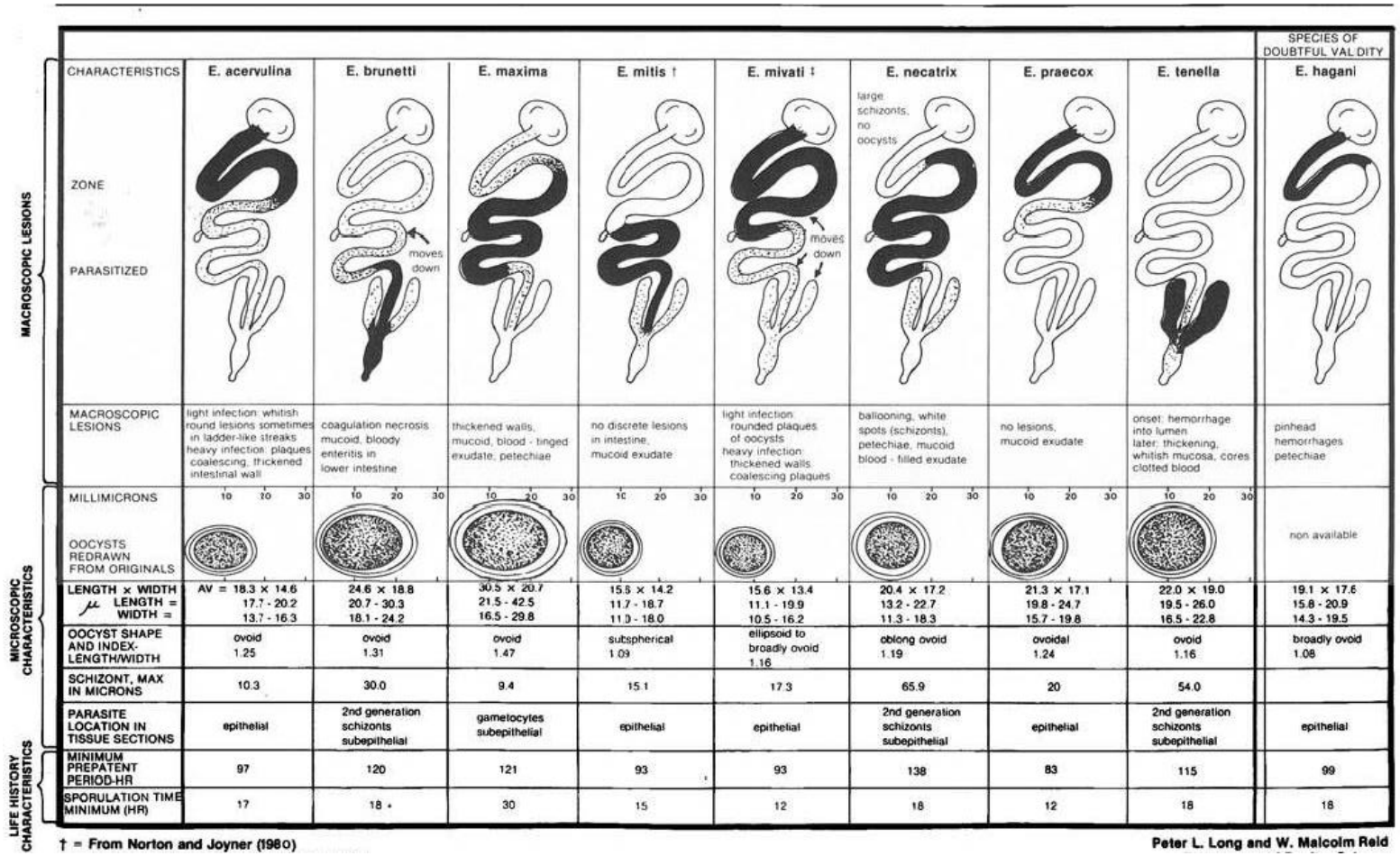
Coccidiosis and nutritional factors

Diets make up over 70 % of poultry production and producers continue to look for ways to produce least cost diets, leading to the use of feed ingredients that compromise the chicken's gut health. Non-starch polysaccharides (NSP) commonly found in cereals such as barley, wheat, rye, and oats are resistant to the host's digestive enzyme, and leads to high viscosity within the intestinal lumen and the digesta. Increased viscosity of the digesta can promote resident bacteria colonization, increased passage time, depressed digestive enzyme action, decrease nutrient digestion, and overall poor performance such as depressed growth rate (Waldenstedt et al., 2000). In addition, feed form have also been shown to affect gut health leading to mortalities associated with a combination of NE and coccidiosis. Finely ground feed form may cause higher mortality attributed to NE compared to coarsely ground feed form (Branton et al., 1987). Feeding whole wheat to broiler chickens increased gut health and nutrient absorption, reduced bacterial counts (*Salmonella Typhimurium* and *Clostridium perfringens*) in the intestinal tract of the birds and increased feed efficiency (Plavnik et al., 2002; Engberg et al., 2004). In addition, high concentrations of animal proteins (fish-meal) and animal fat in broiler diets may lead to high counts of ileal and cecal *C. perfringens* (Drew et al., 2004)

Immunogenicity of coccidiosis

Extensive research has been conducted at the cellular and molecular levels to understand mechanisms of the protective immune responses against coccidiosis infection

in chickens. Following an initial exposure to *Eimeria* oocysts by ingestion, a non-specific immune response is activated by low pH and inflammatory reactions, specifically to limit the number of active sporozoites that reach the specific site of infection.



	CHARACTERISTICS	E. acervulina	E. brunetti	E. maxima	E. mitis †	E. mivati ‡	E. necatrix	E. praecox	E. tenella	SPECIES OF DOUBTFUL VALIDITY E. hagani
MACROSCOPIC LESIONS	ZONE / PARASITIZED		moves down			moves down	large schizonts, no oocysts			
	MACROSCOPIC LESIONS	light infection: whitish round lesions sometimes in ladder-like streaks heavy infection: plaques coalescing, thickened intestinal wall	coagulation necrosis mucoid, bloody enteritis in lower intestine	thickened walls, mucoid, blood - tinged exudate, petechiae	no discrete lesions in intestine, mucoid exudate	light infection: rounded plaques of oocysts heavy infection: thickened walls coalescing plaques	ballooning, white spots (schizonts), petechiae, mucoid blood - filled exudate	no lesions, mucoid exudate	onset: hemorrhage into lumen later: thickening, whitish mucosa, cores clotted blood	pinhead hemorrhages petechiae
MICROSCOPIC CHARACTERISTICS	MILLIMICRONS / OOCYSTS REDRAWN FROM ORIGINALS	10 20 30	10 20 30	10 20 30	10 20 30	10 20 30	10 20 30	10 20 30	10 20 30	non available
	LENGTH x WIDTH μ LENGTH = WIDTH =	AV = 18.3 x 14.6 17.7 - 20.2 13.7 - 16.3	24.6 x 18.8 20.7 - 30.3 18.1 - 24.2	30.5 x 20.7 21.5 - 42.5 16.5 - 29.8	15.5 x 14.2 11.7 - 18.7 11.0 - 18.0	15.6 x 13.4 11.1 - 19.9 10.5 - 16.2	20.4 x 17.2 13.2 - 22.7 11.3 - 18.3	21.3 x 17.1 19.8 - 24.7 15.7 - 19.8	22.0 x 19.0 19.5 - 26.0 16.5 - 22.8	19.1 x 17.6 15.8 - 20.9 14.3 - 19.5
	OOCYST SHAPE AND INDEX-LENGTH/WIDTH	ovoid 1.25	ovoid 1.31	ovoid 1.47	subspherical 1.09	ellipsoid to broadly ovoid 1.16	oblong ovoid 1.19	ovoidal 1.24	ovoid 1.16	broadly ovoid 1.08
	SCHIZONT, MAX IN MICRONS	10.3	30.0	9.4	15.1	17.3	65.9	20	54.0	
	PARASITE LOCATION IN TISSUE SECTIONS	epithelial	2nd generation schizonts subepithelial	gametocytes subepithelial	epithelial	epithelial	2nd generation schizonts subepithelial	epithelial	2nd generation schizonts subepithelial	epithelial
LIFE HISTORY CHARACTERISTICS	MINIMUM PREPATENT PERIOD-HR	97	120	121	93	93	138	83	115	99
	SPORULATION TIME MINIMUM (HR)	17	18	30	15	12	18	12	18	18

† = From Norton and Joyner (1980)

Peter L. Long and W. Malcolm Reid

Figure 2.6 Diagnostic features of different *Eimeria* species found in chicken

Taken from McDougald and Fitz-Coy (2008).

Once sporozoites reach the site of infection and infection is established, the first defensive response elicited by the bird is the formation of specific immunity which include cell-mediated and humoral immune responses (Lillehoj et al., 1999). Although under commercial conditions chickens are often simultaneously infected by multiple species of *Eimeria*, it has been established that a specific immune response to one *Eimeria* species induces little or no protection against challenges from other species (Lillehoj and Lillehoj, 2000). In early studies, it was suggested that cell-mediated immune responses (cellular immunity) play a major role in the development of active protection against *Eimeria,* with humoral immunity playing only a minor role (Rose and Hesketh, 1979; Lillehoj and Choi, 1998; Dalloul and Lillehoj, 2005). Lillehoj (1987) reported that protective immunity against coccidiosis challenge was not significantly affected in bursectomized chickens despite an inability to produce specific antibodies. It was then concluded that T-cell immunity plays a major role during infection with *Eimeria*, whereas antibodies have little or no effect against *Eimeria*. The direct role of T-cell subsets, referred to as cytotoxic $CD8^+$ and $CD4^+$ helper T-cells, has been demonstrated in the development of active immunity in chickens (Lillehoj et al., 2004; Lillehoj et al., 2007; Trout and Lillehoj, 1995). There is evidence that $CD4^+$ cells are involved in primary coccidiosis infections, while $CD8^+$ cells are important in later infections (Lillehoj and Choi, 1998). *Eimeria* species generate specific immune responses in different ways depending on their location within the gut. It was found that both $CD4^+$ and $CD8^+$ T-cells were stimulated at specific sites of infection after a primary infection (Bessay et al., 1996). Cornelissen et al. (2009) showed that infection with *E. acervulina* induced the production of $CD8^+$ T-cells, which is similar to findings showing an increase

in $CD4^+$ and $CD8^+$ T-cells with *E. maxima* and *E. acervulina* infections, respectively (Trout and Lillehoj, 1995). Following infections by *E. acervulina* and *E. maxima*, sporozoites have been reported within $CD8^+$ cells, and occasionally in $CD4^+$ cells. Also, $CD4^+$ cell counts remain elevated during later infections, indicating that IEL cytotoxic $CD8^+$ and $CD4^+$ T-cells provide both active and protective immunity (Trout and Lillehoj, 1995). Furthermore, the roles of cytokines, NK cells, macrophages, and helperT-cells (Th1 and Th2) in cell-mediated protection against *Eimeria* have also been reported. It was shown that increases in interferon-γ (IFN- γ) production by effector T-cells, inhibits parasite invasion, and, therefore, elicits protection (Lillehoj et al., 2004). Cornelissen et al. (2009) noted an increase in macrophages, Th1 (IL-2, IL-18 and IFN- γ), Th 2 (IL-4 and IL-10), and helper T-cells following infections with E. *acervulina*, *E. maxima*, *E. maxima.* The Th1 and Th2 immune pathways, which are important in resolving intracellular and extracellular parasites, respectively, play key roles in enhancing parasite killing by NK cells, T-cells, and macrophages.

In addition to the role of cell-mediated immunity, several studies have also shown that humoral immunity plays a role in protection against coccidiosis infections. Although the role of *Eimeria* specific antibodies in immune responses is limited and has not been well defined, IgM, IgG, and IgA antibodies have been recovered in chickens following their recovery from a coccidiosis infection. Circulating levels of coccidial specific IgY and IgA were detected in birds 7 days after a coccidiosis infection, with peak levels reached by day 14 (Lillehoj, 1987). The type of antibody and the time they are produced (during a primary infection or in a convalescence stage) is dependent on the *Eimeria* species involved in the infection. Trout and Lillehoj (1995) suggested that after a

coccidiosis infection, parasites enter the intestinal epithelium, but parasite development is prevented by humoral immune responses. This suggests that both cell-mediated and humoral immunity is activated following a coccidial infection, although innate immunity plays a major role in the development of immune responses against coccidiosis (Lillehoj et al., 2007).

Impact of coccidiosis on broiler industry

Of all the intestinal pathogens that affect the broiler chicken, coccidiosis is regarded as a parasitic disease with a substantial economic impact on the poultry industry (Williams, 2002). The significant economic impact of coccidiosis is attributed to losses due to poor performance (poor feed efficiency and body weight gain) associated with subclinical infections. Furthermore, high morbidity and mortality are associated with clinical coccidiosis infections, and the cost involved in prophylaxis and curative treatments (Vermeulen et al., 2001; Peek and Landman, 2011). In the United States of America (**US**), it is estimated that over $700 million per year is lost for the control and treatment of coccidiosis (Chapman, 1998). The estimated loss to the worldwide commercial poultry industry due to coccidiosis is approximately $3 billion (Williams, 1999a; Peek and Landman, 2011), while in the United Kingdom, the loss has been estimated to be GB£38.6 million (Williams, 1999a; Shirley et al. 2007). Commercial broiler chicken production in the US involves the raising of large numbers of birds (up to 50,000 chickens) on built-up litter within a single house, with litter changes occurring only between a minimum of 6 flock grow-outs each year (Bell and Weaver, 2002). This intensive method of production provides a good opportunity for the transmission of *Eimeria* parasites between flocks, and between individual birds. Coccidial oocysts can

survive in chicken houses for a very long time, and the presence of the oocyst cell wall makes them resistant to the environment and to disinfectants commonly used in commercial chicken house cleaning. Similarly, sporulated oocysts can survive in the environment for up to 2 years (Reid, 1990). Hence, it is almost impossible to find a commercialized broiler chicken flock that is completely free of coccidia (Williams, 1999a). Although clinical coccidiosis is not commonly found in commercial operations, subclinical coccidiosis continues to be a persistent problem (Williams, 1999b; Lee et. al., 2009). In general, there are 3 classifications for coccidiosis infections, all of which cause some type of adverse effects on economic performance. These 3 classifications are:

1. Clinical coccidiosis: This is characterized by visible pathological changes in the intestinal epithelium leading to diarrhea, bloody feces, blood loss, hypovolemic shock, mortality, and morbidity.
2. Subclinical coccidiosis: This is perhaps the most common form. This type of coccidiosis unlike clinical coccidiosis is not very visible, because there are no obvious signs of disease. However, it causes some pathological changes to the intestinal lining, leading to growth depression, a high feed conversion ratio, and sometimes mortality (due to debilitation of the host).
3. Coccidiasis: This is regarded as a mild infection with no adverse effects on the host (Levine, 1961).

Three species of *Eimeria* are most commonly reported in commercial chicken operations in the US, with each species causing distinctly recognizable diseases. The EM1 vaccine is formulated with these 3 species of *Eimeria*, and can therefore provide immune protection against the 3 species of *Eimeria*. These species are: *E*. *acervulina*, *E*. *maxima*, and *E*. *tenella. E. acervulina* is the most frequently encountered worldwide in commercial poultry (Chapman, 2005). The oocysts are ovoid in shape, with an average size of 18.3 × 14.6 μm. Mild infection may not be prominent, but can result in reduced weight gain, reduced feed efficiency, and loss of skin pigmentation. Heavy infections can

result in diarrhea, a pronounced reduction in weight gain, and even mortality. Lesions may coalesce, resulting in a thickening of the intestinal mucosa and reduced nutrient absorption within the small intestine. Gross pathological signs may be limited to the duodenal loop in light infections, but may extend to the proximal small intestine in heavy infections. Microscopic smears during field posting sessions may indicate coccidia infections at varying developmental stages. Histopathological tests of the duodenal loop and proximal small intestine may reveal coccidial infections at various stages in the villi lining, with subsequent damage to the tips of the villi and thickening of the mucosa.

E. maxima predominantly parasitize the mid-small intestine from the end of the duodenal loop to areas past the yolk sac diverticulum. In heavy infection, lesions may extend throughout the small intestine. The characteristically large oocysts of *E. maxima* are easily recognizable, measuring an average of 30.5 × 20.7 μm. Large infections with up to 200,000 oocysts can result in weight loss, rough feathers, poor weight gain, diarrhea, morbidity, and occasional mortalities. Gross lesions develop approximately 5 to 8 days post-infection when coccidial sexual life cycle stages develop in deeper tissues. Lesions include congestion, edema, thickening of the intestinal mucosa, and yellow-orange fluid accumulations in the mid-gut (ballooning). Microscopic lesions include cellular infiltration, edema, the presence of various life cycle stages of *Eimeria*, and the destruction of intestinal villi structure.

E. tenella infection is the most recognizable type of poultry coccidiosis, because of the lesions it produces in the ceca. Gross lesions include bleeding, and loss of skin pigmentation, and subsequently cause weight loss, anemia, high levels morbidity, and

eventual mortality in birds. The average oocyst size is 22.0 × 19.0 µm, and infections by 1,000 to 10,000 sporulated oocysts can produce clinical disease in birds.

Control of coccidiosis

Since 1948, broad spectrum anticoccidial drugs have been used for the control of coccidiosis in poultry. These anticoccidial drugs can be classified into 2 main categories based on their specific modes of action (Chapman, 1999). These 2 categories are:

1. Synthetic anticoccidials: These compounds are produced by chemical synthesis and are often referred to as 'chemicals'. Their mode of action is generally by affecting parasite metabolism. However, their specific mode of action depends on the actual compound itself. For example, decoquinate and clopidol inhibit the mitochondrial respiration of the parasite, and sulphonamides inhibit the folic acid metabolic pathway and uptake of vitamin B1 by the parasite. Other synthetic anticoccidials with unknown modes of action include halofuginone, nicarbazin, robenidine, and diclazuril.

2. Polyether ionophores (ionophore antibiotics): These anticoccidials are produced by fermentation using Streptomyces or Actinomadura species. Their mode of action is primarily by altering the transport of monovalent and divalent cations (such as Na^{+}, K^{+}, Ca^{++}) across cell membranes and the subsequent disruption of osmotic balance (Kant et al., 2013). They can be classified as monovalent ionophores (e.g. Monensin, Narasin and Salinomycin), and divalent ionophores (e.g. Lasalocid). Ionophores allow for the development of immunity against coccidia (Chapman, 1999), and can have antimicrobial and growth promoting activities, such as the inhibition of gram-positive organisms (Vissiennon et al., 2000).

3. Some anticoccidial products consist of both chemical and ionophore compounds (e.g. Maxiban®, which contains a mixture of Nicarbazin and Narasin), or a combination of two chemical compounds (e.g Lerbek ®, which contains methylclorpindol and methylbenzoquate). These types of anticoccidials are widely used in poultry production for the control of coccidiosis. The various in-feed anticoccidials that are commonly used in broilers are described in Table 2.2.

Anticoccidial drugs remain the main stay for poultry producers in the control of coccidiosis, because they are a good preventative method when added to chicken feed,

and because they are well adaptable for large-scale use (Chapman, 2010). However, the intense and widespread use of anticoccidial drugs has led to the worldwide development of an acquired resistance to the drugs by coccidia (Chapman, 1997). Resistance occurrences have been reported wherever poultry are intensively reared, in the US, South America, Europe, and China (Jeffers, 1975; Chapman 1997; Peek and Landman 2003). Resistance to some chemical anticoccidials can develop quickly, or resistance to ionophores may take several years (Chapman, 1997; McDougald, 2003). Acquired drug resistance to coccidia may be complete (in which increasing doses up to maximum levels that are tolerated by the host are ineffective, e.g. nicarbazin). However, it is possible that drug resistance may allow trickle-like infections to occur, which can lead to the eventual development of immunocompetence (Chapman, 1998; Peek and Landman, 2003). In order to ameliorate the problem of drug resistance, poultry producers have adopted rearing programs that make use of compounds with different modes of action. This rationale is based on the fact that reducing the length of anticoccidial drug exposure tends to increase the sensitivity of coccidia to the various drugs. Shuttle and rotation programs are most commonly employed (McDougald, 2003; De Gussem, 2007). In a shuttle program, different drugs are used in the different feed-type phases during the grow-out period. For example, a chemical (i.e. Nicarbazin) may be added to the starter feed, whereas an ionophore may be added to the grower feed. In a rotation program, different drugs are used in successive flocks. Although there has been a reduction in coccidiosis outbreaks, due in part to an increase in the effectiveness of anticoccidial drugs administered through the shuttle and rotation programs, the use of these programs does not fully prevent drug resistance, because most *Eimeria* isolates found in commercial

poultry practices exhibit varying levels of resistance to more than one drug (Chapman, 1997). Another important factor affecting the use of in-feed anticoccidials are concerns about drug residues in poultry products and the desire of consumers to ban the use of drugs in animal feeds (Young and Craig, 2001; McEvoy, 2001). The use of antimicrobials and antibiotic growth promoters in feed has received enormous criticism, particularly in the European Union, where new legislation is being proposed that would phase-out the use of these drugs (Farrant, 2001). There is, therefore, a pressing need to shift from the use of chemotherapeutics for the control of coccidiosis to other alternative control options.

Alternatives for anticoccidial drugs

The occurrence of anticoccidial drug resistance together with increasing consumer and regulation concerns on the use of anticoccidial drugs in chicken feed has resulted in the search for alternative control strategies. The effects of these alternative compounds for the control of coccidiosis have been extensively reviewed. These include (1) natural products that are rich in fatty acids (Allen and Danforth, 1998), (2) plant extracts (Naidoo et al., 2008), (3) antioxidants (Augustine et al., 1999; Youn and Noh, 2001), (4) pre- and probiotics (Peek and Landman, 2011) and (5) vaccines (Vermeulen, 2001; Williams, 2002; Chapman, 2002, 2014; Tewari, 2011).

Sources rich in fatty acids

Fat sources containing high amounts of n-3 fatty acids (docosahexaenoic acid, eicosapentaenoic acid, and linolenic acid), such as fish oils, flaxseed oil, and whole flaxseed, added to chick starter rations at 1 day of age, have been shown to effectively

reduce the amount of lesions resulting from challenge infections with *E. tenella*. Conversely, these fat sources have not proven effective for challenge infections by *E. maxima* (Allen et. al., 1997a). There is evidence that fish oil and flaxseed oil diets reduce lesions associated with the development of *E. tenella* in the gut. These oils have also been shown to cause the ultrastructural degradation of both the asexual and sexual stages of the organism, which are characterized by cytoplasmic vacuolization and chromatin condensation within the nucleus (Allen et.al., 1997a; Allen and Danforth, 1998). It was concluded that the presence of a high percentage of easily oxidized double bonds in diets rich in n-3 fatty acids would induce a state of oxidative stress that is detrimental to *Eimeria* parasite development.

Table 2.2 Various anticoccidial drugs used in poultry feeds.

Chemical name	Trade name	Class	Company	Biological activity and mode of action
Monensin	Coban, Elancoban	Ionophore	Elanco	Broad spectrum activity. Acts on trophozoites and 1st generation schizonts, within first 2 days of life cycle. Form complexes with sodium and potassium ions in the host and developing parasite. The monensin-cation complex renders membrane permeable to sodium and potassium ions. Effective at 0.01 to 0.121% concentration in the feed
Lasalocid	Avatec	Ionophore	Hoffmann-La Roche	Effective at 0.005 to 0.0075% concentration. Has affinity for divalent cations as well as monovalent ions
Salinomycin	Bio-cox, Salinomax, Sacox	Ionophore	Agri-Bio	Closely related to monensin. It has anticoccidial activity at 0.01% in the feed. Has affinity for sodium and potassium ions
Maduramicin	Cygro	Ionophore	American Cyanamid	Most potent among the polyether Ionophores. Aministered at 5 to 6 ppm in feed. Has ionic affinity. May cause severe cardiovascular defects in host cells
Narasin	Monteban	Ionophore	Elanco	Has anticoccidial activity at 54 to72g/T in feed, and affinity for sodium and potassium ions
Robenidine	Robenz, Cycostat	Chemical	American Cyanamid	Inhibits oxidative phosphorylation in late first generation and second stage schizonts, but mostly effective against the maturing first generation schizonts.It is effective at 0.0066% mixture in the feed. It is used to a less extent in Europe and South America, but not in USA
Amprolium	Amprol	Chemical	MSD-AGVET	Thiamine antagonist, blocks the thiamine receptors and makes thiamine unavailable to the parasite. It is effective against 1st generation of trophozoites and schizonts and shows peak activity early in day 3 of cycle. It is active against *E. tenella*, *E. necatrix* and *E. acervulina* and to lesser extent *E. maxima*. Effective at 0.0125 to 0.025% mixture in the feed
Diclazuril	Clinacox	Chemical	Schering-plough	Effective at 1 ppm mixture in the feed

Chart information taken from Mathis et al. (1984), McDougald and Fitz-Coy, (2008), Chapman, (2010), and Kant et al. (2013).

Table 2.2 (continued)

Chemical name	Trade name	Class	Company	Biological activity and mode of action
Nicarbazin	Nicarb	Chemical	Merck	Has broad spectrum activity and effective against all *Eimeria* spp. This compound has coccidiocidal activity, mainly against the 2nd generation schizonts. It alters the intracellular energy-supplying ATP of the coccidial parasite, and effective at 0.0125% concentration in feed. It should not be used in egg laying birds because it reduces egg production and number of fertile eggs. It also causes depigmentation of eggs, malformed egg yolk, and poor hatchability.
Decoquinate	Deccox	Chemical	Rhone-Poulenc	Has broad spectrum coccidiostat activity and inhibits sporozoite development. It is used at a concentration of 0.003% in the feed. Has no withdrawal requirements
Halofuginone	Stenorol	Chemical	Agri-vet	Has potent broad spectrum coccidiocidal and coccidiostatic activity against 1st and 2nd generation schizonts. Effective at a feed concentration of 3 ppm. Use in young birds upto 12 weeks of age but not be used in egg laying birds
Amprolium + Ethopabate	Amprol Plus	Chemical + Chemical	Merck	Effective at a feed concentration of 0.0125% amprolium and 0.004% Ethopabate
Narasin + Nicarbazin	Maxiban	Ionophore + Chemical	Elanco	Synagestic effect of an ionophore and chemical anticoccidial. Effective at a feed concentration of 54-90 g/T
Sulphaquinoxaline	SQ	Chemical	Merck	Has an inhibitory effect on schizogony at feed concentration of 0.025 to 0.033% against *E. acervulina*, *E. necatrix,* and *E. tenella.* Feed concentration of 0.1% inhibited invasion by the sporozoites. Synergestic effect with pyrimethamine gave better control of *E. acervulina,* and *E. maxima*

Plant extracts

Different herbal substances have been studied for their potential use as dietary supplements to control coccidiosis. Artemisinin, a Chinese herbal extract obtained from *Artemisia annua* and *Artemisia sieberi,* is a naturally occurring endoperoxide with antimalarial properties, and has been shown to be effective in reducing oocyst output of *E. acervulina* and *E. tenella* infections, but not *E. maxima*, when incorporated in starter diets (Allen et al. 1997a, b). Its mode of action is thought to be by the induction of oxidative stress in the parasites. It is most effective against *E. acervulina* and *E. tenella* infections when fed at levels of 8.5 ppm and 17 ppm, respectively (Allen and Danforth, 1998). In a study by Youn and Noh (2001), the anticoccidial activity of 15 Asian herb extracts was evaluated following challenge infections with *E. tenella*. Although all 15 herbal extracts showed varying degrees of effect, extracts from *Sophora flavescens Aiton* was the most effective in maintaining body weight gain, improving survival rates, and reducing bloody diarrhea, lesion scores, and oocyst production. Extracts from *Sophora japonica*, *Torreya nucifera* affected weight gain, clinical signs of disease, survival rates and lesion scores less, when compared to an infected control group. It can therefore be concluded that plant extracts can vary in their mode of action, and effects on coccidiosis control.

Antioxidants

Use of plant extracts with antioxidant activities for the control of coccidiosis has also been studied. Allen et al. (1997a) showed that chick feed supplemented with the potent antioxidant Gamma-tocopherol, found abundantly in seed oils such as flax, wheat,

corn and soybean, at a level of 8 ppm improved chick weight gain, and reduced lesion scores and oocyst output in the upper and mid area of the small intestine that were infected by *E. acervulina* and *E. maxima*. However, this potent antioxidant had little effect in chickens infected with *E. tenella*. The osmoprotective effect of Betaine (a sweet crystalline alkaloid found in sugar beets) was examined against a coccidiosis infection (Augustine and Danforth, 1999). Betaine fed at 0.15 % in combination with 66 ppm Salinomycin significantly improved the weight gain and feed efficiency of birds infected with *E. acervulina, E. maxima*, and *E. tenella*. Betaine and Salinomycin either alone or together inhibited the gut invasion of *E. acervulina*. It was concluded that their combination may improve the performance of chickens infected with coccidiosis, either directly by inhibiting the development of second generation *E. acervulina* schizonts or by providing an osmoprotective effect on intestinal structure. Using toltrazuril as a positive control, plant extracts demonstrating antioxidant activity, such as *Tulbaghia violacea* (35 mg/kg), *Vitis vinifera* (75 mg/kg) and *Artemisia afra* (150 mg/kg) were examined for their anticoccidial activities. The effects of these plant extracts on feed conversion ratio were similar to toltrazuril. In addition, *T. violacea* significantly decreased oocyst shedding in the birds. From this study, it was concluded that plant extracts rich in antioxidants have potential positive benefits in birds infected by coccidia infections (Naidoo et al., 2008). Several other natural products that have been tested in birds with coccidiosis infections include mushrooms and their extracts (Guo et al., 2004; Guo et. al., 2005; Dalloul and Lillehoj, 2006). It was found in all these studies that when birds were challenged with *E. acervulina,* and *E. tenella*, lectin (mushroom extract) induced cellular and humoral-based immunities, thereby improving performance and reducing oocyst

output. Essential oil blends have also been shown to reduce lesions and oocyst shedding, while maintaining growth performance during mixed coccidial infections (Oviedo-Rondón et al., 2006). Allen and Fetterer (2002) suggested that natural products that elicit oxidative stress responses (e.g. n-3 FA and artemisinin), can be particularly effective against *E. tenella*; whereas products that have antioxidant properties (e.g. γ-tocopherol), can be more effective against *E. maxima* and *E. acervulina*. Although the use of natural products alone or in combination with current coccidiosis control programs have tremendous potential, several studies needs to be conducted to determine the safe dosages and efficient ways of application of these natural products.

Pre- and probiotics

Gibson and Roberfroid (1995) defined a prebiotic as "*a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health*". Mannanoligosaccharides (MOS), derived from the cell wall of the yeast *Saccharomyces cerevisiae*, have been described as a prebiotic that is used in animal feed to promote gastrointestinal health and improve performance. MOS competes with the mannose-specific binding of pathogens, resulting in a reduction of their colonization (Ofek et al., 1977). MOS are also thought to block the binding of pathogens to mannan receptors on the intestinal mucosa surface (Spring et al., 2000). Dietary MOS administered at rates of 1g/Kg and 10g/Kg in feed have reduced the severity of *E. tenella* and *E. acervulina* infections, respectively (Elmusharaf et al., 2007). Prebiotics function in targeting the bacteria already present within and adapted to the gut environment. Studies have shown

that the effect of MOS on the control of coccidiosis may be dependent of the amount of dietary MOS incorporated into the feed (McCann et al., 2006).

A probiotic is defined as "*a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance*". Probiotics consist of beneficial live bacteria or yeast that are added to diets. The most commonly used probiotics in livestock are *Bacillus*, *Enterococcus*, and the yeast *Saccharomyces*. Probiotics ensures gut health and a balanced intestinal microflora by modulating the immune system and enhancing host resistance to enteric infections. The benefit of probiotics in developing immunity against a coccidiosis challenge has been demonstrated in several studies (Jin et al., 1996; 1998; 2000; Zulkifli et al., 2000, Mathis et al., 2014).

Anticoccidial vaccines

Coccidia live vaccines comprised of oocysts of various *Eimeria* species, are the preferred alternative to anticoccidial drugs for the control of coccidiosis in poultry. The commercialization of anticoccidial vaccines began with the production of the Coccivac® in the 1950s (Williams, 2002). It is thought that the administration of low doses of coccidia oocysts early in the life of the bird can induce protective immunity (after 2 to 3 cyclic infections) before the occurrence of a field challenge (Long et al., 1986). For a long time, the use of coccidia vaccines were limited to broiler breeders and replacement layer stocks (William, 2002; Shapiro, 2001), but 2 major factors have enhanced the use of live oocyst vaccines for the control of coccidiosis in broilers. These include: (1) understanding that protective immunity can be produced following immunization with low doses of oocysts in day old chicks, (2) the development of new methods that facilitate vaccine application in the hatchery (Chapman, 2002).

Development of protective immunity from a coccidiosis vaccine

The process of developing an immune response following the administration of a coccidial vaccine is similar to that previously described for a challenge infection. Coccidiosis vaccines provide low controlled numbers of *Eimeria* parasites that ensures the development of substantial immunocompetence after two to three consecutive cycles of infections. The asexual developmental stage (multiplication of trophozoites) plays a key role in generating a protective immune response, and immunity develops primarily through a cellular response (Jenkins et al. 1991; Dalloul and Lillehoj, 2005). T cells and cytokines play important roles in protective immunity against *Eimeria*. Th1-type cytokines function to limit the multiplication of *Eimeria* parasites within the gut, during the early stages of infection (vaccine application). Regulatory cytokines and cellular responses are important in limiting proinflammatory cytokine-associated pathologies (Roberts et al. 1996). Immunity is specie specific (i.e. immunity against one species of *Eimeria* will not provide protection against a different species). Protective immunity is generally regarded as the absence of oocyst production, and the absence of clinical signs in birds infected with coccidiosis (Price, 2012). All commercial anticoccidial vaccines used in the field today can generally be classified into 2 categories. These 2 categories are: (1) nonattenuated live oocyst vaccines, and (2) attenuated live oocyst vaccines.

Live nonattenuated vaccines consist of a controlled but variable number of wild-type *Eimeria* species oocysts (Lee, 1987). Examples of this type of vaccine include Inovocox®, Immucox®, Coccivac®-B and Coccivac®-D (Table 2.3; Chapman, 2014). Because live nonattenuated vaccines only contain a small but sufficient number of *Eimeria* oocyst species, their effectiveness is dependent on the recycling of the initial low

dose that is administered. This leads to the gradual buildup of solid immunity (Dalloul and Lillehoj, 2005). The first commercial live nonattenuated vaccine (Coccivac® D) launched in 1952 contained oocysts of the 8 wild-type *Eimeria* species, and was used to vaccinate broiler breeders and replacement pullets (Williams, 2002). Currently, anticoccidial vaccines used in the broiler industry contain oocysts of various types of *Eimeria* species. Such vaccines with combinations of various species include: *E. acervulina*, *E. maxima* and *E. tenella* (Inovocox®), or 4 *Eimeria* species; *E. acervulina*, *E. maxima, Eimeria necatrix,* and *E. tenella* (Immucox® C1); *E. acervulina*, *Eimeria mivati, E. maxima,* and *E. tenella* (Coccivac® B) (Vermeulen et al., 2001). Birds are vaccinated within the first week of life using vaccination methods that ensures uniformity of vaccine application and the uptake of an adequate amount of vaccine oocysts for sufficient cycling (Dalloul and Lillehoj, 2005). Although vaccination with a nonattenuated live oocyst vaccine has proven to be effective for the development of protection against subsequent *Eimeria* challenges (Williams, 2003), there has been a skepticism regarding the use of vaccination programs for US broiler production, especially in broilers subjected to short grow-out periods. This is largely due to reports noting negative effects of nonattenuated live oocyst vaccine on grow-out performance (Allen and Fetterer, 2002). Nonattenuated vaccines are formulated to introduce a controlled coccidial infection during early grow-out and have been shown to decrease BW gain and lead to a poorer feed efficiency in broilers (Danforth, 1998).

Attenuated live oocyst vaccines have the advantage of exhibiting reduced pathogenicity but being capable of inducing protective immunity with minimal potential for clinical disease (McDougald, 2003). Attenuated strains are selected via several

passages through embryonated eggs or by the selection for precocious (early) development (Williams, 2002). Examples of these types of commercial vaccines include Paracox® (Williams, 1994) and Livacox® (BedrnI´k, 1993). The second generation schizonts and sexual development are completely absent in the attenuated vaccines, which results in a loss of virulence with only moderate tissue damage (McDougald and Jeffers, 1976).

Table 2.3 Live attenuated and nonattenuated anticoccidial vaccines developed for various classes of chicken

Vaccine Trade name	Bird type	Eimeria Species	Attenuation	Route of administration	Age of birds	Manufacturer/Distributor
Coccivac® -B[1,2,3]	Broilers	*E. acervulina*, *E. maxima*, *E. mivati*, *E. tenella*	None	Hatchery spray, ocular, drinking water, feed spray	Single dose at 1 to 14 d	Schering-Plough Animal Health (USA)
Coccivac®-D[1,2,3]	Breeders, Layers	*E. acervulina*, *E. brunetti*, *E. hagani*, *E. maxima*, *E. mivati*, *E. necatrix*, *E. praecox*, *E. tenella*	None	Hatchery spray, ocular, drinking water, feed spray	Single dose at 1 to 14 d	Schering-Plough Animal Health (USA)
Immucox® C1[1,2,3]	Broilers, Roasters	*E. acervulina*, *E. maxima*, *E. necatrix*, *E. tenella*	None	Drinking water, oral gel	Single dose 1 to 4 d	Vetech Laboratories (Canada)
Immucox® C2[1,2,3]	Breeders, Layers	*E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. tenella*, *E. mivati*, *E. praecox*	None	Drinking water, oral gel	Single dose 1 to 4 d	Vetech Laboratories (Canada)
Inovocox®[1,2,3]	Broilers	*E. tenella*, *E. acervulina*, 2 strains of *E.maxima*	None	In ovo	Single dose in ovo	Zoetis Animal Health (USA)
Inovocox®EM1[1,2,3]	Broilers	*E. tenella*, *E. acervulina*, 1 strain of *E.maxima*	None	In ovo	Single dose in ovo	Zoetis Animal Health (USA)
Advent®[2]	Broilers	*E. acervulina*, *E. maxima*, *E. tenella*	None	Hatchery spray, feed spray	Spray single dose in hatchery or on first feed	Novus Int. (USA)
Livacox® D[3]	Caged chickens	*E. acervulina*, *E. tenella*	Yes	Drinking water	Single dose at 1 to 10 d	Biopharm (Czech Republic)
Livacox® T[3]	Broilers/Breeders	*E. acervulina, E. maxima, E. tenella*	Yes	Drinking water, ocular	Single dose at 1 to 10 d	Biopharm (Czech Republic)
Livacox® Q[3]	Broilers	*E. acervulina*, *E. brunetti*, *E. maxima*, *E. tenella*	Yes	Drinking water, ocular	Single dose at 1 to 10 d	Biopharm (Czech Republic)
Paracox®[3]	Broilers, breeders, Layers	*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mivati*, *E. necatrix*, *E. praecox*, *E. tenella*	Yes	Drinking water, feed spray	Single dose at 1 to 9 d	Schering-Plough Animal Health (UK)
Paracox®-5[3]	Broilers	*E. acervulina*, *E. maxima, E. mivati*, *E. tenella*	Yes	Hatchery spray, drinking water, feed spray	Single dose at 1 or 3 d	Schering-Plough Animal Health (UK)

Taken from Williams (2002), Pfizer Animal Health (2011), and Vermeulen (2001).
[1]Currently approved for use in Canada;
[2]Currently approved for use in the United States;
[3]Currently approved for use in countries outside of Canada and the United States

Methods of vaccine administration

Improvements in the methods of vaccine delivery have improved the efficiency and efficacy of the coccidiosis vaccine as a means for control in coccidiosis (Lee et al., 2009). Williams (2002) described various methods by which commercial live coccidiosis vaccines are administered to poultry. Uniform distribution of live vaccine oocysts is crucial for the induction of protective immunity. The development of hatchery-based delivery methods, which ensures uniform vaccine delivery, has avoided the need to vaccinate birds in the poultry house, and has been embraced by commercial broiler producers (Williams, 2002). Early formulations of live vaccines have been administered to birds via drinking water and feed, but it soon became evident that the induction of immunity was not maximized by these routes of administration. Vaccine delivery through feed and water reduces the chances of individual birds receiving a full dose of vaccine in equal proportion. It has been a common problem that some birds received higher vaccine doses than others through their drinking water, thereby creating uneven vaccine distribution and poor development of immunity in the flock as a whole (Williams, 2002). A similar scenario has been found when birds were immunized through feed. Colored edible gels containing oocysts (Immucox®) have been provided to 1 day old birds. The gels were placed in feed trays at the poultry house or in chick crates at the hatchery. Although these methods of vaccine administration to poultry, especially in breeder flocks, are still being used in some countries, these methods have been replaced with hatchery based vaccinations in the US. The two most widely used methods in hatcheries in the US are spray vaccination and in ovo administration.

In spray vaccination, the oocysts are suspended in colored water (mostly bright colors, such as red) and sprayed over the chicks in open trays using a spray cabinet. This method is commonly used for Coccivac®, Paracox®-5, ADVENT®, and Viracox®. The color promotes preening, and provides for a visual evaluation of vaccine distribution and ingestion. Chicks likely ingest the oocysts by direct oral and ocular routes, but ingestion mainly occurs by the self-preening and pecking of drops of diluted vaccine oocysts off a neighboring chick (Chapman et al., 2002; Dalloul and Lillehoj, 2005). Factors which may influence preening include the application of biologically active materials, increases in sound and light intensity, and decreases in temperature (Caldwell et al., 2001b). Because the oocysts are suspended in water, the vaccinated chicks become wet, but the water eventually dry off from the chicks' body. It has been shown that over 94% of spray vaccinated birds ingest the oocysts (Schetters et al., 1999).

In ovo injection is a more recent and well embraced method of vaccination, which is the inoculation of embryonated eggs with vaccine oocysts. The in ovo injection concept was first developed by Sharma and Burmester (1982). Although *in ovo* vaccination was initially developed for vaccination against Marek's disease, it has become widely utilized for vaccination against numerous poultry disease including coccidiosis. Today, *in ovo* vaccination is being used in both research and commercial applications (Williams, 2007). Conventional vaccine application methods have included posthatch feed and drinking water supplementation, hand vaccination, ocular instillation. However, these methods are time and labor intensive, and inefficient, and lead to inconsistent vaccine delivery. In ovo vaccination presents several advantages over these methods. These include uniform and fast delivery, increased efficiency, and early

development of immunity (Williams, 2007). Today, the US broiler industry employ in ovo injection technology for vaccinations against Marek`s disease (Williams and Hopkins, 2011), coccidiosis (Mathis et al., 2014), and infectious bursal disease (Moura, 2007). Commercial in ovo technology has expanded its capabilities from laboratory single egg injectors to multi-egg injectors capable of injecting between 50,000 to 70,000 eggs per hour (William, 2007). Because in ovo injection requires the injection of embryonated eggs, the stage of development of the embryos is critical in determining the correct time and site of injection. Successful in ovo vaccinations are dependent the timing of the injection in relation to the stage of the embryo development (William, 2007). It has been shown through various studies that the optimal time for in-ovo injection is during late stage incubation. This is typically between 17.0 and 19.0 day of incubation (Keralapurath et al., 2010; Zhai et al., 2011a, b, and c; Jochemsen and Jeurissen, 2002). This time corresponds to the period between the beginning of yolk sac absorption into the body and the positioning of the head of the bird under its right wing, to the beginning of internal and external pipping, with active imbibition of the amniotic fluid (Williams, 2007; Jochemsen and Jeurissen, 2002).

There are 5 basic compartments in a developing embryo that can be potentially accessed by in ovo injection. These areas include the air cell, allantoic sac, amniotic sac, the embryo body, and the yolk sac (Fig. 2.7; William and Hopkins, 2011). Because these compartments rapidly change during the late stages of incubation, it is critical to identify the ‘safe’ window for injection between days 17.0 and 19.0 of incubation. Although all the compartments can be accessed during *in ovo* injection, it is important to understand that each area represents a distinct route for specific vaccine administration and for a

subsequent specific for immune response. It is essential to ensure that specific vaccines are deposited in appropriate correct locations inside the egg. In addition to the commercial in ovo application of vaccines, substances that have been injected in ovo include carbohydrates (Tako et al., 2004; Uni et al., 2005; Zhai et al., 2011a, b, and c), electrolyte solutions (McGruder et al., 2011), amino acids (Kadam et al., 2008), stimulants (Keralapurath et al., 2010; Zhai et al., 2008), and soluble and particulate substances (Jochemsen and Jeurissen, 2002). The Embrex Inovoject® system manufactured by Zoetis Animal Health (Durham, NC), is one type of in ovo injection machine used in commercial application. The Inovoject® system has an injection depth of approximately 2.54 cm, which targets the amnion of the embryo for coccidiosis vaccine application (Fig. 2.7; William and Hopkins 2011).

In ovo injection of late stage embryo with coccidiosis Inovocox® EM1 vaccine

Using the Embrex Inovoject® system, the Inovocox® EM1 vaccine (**EM1**) is recommended for the vaccination of 18- to 19- day-old chicken embryos for the prevention of coccidiosis cause by *Eimeria acervulina*, *E. maxima*, and *E. tenella*. For correct delivery, the EM1 vaccine should be deposited within the amniotic sac, or in the subcutaneous and intramuscular regions of the embryo. The preferable site of intramuscular delivery is in the right breast muscle of the embryo's body (Williams and Hopkins, 2011). Injection of the EM1 vaccine into the air cell, allantoic sac, abdomen, cranium, eye, and thorax of the embryo are regarded as inferior delivery sites and will have subsequent negative effects on vaccination efficacy and embryo survival (Williams and Hopkins, 2011). Research has shown that the in ovo administration of live coccidia oocyst vaccines to late-stage chicken embryos is safe (Weber, et al., 2004). Today, in ovo

vaccination occurs routinely in approximately 98 % of commercial broiler hatcheries in the US. As with most in ovo vaccinations, in order to ensure that the EM1vaccine is delivered into the amnion, an understanding of the developmental events in late stage embryos is critical (these stages have been previously described in the avian embryogenesis section of this chapter). Although most literature categorically describes the physiological state of embryos on day 18 and 19 of incubation, common commercial practice is to inject embryonated eggs at the time of transfer from the setter to the hatcher, which is commonly at day 18.5 or 19.0 of incubation. The development of embryo at d 18.5 of incubation is, therefore, described in this section.

During the late stages of incubation, the embryo prepares itself for hatching. This preparation involves the folding and tucking of the embryo's head under the air cell membrane and vigorously moving the head and beak into positon for the hatching process (Tong et al., 2013). On embryonic day (**ED**) 18.5, the following changes begins to occur in the embryo: (1) head is tilted to the right and under the right wing, (2) the allantois starts to dry up, (3) the amniotic fluid is present but is beginning to regress due to the embryo's swallowing reflexes, and (4) absorption of the yolk sac into the embryo begins (Parkhurst and Mountney, 1988). In ovo vaccination is optimized at this time due to the presence of the amnion, which is actively imbibed by the embryo. The immune response to the EM1 vaccine is largely mediated by a T-cell reaction. This same reaction was previously described for nonattenuated live oocyst vaccines. The nature of this T-cell response can be influenced by the age of the bird, the type of *Eimeria* species, and the number of parasites present.

Prehatch and posthatch factors that participate in the immune response to an *Eimeria* infection

In day 18.5 embryos, the structural integrity, length, and width of the intestine is developing. At this time, the relative weight of the small intestine is approximately 3.5 % (Zhai et al., 2011a,b; Uni et al., 2003). Furthermore, associated gut lymphocyte populations are beginning to differentiate, and a relatively low level of T-cell infiltration is occurring at the time of in ovo injection (Holling et al., 2004). This low T-cell infiltration may be associated with the immaturity of the embryo immune system. It may also be associated with a lower level of reactive T-cell stimulation by foreign antigens (Holling et. al., 2004). At hatch, the immature small intestine undergoes significant changes within the first 24 hours undergoing morphological, biochemical, and molecular changes. There is significant influx of $CD4^+$ and $CD8^+$ cells into the gut of chickens inoculated with sporulated *Eimeria* oocysts (Swinkels et al., 2006). CD4+ cells or helper T cells are produced in response to infections and send signals to other types of immune cells, including $CD8^+$ killer cells. $CD4^+$ cells send signal and $CD8^+$ cells destroy the infection. The $CD4^+$ and $CD8^+$ cells are produced at the beginning of the *Eimeria* life cycle in order to limit the reaction to vaccinal oocysts or to clear the infection (Swinkels et al., 2006). By day 7 posthatch, the proportion of $CD8^+$, MHC II (Major Histocompatibility Complex II) and TCR (T cell Receptors recruited from the spleen) cells increase in response to the *Eimeria* antigen (Holling et al., 2004; Swinkels et al., 2006; Wallach, 2010). The main function of MHC class II molecules is to present antigens to $CD4^+$ T-lymphocytes. MHC class II molecules are important for the initiation of the antigen-specific immune response (Holling et al., 2004).

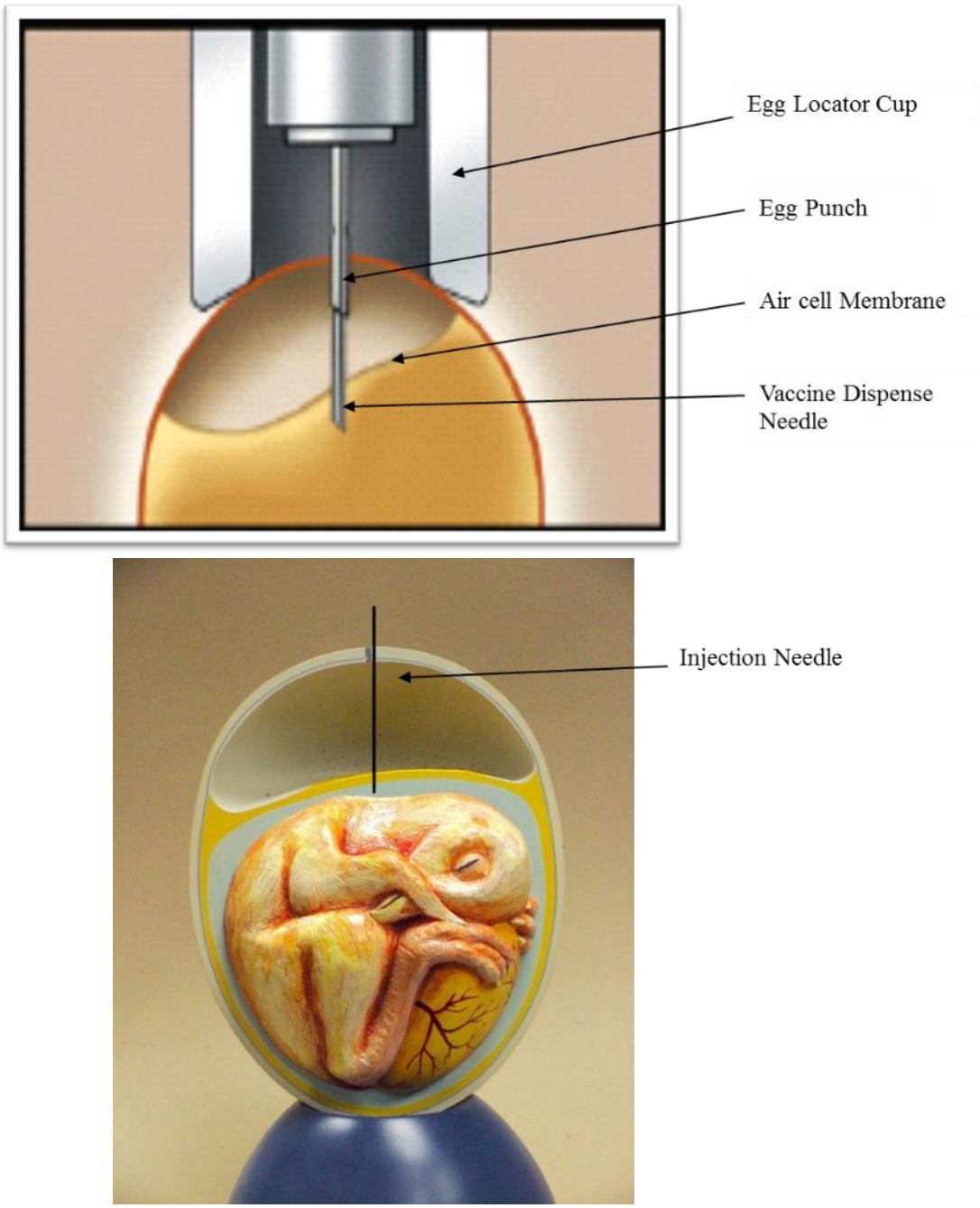


Figure 2.7 Individual injection tool of the Inovoject® multi-egg injector system showing the site (amnion) of injection in day 18 embryo

Images taken from Embryo Development 101; Zoetis Animal Health, Poultry Health Division.

Similarly, the uptake of oocysts by macrophages results in the activation and up regulation of MHC II expression. By day 14 posthatch, Interleukin-8 (IL-8) levels are significantly increased. IL-8 is a chemotactic factor that attracts neutrophils, basophils, and T-cells. Chemotactic factor IL-8 is released in response to epithelial necrosis caused by the reproductive life cycle stages of *Eimeria*. The neutrophils and macrophages recruited in response to IL-8 help to reduce the rate of infection (Swinkels et al., 2006). Technical information on the biology of the EM1 vaccine is limited in the scientific literature. Therefore, the goal of this study was to evaluate the physiological effects of the EM1 vaccine in Ross × Ross 708 broiler chickens when used for the control of coccidiosis.

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CHAPTER III

EFFECTS OF COCCIDIOSIS VACCINE ADMINISTERED BY IN OVO INJECTION ON THE HATCHABILITY AND HATCHING CHICK QUALITY OF BROILERS

Abstract

Effects of the in ovo injection of a commercial coccidiosis vaccine on the hatchability and hatching chick quality parameters of Ross × Ross 708 broilers were examined. There were 4 treatment groups arranged on each of 7 replicate tray levels of a single-stage incubator (28 treatment-replicate groups). Each treatment- replicate (TR) group contained 63 eggs (1,764 eggs total). On d 18.5 of incubation, eggs were subjected to one of 4 treatments using a commercial multi-egg injector. Three control groups (non-injected, dry-punch, and diluent-injected) and one treatment group (injected with diluent containing Inovocox EM1 vaccine) were used. On d 18.5 of incubation, the site of injection (SOI) and stage of embryo development (ES) were determined. On d 21.0 of incubation, hatchability of injected eggs (HI), chick BW, yolk sac, and liver weights were determined. On d 21.0 of incubation (d 0 posthatch), 20 chicks from each of the 28 TR groups (560 birds total) were placed in corresponding isolated wire-floored battery cages. On a daily basis, from d 0 to 14 posthatch, pooled fecal samples from each individual replicate cage were collected for oocyst output determination. There was no significant difference among treatments for HI or chick BW on d 21 of incubation. In the non-

injected control and treated groups, mean HI were 93.1 and 89.4 %, respectively, and chick BW were 43.8 and 43.1 g, respectively. Mean embryonic stage score was 2.09, and 91.2 % of SOI were in the amnion. Oocyst shedding began 4 d posthatch (d 6 post-injection), and reached a peak at d 7 posthatch (d 10 post-injection). It was concluded that the in ovo injection of Inovocox EM1 vaccine does not have a detrimental effect on broiler embryogenesis or hatching chick quality.

Keywords: chick quality, embryo, hatchability, Inovocox EM1 vaccine, in ovo injection

Introduction

The conventional posthatch methods of vaccination such as subcutaneous injection, spray, drinking water, or feed applications may result in a lack of consistent vaccine delivery and subsequent poor vaccine efficacy. Where possible these methods are being replaced by in ovo injection technology, which is a faster, more effective, and uniform method of vaccine delivery (Williams, 2007). Hatchery-applied vaccines for Marek's disease virus (MD), Infectious Bursal Disease Virus (IBDV), and coccidiosis vaccines can be administered to late-stage chicken embryos via in ovo injection (Jochemsen and Jeurissen, 2002; Weber and Evans, 2003). The in ovo vaccination of broiler hatching eggs occurs widely in commercial hatcheries in North America, South America, Europe, and Asia (Jochemsen and Jeurissen, 2002). Avian coccidiosis is a parasitic disease of poultry caused by protozoan parasites of the genus *Eimeria* (phylum Apicomplexa). *Eimeria* infects the intestinal tract and is transmitted between birds by a fecal-oral route. Annual economic losses to the commercial poultry industry due to coccidiosis are estimated at $800 million worldwide, and $127 million in the U.S.

(Chapman, 2009). These losses include costs incurred by in-feed medication, mortality, poor feed efficiency, and impaired performance (Williams, 1998). Coccidiosis is therefore considered as one of the top diseases of concern to the broiler industry worldwide.

In the U.S., the coccidiosis vaccine has become widely used as a means of controlling coccidiosis in broiler chickens. Coccidiosis vaccines are used as alternatives to chemotherapeutic agents for the control of coccidiosis (Chapman, 2000). These vaccines can be used either alone or as part of a rotation program in combination with other anticoccidial programs, depending on the season of the year (Chapman, 2000). The Inovocox EM1 (**EM1**) vaccine (Zoetis Inc., Kalamazoo, MI) contains live oocysts of 3 common species of coccidia*: Eimeria acervulina, E. maxima*, and *E. tenella*. The EM1 vaccine is recommended for in ovo vaccination of late stage [18 to19 days of incubation (**doi**)] embryonated chicken eggs for the prevention of coccidiosis caused by these 3 species of coccidia. The success of in ovo vaccination and the subsequent efficacy of the vaccine are influenced by the application technique used, the timing of the injection relative to the stage of embryonic development, and the exact site of vaccine deposition (site of injection; **SOI**) in the developing embryo. An in ovo vaccination technique must be easy to apply, achieve solid vaccine efficacy, support embryo health, and ensure chick quality (Williams and Zedek, 2010). The in ovo injection machine developed by Embrex (Embrex Inovoject system; Zoetis Inc.), has evolved into a commercial applicator that is capable of simultaneously injecting over 50,000 eggs per hour, making in ovo vaccination a more reliable method of vaccine application (Williams, 2007). The timing of in ovo injection must synchronize with the appropriate stage of embryonic

development in order to achieve the desired vaccine outcome. The stage of development that is most appropriate is one that allows for the targeted embryonic sites to become optimum in size and position for precise and accurate injection. Chicken embryonic development starts as early as 44 h into incubation, beginning with the development of the heart and vascular system (Tong et al., 2013). By 2 and 3 doi, there is the development of the central nervous system and the appearance of functional systems such as the limb buds and auditory pit. The embryo begins to move into hatching position by 14 doi, entering the growth phase prior to hatching (Mellor and Diesch, 2007). The entire developmental period of the chicken embryo occurs within a 21 d period, and its stages are generally divided into 3 developmental phases based on external features: the early, middle, and late stages. The various organs and associated nervous, circulatory, and reproductive systems are formed during the first 2 stages, while growth and maturation of these organs occur in the third stage.

Although several methods of staging chicken embryonic development have been described (Tong et al., 2013), the use of developmental stage scores provides a more practical, field-based approach that can be used to determine embryonic physiological development. This developmental stage scoring system utilizes predictable physiological markers and developmental features to generate an additive score that ranges from 1 to 7, depending on the stage of embryonic development (unpublished data, Embrex SOP #EMB-007, 'Site of Injection and Staging Embryos'). This embryonic stage score (**ES**) can then be used as a predictable marker for determining the age of the chicken embryo. The age and stage of development of the embryo is important in determining the accuracy of vaccine deposition or SOI**.** The SOI can occur in 5 different locations in the egg of the

developing late stage embryo. These areas are the air cell **(AC)**, amnion **(AM)**, allantois **(AL)**, embryo body **(EM)**, and the yolk sac (Williams, 2007). The injection of vaccines into any of these compartments within the egg may influence the efficacy of a vaccine, the survival of the embryo, and the response of the embryo to the vaccine. A precise deposition of the vaccine into the correct in ovo site is needed in order to obtain an optimal response by the embryo to the vaccine (Williams and Hopkins, 2011). Information on the effects of the in ovo injection of the commercial EM1 vaccine on chicken embryos at 18 through 19 doi, and on subsequent hatching chick quality is limited in the scientific literature. A more precise and practical approach for evaluating the success of in ovo injection under commercial hatchery settings is also currently unavailable. Therefore, the objective of this study was to investigate effects of the commercial EM1 vaccine on the hatchability and chick quality variables of broilers embryos injected as embryos at 18.5 doi, and to provide a stepwise and practical approach for evaluating the outcome of in ovo coccidiosis vaccination under commercial hatchery practice.

Materials and Methods

General

This study was conducted under a protocol that was approved by the Institutional Animal Care and Use Committee of Mississippi State University. A total of 2,520 Ross × Ross 708 broiler hatching eggs collected from a single 48-wk-old commercial broiler breeder flock were held for 2 d under standard storage conditions and were then individually weighed before being set. A total of 1,764 embryonated eggs that were of good quality and within ±10 % of the mean weight of all eggs weighed (60 ± 6.0 g), were

randomly assigned to 28 treatment-replicate groups (4 treatment groups on each of 7 incubator tray levels) each containing 63 eggs, in a Jamesway model PS 500® setter unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada) (Pulikanti et al., 2012). Each tray level served as a replicate unit (experimental block). On d 16 of incubation, eggs were candled as specified by Ernst et al. (2004), and eggs with cracked shells and those that were unfertilized or contained dead embryos were removed and discarded. After candling, a total of 1,594 embryonated eggs were retained, and were randomly assigned to all 4 treatment groups, each containing approximately 56 eggs, on each of the 7 tray levels in the setter. All eggs were incubated under standard conditions (Peebles and Brake, 1987).

Injection and experimental layout

On 18.5 doi, eggs were injected using an Embrex Inovoject multi-egg injection system capable of simultaneously injecting a tray of 42 eggs. In order to achieve the manufacturer's vaccination recommendation, 3 vials containing 8,000 doses each of EM1 vaccine were reconstituted in 1,200 mL of sterile commercial MD vaccine diluent (Merial Co., Duluth, GA) and administered at the rate of 50 µL per egg. Vaccinal oocyst count was conducted following vaccine reconstitution to determine the number of oocysts contained per dose administered into each individual embryonated egg. During the injection process, 50 µL vaccine samples were collected directly from the injection needle into a quality control plate. The vaccine samples containing viable oocysts were then placed in vials and transported in cold condition to the laboratory. Aliquots of samples were placed onto microscope slides and the number of oocysts per field counted. Each dose of EM1vaccine consisting of 375 oocysts from each of the *acervulina,*

maxima, and *tenella* species of *Eimeria,* was injected into each individual embryonated egg, with each embryonated egg receiving equal number of oocysts. During the injection process each egg was subjected to one of the following treatments: non-injected (treatment 1), dry-punch (treatment 2), diluent-injected [treatment 3 (50 µL of commercial diluent)], and EM1 vaccine-injected [treatment 4 (50 µL of commercial diluent containing EM1vaccine)]. Eggs belonging to the non-injected group were subjected to the same procedures as the other treatment groups, except that they were not injected. Following the completion of the entire injection process, all eggs were subsequently transferred to a Jamesway model PS 500 hatcher unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada), in which eggs in their respective treatment replicate groups were assigned to a hatcher basket which corresponded to their positions in the setter.

Site of injection and embryo staging

During the in ovo injection process on 18.5 doi, a total of 120 embryonated eggs were injected with coomassie brilliant blue G-250 (colloidal) dye. The dye was injected alongside the EM1vaccine using 2 designated injection lines on the Embrex Inovoject System. The concurrent in ovo injection of the dye with the EM1vaccine was used to validate the localization of the vaccine by evaluating the location of the dye within the injected embryonated eggs. The SOI and ES of each embryonated egg were subsequently determined following the injection on 18.5 doi.

Data collection

On 0 doi, individual egg weights were recorded. On 21 doi (d of hatch), hatchability of injected eggs (**HI**) and hatchling BW (**HBW**) were determined. Mean HI and HBW were obtained for each of the 28 treatment-replicate groups. Furthermore, on d of hatch, 3 chicks from each treatment replicate group (84 total) were euthanized, weighed, and necropsied for intestine, yolk sac and liver extraction. Absolute chick BW, intestine (**IW**), yolk sac (**YSW**), liver weights (**LW**), and yolk-free BW (**YFBW**) were determined. Subsequently, intestine (**RIBW**), yolk sac (**RYBW**) and liver weights (**RLBW**) relative to BW; and intestine (**RIYFW**) and liver (**RLYFW**) weights relative to YFBW were calculated. Similarly, chick yolk free body mass (**YFBM**) and chick BW relative to set egg weight (**RBSW**) were calculated. All relative values were expressed as percentages. The YFBM was also expressed as a percentage and was calculated by dividing YFBW by BW, and multiplying the result by 100. Hatch residue analysis was conducted according to the procedures of Ernst et al. (2004) to determine post-injection embryonic mortality (**PIM**). Only embryos that were observed to have died after injection on 18.5 doi were included in the determination of PIM.

Embryo euthenization and evaluation of vaccine deposition

Embryo euthenization and SOI evaluation were conducted according to methods described by Williams and Hopkins (2011). Following the injection of coomassie blue dye, test eggs were retained in their incubation flats and were placed in air-tight bags according to treatment. Embryos contained in their shells were euthanized using CO_2 gas, and were then stored cold (4^{o}C) for 4 hours. After euthenization, each egg was carefully dissected to determine its SOI (dye deposition site) and ES. Only normally developed

embryos were included in the determination of SOI and ES. The SOI of each egg was designated as either AC, AM, AL, or EM, with EM injections subdivided into intramuscular (**i.m**) and subcutaneous (**s.c**) injections. The correct SOI for optimal EM1 vaccine efficacy were those that delivered vaccine into the AM (Jochemsen and Jeurissen, 2002; Williams, 2007) or EM intramuscular/subcutaneous (Williams and Hopkins, 2011). For the evaluation of ES, the following physiological parameters and scoring system were used: Internal pip: 0 or 1; external pip: 0 or 1; head of the embryo located on its right side: 0 or 1, with 0 being absent and 1 being present. Furthermore, yolk absorption was scored from 1 to 4: 1 = yolk stalk and intestine present within the yolk sac; 2 = no yolk stalk present and the yolk sac bi-lobed in shape; 3 = yolk sac loses its bi-lobed shape; and 4 = yolk is completely absorbed and there is no discernible shape. The ES scores were additive, with a maximum score of 7. For example, if an embryo had a score of 1 for internal pip, 1 for external pip, 1 for head to the right, and a score of 2 for yolk absorption, this produced an ES of 5 out of 7. The higher the ES, the more advanced was embryonic development. In general, by evaluating embryonic developmental features (ES), it is possible to determine the approximate physiological age of the embryo at the time of in ovo injection, and subsequently to determine the success of the in ovo injection.

Chicks placement and determination of oocysts output

At hatch, 20 chicks from each of the 28 treatment-replicate groups were weighed and placed in isolated wire-floored battery cages and given feed and water ad libitum for 14 d. The ration used met or exceeded National Research Council (1994) requirements for broiler chickens. Chicks used in all experiments were straight-run. From d 0 to 14

posthatch, the entire (pooled) fecal output of all the birds in each individual pen was collected daily. The determination of oocysts per gram of feces (**OPGF**) was performed as previously described by Weber and Evans (2003), and Ryley et al. (1976). Briefly, each entire fecal output was weighed, and an aliquot of approximately 2 g of feces was mixed with an amount of water equivalent to approximately 6 × the volume of feces and homogenized using a hand-held spatula in order to create a suspension. The suspensions were then placed in centrifuge tubes, and centrifuged at 10,000 ×g for 8 min. The supernatant was then discarded and the process was repeated for a second wash. Following the second wash and centrifugation at 10,000 ×g for 8 min, the supernatant was discarded and the precipitant was re-suspended in sugar solution to increase the specific gravity of the fecal sample solution, so that the oocysts would float to the surface and be trapped onto a cover slip placed over the test tube. The cover slip was then removed and placed on a slide and was viewed under the microscope for enumeration of oocysts. The number of oocysts per pen was determined and the mean oocyst per gram of feces within each treatment was calculated.

Statistical description

A randomized complete block design was used, with each of the 7 tray levels of the setter and each of the 7 hatching basket levels in the hatcher representing a block. All 4 treatment groups were randomly arranged and represented within each level (block). All variables were analyzed using the MIXED procedure of SAS software 9.3 (SAS institute, 2012). Treatments were viewed as fixed effects and blocks as random effects. A one-way ANOVA was used to analyze treatment differences for all the parameters investigated. Least-square means were compared in the event of significant global

effects. Global and least-square means differences were considered significant at $P \leq 0.05$.

Results

There were no significant treatment effects on HI ($P = 0.08$) or HBW ($P = 0.43$) at 21.0 doi (Fig. 3.1 and 3.2). However, HI at 21.0 doi in the non-injected control group was numerically higher compared to that in the diluent-injected control and the vaccine-injected treatment groups. The HI means in the non-injected, dry punch, diluent-injected, and vaccine-injected treatment groups were 93.1, 91.7, 90.6, and 89.4 %, respectively (Pooled SEM = 0.98 %). The HBW means in the non-injected, dry punch, diluent-injected, and vaccine-injected treatment groups were 43.4, 43.8, 43.8, and 43.8 g, respectively (Pooled SEM = 0.23 g). Similarly, there were no significant treatment effects on any of the other hatching chick quality variables evaluated in this study. Nevertheless, the treatment means for each of these hatching chick quality parameters are provided in Table 3.1 for observation. Mean RBSW ranged from 71.9 % in the diluent-injected group to 72.9 % in the non-injected control group, with the vaccine-injected group being intermediate at 72.2 %. Mean YFBW of chicks ranged from 38.0 g in the vaccinated group to 38.6 g in the diluent-injected group. Furthermore, YFBM was 87.3 % in the non-injected control group, 88.8 % in the diluent-injected group, and 88.2 % in the vaccine-injected group.

Posthatch oocyst output following the in ovo injection of the EM1 vaccine is shown in Figure 3.3. The oocysts of all 3 species of *Eimeria* (*acervulina*, *maxima*, and *tenella*) were recovered from fecal samples, confirming that the embryos became infected subsequent to the in ovo injection of the coccidiosis vaccine. Oocyst output was greatest

on d 7 posthatch, when embryos received *E. acervulina*, E. *maxima*, and *E. tenella* in ovo on 18.5 doi. This peak was followed by a second, smaller peak at d 10 posthatch. Beginning at d 4 and 5 posthatch, small (approximately 20×16 μm) and large (approximately 32×28 μm) sized oocysts that are, respectively, indicative of *E. acervulina and E. maxima* infection, were observed in the feces of most birds that received the EM1vaccine. Peak oocyst output observed on d 7 posthatch suggests that all *Eimeria spps* were present. A second, smaller peak of oocyst output detected by d 10 posthatch suggests that there was a delayed output of oocysts in a few chicks. Chicks hatched from control eggs that received no injection, a dry-punch injection, or a diluent injection, did not shed a detectable numbers of oocysts during the 2 wk posthatch period. Mean ES for 118 embryonated eggs at 18.5 doi was 2.09 ± 0.43. Furthermore, the SOI evaluation of those same eggs at 18.5 doi indicated that 84.7, 15.3, 6.8, and 8.5 % of the eggs received vaccine in AM, EM, i.m, and s.c sites, respectively (Table 3.2).

Discussion

The HI of eggs that were injected with the EM1vaccine was largely unaffected. Although there was a numerical decrease in HI in the vaccine-injected group, there were also similar decreases observed in the dry punch and diluent-injected control groups in comparison to the non-injected control group. This numerical decrease in HI may have resulted from the creation of injection holes in the embryonated eggs belonging to these groups (dry punch, diluent-injected and vaccine-injected groups). Nevertheless, treatment had no significant effect on mean HI at 21 doi, and most notably, in ovo injection of the EM1 vaccine did not cause any detrimental effects on hatchability. Similarly, treatment had no significant effect on mean chick BW at 21 of doi. Furthermore, there were no

statistical differences between the vaccine-injected and control groups for all the chick quality parameters determined in this study. The YFBM, which is YFBW divided by BW, is an indicator of chick development during incubation and has been positively correlated with subsequent chick performance (Molenaar et al., 2011). The in ovo injection of the EM1 vaccine, therefore, did not adversely affect embryonic development or survivability when assessed at 21 doi, and likewise exerted no subsequent negative effects on hatchling quality.

The success of any in ovo vaccination depends on at least 2 major factors. These major factors include the stage of development that the embryo is at when injected, and the accuracy of vaccine deposition in the appropriate SOI. The stages of chicken embryo development are generally divided into three major phases (early-, mid- and late- phase) and are practically classified into embryonic days (**ED**) based on a 24 h calendar time period (Tong et al., 2013). The ES system, however, is a more precise method of evaluating and classifying embryonic development by utilizing physiological markers and developmental features of the embryos that follow a predictable sequence. The ES scoring system utilizes an additive scale that increases with developmental maturation to a maximum of 7. The points are added when specific criteria are met as the embryo develops towards hatching (unpublished data, Embrex SOP #EMB-007, 'Site of Injection and Staging Embryos'). Embryos with a high ES are more developed than are embryos with a low ES. The ES is a predictor of the actual physiological age of an embryo and is influenced by factors such as incubation time and conditions, flock breed and genetics, and maternal nutrition. Accurate information regarding embryonic development is important for SOI accuracy and eventual vaccine efficacy. Previous extensive work

conducted on in ovo vaccination under commercial standard practice (Williams and Zedek, 2010; Williams and Hopkins, 2012) suggests that the deposition of in ovo-injected vaccine in the appropriate location is critical for the protective efficacy of the vaccine against Marek's disease (MD). The 5 regions of embryonated eggs that are primarily involved with in ovo injection include the AC, AL, AM, EM and yolk sac (**YS**). The EM region can be further divided into s.c, i.m, intra-cranial, intra-orbital, or intra-abdominal regions. It has been suggested that the EM1vaccine must be delivered into the AM or EM (i.m or s.c) regions to achieve a successful vaccination. Vaccines or other solutions deposited in the AM are imbibed or aspirated by the embryo prior to hatching (Jochemsen and Jeurissen, 2002). Delivery into the AC, AL, intra-cranial, intra-orbital, or intra-abdominal regions are regarded as unsatisfactory vaccine deliveries, and will lead to an ineffective vaccination and possibly the death of the embryo. In a previous study in which the relationship between ES and SOI (unpublished data) was evaluated, it was shown that when ES was between 1 and 3, there was an 80 to 94 % chance of vaccine deposition occurring in the AM, and 6 to 20 % chance of vaccine deposition occurring in the EM. An ES of 2 ensures that the percentage likelihood for an injection occurring in the AM, EM, and AL was 94, 4, and 2 %, respectively. However, with an ES of 4 to 7, the percentage likelihood for injections occurring in the EM is 70 to 90 %, and the percentage likelihood for injections occurring in the AM is 5 to 30 %. Another earlier study (unpublished data) was conducted to show the relationship between ES and embryonic age at ED 17.5, 18.0, 18.5, and 19.0. Data was obtained using eggs from 6 flocks of 3 breed crosses (5,341 total eggs) that were set in a Jamesway incubator at different times. The results showed that there was a significant ($P = 0.05$) distribution of

ES values across all 4 embryonic ages. In brief, ED 17.5, 18.0, 18.5, and 19.0 corresponded to ES 1, 2, 3, and 4, respectively, in approximately 60 % of the embryo population. Based on these results, the SOI and ES in a test population of the current study that occupied approximately 10 % of the total embryo population were evaluated using protein-staining blue dye to determine the efficacy of the EM1vaccine. Results showed that mean ES was of 2.09 ± 0.43 (mean ± SD) and that the percentage injections in the AM and EM were 84.7 and 15.3 % (i.m., 6.8 %; s.c., 8.5 %), respectively. The ES and SOI results in this study showed that the embryos were at a physiological stage of development that corresponded to ED 18.0 and 18.5, and that 91.5 % of vaccine deliveries were in the AM and EM. Vaccine deposition in the AM, i.m or s.c (right breast area) of the developing embryo is the specified target site for optimal uniform vaccination. The physiological markers for these embryos at ED 18.0 and 18.5 were: head at the right wing, internal pip may be present or absent, external pip is absent, and with yolk absorption scores between 1 and 2. This stage of physiological development corresponds to a time that is recommended for injection of the EM1vaccine for optimal vaccine delivery and subsequent vaccine uptake by the embryo. In addition, the ES showed uniformity of the embryo population (based on the SD). This uniformity ensures that a great percentage of the embryos received the vaccine in similar manner and can influence the subsequent outcome of the vaccination during field challenge.

Following the in ovo injection of the EM1vaccine, the success of vaccination was further determined by the evaluation of fecal oocyst recovery from the posthatch chicks. The assessment of vaccinal oocyst output was used to ensure that the birds vaccinated with the EM1vaccine actually received vaccinal oocysts by way of in ovo injection.

Post-injection oocyst output evaluation indicates that the chicks were in fact infected with all 3 *Eimeria spps* when EM1was administered in ovo. Moreover, there were no oocysts in the feces of chicks from the non-injected and diluent-injected control groups. Peak oocyst output was observed at d 7 posthatch, indicating that the coccidia life cycle started at hatch. This is consistent with previous work, in which the in ovo injection of oocysts resulted in a shedding peak at approximately 7 d posthatch (Weber and Evans, 2003). It has been suggested that following an in ovo injection of coccidial oocysts, oocysts may remain inactive in the embryo's intestine after being ingested from the amniotic fluid with no significant life cycle changes occurring until the chicks hatch (Weber et al., 2001). As with previous studies (Weber et al., 2004), the current evidence suggests that the initiation of immunity in response to the EM1 vaccine oocysts occurs at hatch. This evidence is based on the time that oocysts of *E. acervulina* (4 d posthatch) and *E. maxima* (6 d posthatch) were observed in their feces in relation to their known prepatent life cycle period (Conway and McKenzie, 1991). Oocysts or intestinal lesions were histologically undetected at hatch for all 3 *Eimeria spps*. This suggests a mild vaccinal reaction to infection that is capable of inducing the development of an immune response.

In conclusion, the in ovo injection of the EM1vaccine on 18.5 doi in Ross × Ross 708 broilers had no detrimental effect on hatching chick quality. It also did not adversely affect embryonic survivability, as evidenced by the lack of any adverse treatment effects on HI. Furthermore, the injection of the EM1vaccine produced a vaccine-induced infection that was made evident by the OPGF counts occurring between 1 and 14 d posthatch in the vaccine-injected group. The use of qualitative procedures, namely SOI and ES, ensured that the injection of the EM1vaccine between 18 and 18.5 doi provided

accurate delivery of the vaccine into the AM and EM (i.m or s.c), and the recovery of oocysts during the posthatch period was indicative of a stimulation of immunity necessary for protection against coccidial challenge.

Table 3.1 Somatic and yolk parameter means in the non-injected, dry-punch and diluent-injected controls, and eggs injected with EM1 vaccine, determined on 0 d posthatch.[1,2,3]

Treatments	BW (g)	YFBW (g)	RBSW (%)	RYFWSW (%)	IW (g)	RIBW (%)	RIYFW (%)	LW (g)	RLBW (%)	RLYFW (%)	YSW (g)	RYBW (%)	YFBM (%)
Non-injected control	43.75	38.16	72.87	63.57	1.67	3.84	4.39	1.04	2.38	2.72	5.59	12.73	87.27
Dry-punch control	43.65	38.37	72.66	63.89	1.73	3.98	4.51	1.07	2.45	2.78	5.29	12.04	87.96
Diluent-injected control	43.55	38.61	71.85	63.74	1.74	4.02	4.52	1.04	2.41	2.71	4.93	11.25	88.75
Vaccine-Injected	43.09	37.98	72.15	63.59	1.67	3.90	4.42	1.06	2.46	2.79	5.12	11.85	88.15
SEM	0.55	0.43	0.49	0.47	0.05	0.13	0.13	0.03	0.07	0.07	0.28	0.58	0.58
P-value	0.84	0.75	0.45	0.95	0.60	0.78	0.87	0.89	0.83	0.79	0.43	0.37	0.37

[1]Three birds in each of 7 replicate units (21 birds per treatment group) were used to calculate each treatment mean.
[2]Body Weight (BW), yolk-free BW (YFBW), BW as a percentage of set egg weight (RBSW), yolk-free BW as a percentage of set egg weight (RYFWSW), intestine weight (IW), intestine weight as a percentage of BW (RIBW), intestine weight as a percentage of yolk-free BW (RIYFW), liver weight (LW), liver weight as a percentage of BW (RLBW), liver weight as a percentage of yolk-free BW (RLYFW), yolk sac weight (YSW), yolk sac weight as a percentage of BW (RYBW), and yolk-free body mass (YFBM). [3]No significant difference was observed among treatment groups for all parameters.

Table 3.2 Site of injection (SOI) evaluation [Amnion (AM), Embryo (EM), Intramuscular (IM), Subcutaneous (SQ), and embryo staging (ES; 1-7 and total) of the selected embryo population vaccinated with EM1vaccine on d 18.5 of incubation.[1,3]

Site of Injection (SOI)							Developmental Embryonic Staging (ES)									
Treatment	AM (%)	EM (%)	TOTAL (%)	IM (%)	SQ (%)	N	Stage Score	1	2	3	4	5	6	7	Total	Mean ± StdevP
All treatment groups[2]	84.75	15.25	100	6.78	8.47	118	Number of Embryos	5	98	14	1	0	0	0	118	
							Ratio of embryo for each stage score (%)	4.24	83.05	11.86	0.85	0	0	0	100	2.09 ± 0.43

[1] A total of 118 embryos were used to calculate the mean ES and SOI.

[2] Embryo population selected from all treatment groups (non-injected, dry-punch, and diluent-injected controls, and EM1 vaccine).

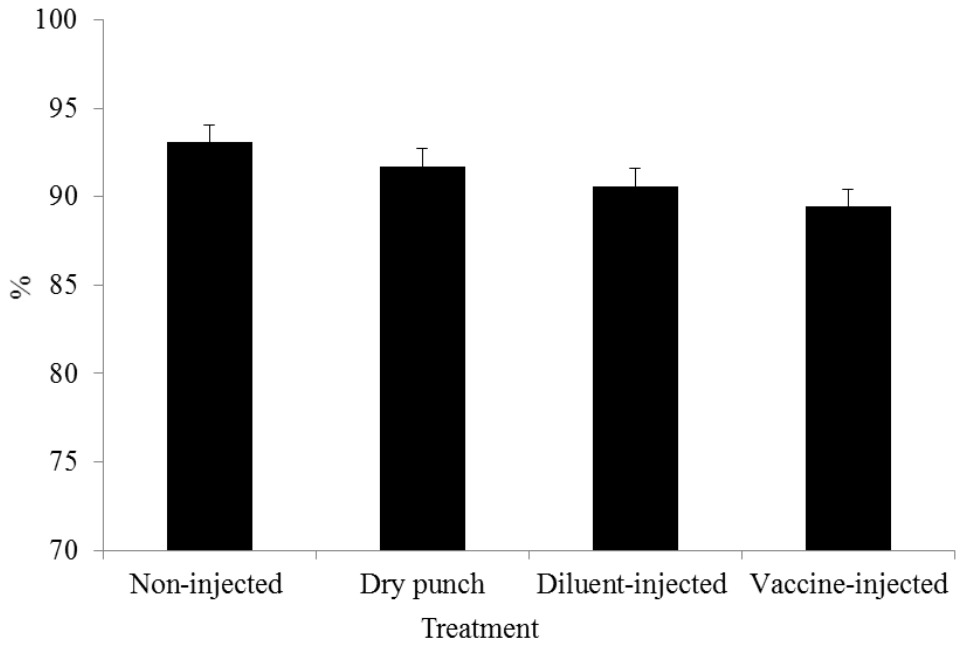

Figure 3.1 Hatchability on d 21.0 of incubation as a percentage of embryonated injected eggs

In non-injected, dry punch, and diluent-injected (50 µL) controls and eggs injected with the EM1vaccine in 50 µL of diluent.[1,2]
[1]Data from 7 replicate units was used for calculation of means for each treatment group.
[2]No significant difference among treatment groups for hatchability ($P = 0.08$).

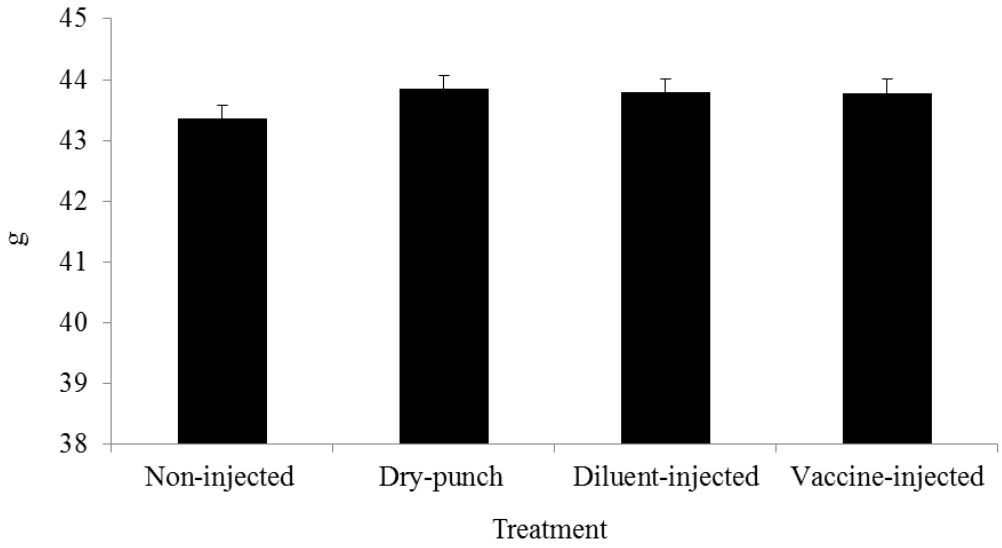

Figure 3.2 Hatching BW on d 21.0 of incubation

In non-injected, dry punch and diluent-injected (50 µL) controls, and eggs injected with the EM1vaccine in 50 µL of diluent.[1,2]
[1]Data from 7 replicate units was used for calculation of means for each treatment group.
[2]No significant difference among treatment groups for hatching BW (P = 0.43).

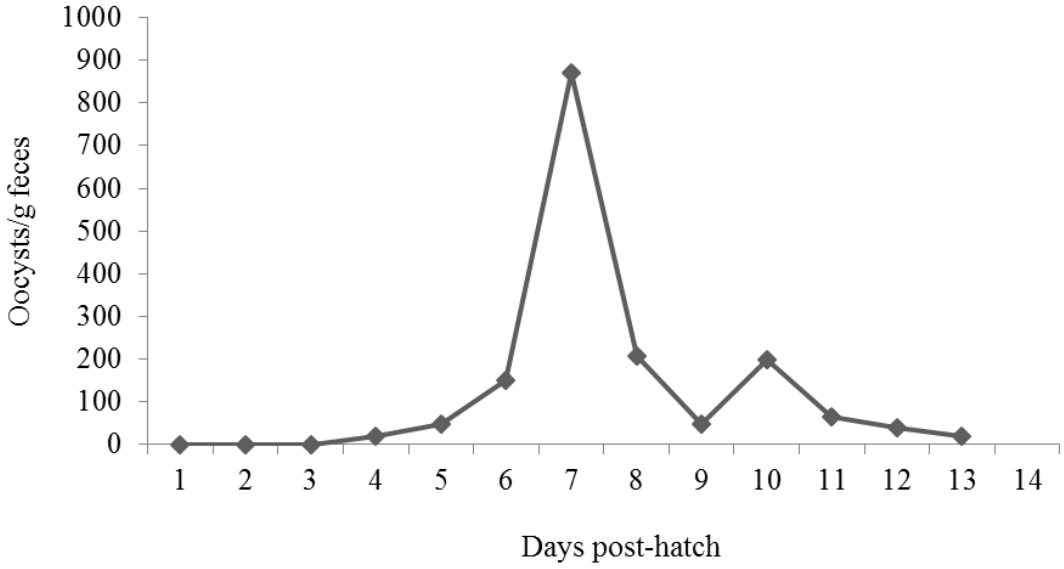

Figure 3.3 Posthatch oocysts shedding by broiler chicks after *in ovo* injection with the EM1 vaccine on d 18.5 of incubation

Seven replicate pens were used to calculate mean number of oocysts per gram of feces.

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CHAPTER IV

EFFECTS OF COCCIDIOSIS IN OVO VACCINATION ON BROILER PERFORMANCE

Abstract

Effects of the in ovo injection of a commercial coccidiosis vaccine on hatching chick quality parameters and 14 d posthatch oocyst shedding pattern has been previously examined. This study was designed to examine broiler performance during the 14 d posthatch period of oocyst shedding following the in ovo injection of a commercial coccidiosis vaccine. On each of 7 replicate tray levels of a single-stage incubator, a total of 4 treatment groups were randomly represented, with each treatment (TR) group containing 63 eggs. Treatments were administered using a commercial multi-egg injector on 18.5 d of incubation (doi). The treatments included 3 control groups (non-injected, dry-punch, and diluent-injected) and one treatment group (injected with diluent containing Inovocox EM1 vaccine). On 21 doi, 20 chicks from each of the 28 treatment replicate groups were placed in corresponding wire-floored battery cages. Feed intake (FI), BW gain (BWG), and feed conversion ratio (FCR) were determined for the 0 to 7, 7 to 14, and cumulative 0 to 14 d of age (doa) intervals. There was no significant treatment effect on BW at 0 and 7 doa, but there was significant treatment effect on BW at 14 doa. There was significant treatment effect on BWG, FI, and FCR in the 7 to 14 and 0 to 14 doa intervals, but there was no significant treatment effect on BWG, FI, and FCR in the 0

to 7 doa interval. The performance parameters measured in birds belonging to the diluent-injected and vaccine-injected groups were significantly different from those in birds belonging to the non-injected and dry-punch control groups. However, the performance parameters were not significantly different between the diluent-injected and vaccine-injected groups. It was concluded that use of the Inovocox EM1 vaccine in commercial diluent has no detrimental effect on the overall post-hatch performance of broilers.

Key words: Inovocox EM1 vaccine, chicks, in ovo injection, performance, post-hatch

Introduction

Among the world's poultry producers, coccidiosis continues to be one of the most commonly reported diseases (Biggs, 1982; Williams 1999), with subclinical coccidiosis being the most commonly reported form. Without the demonstration of overt clinical symptoms, subclinical coccidiosis is characterized by weight loss, reduced weight gain, and poor feed efficiency (Williams, 1999). Due to historic resistance to in-feed anticoccidials against strains of *Eimeria* parasites, and to increasing public awareness of drug residues in poultry products, the use of live coccidia vaccines has become an option of greater interest for the control of coccidiosis (William, 2002; McEvoy, 2001). The life span of the meat-type bird averages only 42 d (William, 2002). Therefore, early vaccination is a practicable method of controlling coccidiosis because it allows for an earlier protection against coccidiosis infection. In ovo vaccination of embryos with live parasites ensures the controlled delivery of a precise dose of vaccinal oocysts to each

embryo, which results in the early development of more uniform protection (William, 2002).

Although the use of vaccination for control of coccidiosis infection has become widely accepted (William, 2002), adverse effects on early broiler growth performance has been reported (Lehman et al, 2009). Studies have shown that under commercial conditions, early temporary reductions in BW gain (**BWG**) and feed efficiency between 14 and 28 d of age (**doa**), can occur as a result of vaccination with live non-attenuated coccidial oocysts (William, 2002; Chapman et al., 2002). This temporary reduction in BWG is usually followed by a compensatory increase in BWG by 35 to 42 doa (Mathis, 1999; Mathis and Lang, 2001). While the effect of vaccination programs on broiler grow-out performance has been examined (Mathis, 1999), only a few studies have examined the performance of broilers during the early post-vaccination peak of oocyst shedding.

The objective of this current study was to evaluate the growth performance (feed intake, BWG, and feed efficiency) of broilers during the first 14 d of vaccine-derived coccidial oocyst shedding, without being concurrently challenged with an *Eimeria* infection. The birds that were evaluated for their performance in this study had been previously vaccinated with the EM1 vaccine via in ovo injection on 18.5 d of incubation (**doi**) and had displayed coccidial oocyst shedding from 0 to 14 doa (Sokale et al., 2015a). To the authors' knowledge, this is the first study that describes the performance characteristics of broilers during the first 14 d of oocyst shedding following an in ovo vaccination with the Inovocox EM1 (**EM1**) vaccine.

Materials and Methods

General

This study was conducted according to a protocol that was approved by the Institutional Animal Care and Use Committee of Mississippi State University. A total of 2,520 broiler hatching eggs (Ross × Ross 708) collected from a commercial breeder flock at 48 wk of age, were held for 2 d under standard storage conditions after collection. Prior to setting, eggs were individually weighed and only eggs that had normal appearance and that weighed within 10 % of the mean weight of all eggs (60 ± 6.0 g) were set (Pulikanti et al., 2012). A total of 1,764 eggs were incubated under standard conditions (Peebles and Brake, 1987; Zhai et al., 2011) in a Jamesway model PS 500 setter unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada). There were 28 treatment-replicate groups (4 treatment groups on each of 7 replicate tray levels), with each containing 63 eggs, arranged in the setter. On 18 doi, eggs were candled to remove those that were unfertilized or contained dead embryos as described by Ernst et al. (2004). On 18.5 doi, eggs were injected according to one of the 4 treatments specified below.

Experimental layout

Eggs were injected using an Embrex Inovoject injector system (Zoetis Animal Health, Research Triangle Park, NC). The methodology for confirmation of site of injection, egg handling, and the injection procedure were as described in detail by Sokale et al. (2015a). Briefly, EM1 vaccine was reconstituted in sterile commercial Marek's Disease vaccine diluent (Merial Co., Duluth, GA) and administered at a rate of 50 µL per egg on 18.5 doi. The number of oocysts contained per dose administered into each individual embryonated egg was determined in a previous study (Sokale et al. 2015a). A

total of 375 oocysts from each of the *acervulina, maxima*, and *tenella* species of *Eimeria,* were injected into each individual embryonated egg. Treatments included non-injected, dry-punch, and diluent-injected (50 µL of commercial diluent delivered to each egg) controls. All eggs were subsequently transferred to a Jamesway model PS 500 hatcher unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada), in which eggs in their respective treatment replicate groups were assigned hatcher basket positions which corresponded to their arrangement in the setter.

Data collection

On 21 doi, 20 straight-run chicks from each of the 28 treatment replicate groups were randomly selected, wing-banded, weighed, and placed in each of 28 isolated wire-floored battery cages of a light-controlled research facility. Chicks were placed in the battery cages using the same experimental design that was used for the arrangement of eggs in the hatcher unit. Pen conditions, including brooding environment, were monitored twice daily throughout the grow-out period to ensure their conformity to commercial standards. Chicks were given feed and water ad libitum for 14 d. All birds were fed a standard Mississippi State University broiler basal starter diet which was formulated to meet or exceed NRC (1994) recommendations throughout the 14 d period (Sokale et al., 2015a). Although housed in the same facility and under the same conditions, the groups which did not receive vaccine were kept separate from the vaccinated groups to reduce the risk of cross-contamination (Sokale et al., 2015a). Bird numbers, BW, and feed weights were determined for each pen on a weekly basis from 0 to 14 doa. Furthermore, BWG, feed intake **(FI)**, feed conversion ratio (**FCR**), and

percentage mortality **(PM)** were determined for the 0 to 7, 7 to 14, and cumulative 0 to 14 doa intervals.

At 0, 3, 5 and 7 doa, one bird from each of the 7 replicate groups in the non-injected, diluent-injected, and vaccine-injected treatment groups (7 birds per treatment) were euthanized, and their intestinal tracts were collected and fixed in 10 % buffered neutral formalin solution for subsequent coccidia scoring by histopathological examination. The formalin-fixed intestine tissues (duodenum, mid-intestine, and cecum) were routinely processed for histopathology and evaluated for the presence of coccidia. The tissues were collected and trimmed such that complete sagittal circumferential sections of the duodenum, mid-intestine, and cecum were made available for examination. Based on coccidia numbers, samples were assigned one of the following quantitative observational scores: Score 1: no coccidia; Score 2: 1 to 30 coccidia; Score 3: 31 to 100 coccidia; Score 4: more than 100 coccidia. Mean coccidia scores for each treatment are presented in Table 4.2.

Statistical description

A randomized complete block design was used during the incubation and grow-out periods. Treatment was viewed as a fixed effect and block as a random effect in the one-way ANOVA of absolute BW, weekly BW gain, FI, FCR, PM, and coccidia count. All variables were analyzed using the MIXED procedure of SAS software 9.3 (SAS institute, 2012). Least-square means were compared in the event of significant global effects. Global and least-square means differences were considered significant at $P \leq 0.05$.

Results and discussion

The performance characteristics of broiler chickens that were administered the EM1 vaccine on 18.5 doi was measured during the first 14 d of oocyst shedding. This performance evaluation was conducted in order to determine any effect on broiler performance during the first few days of oocyst shedding when initial infection is being established for the development of immunity against coccidiosis (Weber et al., 2004). There were no significant effects due to treatment for mean BW at 0 and 7 doa, or BWG, FI, and FCR in the 0 to 7 doa interval. However, there were significant effects due to treatment for mean BW at 14 doa ($P < 0.001$), for BWG ($P < 0.001$), FI ($P = 0.004$), and FCR ($P = 0.002$) in the 7 to 14 doa interval, and for BWG ($P < 0.001$), FI ($P = 0.005$), and FCR ($P = 0.001$) in the 0 to 14 doa interval. For reference, treatment means for BW at 0, 7, and 14 doa, and for BWG, FI, and FCR in each of these age intervals are provided in Table 4.1. The BW of birds at 14 doa was higher in birds belonging to the dry-punch and non-injected control groups in comparison to those belonging to the vaccine-injected and diluent-injected groups. In the 7 to 14 and 0 to 14 doa intervals, the BWG and FI of birds belonging to the dry-punch and non-injected control groups were significantly higher in comparison to those in the vaccine-injected and the diluent-injected groups. In the 7 to 14 and 0 to 14 doa intervals, the FCR of birds belonging to the diluent-injected and vaccine-injected groups was significantly higher in comparison to those in the dry-punch and non-injected control groups. There were no significant treatment effects on the PM of birds in the 0 to 7 and 7 to 14 doa intervals. Furthermore, the cumulative (0 to 14 doa) PM **(CPM)** of birds was not significantly affected by treatment. The CPM of birds

in the non-injected, dry-punch, and diluent-injected control groups, and in the vaccine-injected treatment group is presented in Fig. 4.1.

Histopathological examination of the intestinal tissue samples obtained from birds vaccinated via in ovo injection of the EM1 vaccine confirmed the presence of coccidia parasites from all 3 species of *Eimeria*, at 3, 5 and 7 doa, with peak counts observed between 5 and 7 doa (Table 1). This outcome is a similar to the oocyst per gram of feces (OPGF) count previously examined in these same birds, which showed peak oocyst output occurring at 7 and 10 doa (Sokale et al., 2015a). In general, coccidia counts were low in most of the intestinal sections examined. The predominant species in the duodenum and mid-intestine were *Eimeria acervulina* and *Eimeria maxima*, respectively. In some sections of the duodenum and mid-intestine, it was not possible to definitively identify the species of coccidia based on morphology; however, the coccidia did not exhibit clustering that is typical of *Eimeria acervulina*, and were likely *Eimeria maxima*. In the cecum, the morphology of the coccidia and their location within the mucosa were consistent with that of *Eimeria tenella*. The colonization of the gut by coccidial parasites, and the pattern of oocyst shedding in the feces, is consistent with the normal life cycle of *Eimeria*. The sporozoites from the sporulated vaccinal oocysts are released by the grinding activity of the gizzard, and subsequently penetrate the intestinal mucosa to begin asexual development (schizogony). Sexual development (gametogony) eventually occur which ultimately resulted in the release of fecal oocysts. The duration of this first phase of the organism's life cycle lasts approximately 7 d (Chapman, 2002), which corresponds with the peak of initial coccidial cycling as observed in this study, and that of fecal

oocyst output as demonstrated by Sokale et al., 2015a. By 14 doa, initial oocyst production is commonly reduced.

No difference in BW among treatment groups was observed at 7 doa. More specifically, birds that were administered the EM1 vaccine did not show any decrease in BW during the period of peak oocyst cycling (7 doa) when compared to those in the non-vaccinated group. However BW was significantly decreased in the vaccine-injected and diluent-injected groups at 14 doa (Table 4.1). Although the BW of the birds in the dry-punch and non-injected control groups were higher in comparison to those in the vaccine-injected and diluent-injected groups, there was no difference in BW between the vaccine-injected group and the diluent-injected control group at 14 doa. A similar trend was observed in the other performance parameters that were measured in this study. In the 7 to 14 and 0 to 14 doa intervals, BWG and FI were higher in birds belonging to the dry-punch and non-injected control groups in comparison to those belonging to the vaccine-injected and diluent-injected groups. In addition, in the 7 to 14 and 0 to 14 doa intervals, FCR was higher in birds belonging to the diluent-injected and vaccine-injected groups in comparison to those belonging to the non-injected and dry-punch control groups. Nevertheless, in the 7 to 14 and 0 to 14 doa intervals, the FI, BWG, and FCR of the birds in the vaccine-injected and diluent-injected groups were not significantly different. As in this study, Bello et al. (2014) showed that in the 7 to14 doa interval, BWG was numerically higher in a non-injected control group in comparison to that in a diluent-injected control group. The results of this study indicate that the injection of diluent affected the performance parameters of broiler embryos. They further suggest that increased hatchling BW in response to diluent injection may result in a lower feed

consumption and BWG during early posthatch life. The diluent used in this study is widely used commercially as a carrier for Marek's disease vaccine. Although the precise constitution of the commercial diluent is proprietary, it is suggested that the diluent may increase the relative water content of the bodily tissue of the birds, thereby increasing chick BW at hatch, and may provide nutrients that stimulate growth. Although this effect may be of a limited duration during the chicks' posthatch life, it affects the FI, BWG, and FCR of the chicks during that time. On the other hand, although this study did not specifically show a reduction in the early growth performance of broilers vaccinated with the EM1 vaccine, a transient reduction in early growth during the peak of vaccinal oocyst infection has been shown in several other studies. Parker et al. (2007) and Walk et al. (2011) showed a significant reduction in the FI and BWG of vaccinated birds up to 17 and 18 doa, respectively, when compared to non-vaccinated birds. However, Weber et al. (2004) showed that the BWG of chicks immunized with 1×10^5 sporulated *Eimeria praecox* was not significantly different from that of non-immunized birds through 14 doa. The CPM of birds in the 0 to 14 doa interval was not significantly different among treatments, indicating that addition of the EM1 vaccine in the commercial diluent exerted no adverse effects on the growth performance of the broilers.

In conclusion, in ovo injection of the EM1 vaccine in 50 μL of commercial diluent on 18.5 doi had no detrimental effect on the hatchability of broiler hatching eggs, or the subsequent quality of hatchlings, as earlier demonstrated by Sokale et al. (2015a). Likewise, it had no detrimental effect on posthatch survivability or on 14 d grow-out performance, as demonstrated in the current study.

Table 4.1 Growth performance parameter means in the non-injected, dry-punch, and diluent-injected control groups, and in the vaccine-injected treatment group.[1]

	BW1 (kg)	BWG1 (kg)	FI1(kg)	FCR1	BW2 (kg)	BWG2 (kg)	FI2(kg)	FCR2	BW3 (kg)	BWG3 (kg)	FI3(kg)	FCR3
Non-injected control	0.043	0.080	0.101	1.26	0.124	0.172[a]	0.235[a]	1.37[b]	0.296[a]	0.252[a]	0.336[a]	1.33[b]
Dry-punch control	0.044	0.081	0.102	1.26	0.125	0.173[a]	0.236[a]	1.37[b]	0.298[a]	0.254[a]	0.337[a]	1.33[b]
Diluent-injected control	0.044	0.075	0.096	1.28	0.119	0.139[b]	0.214[b]	1.55[a]	0.257[b]	0.214[b]	0.3101[b]	1.46[a]
Vaccine-injected	0.044	0.077	0.096	1.26	0.121	0.142[b]	0.212[b]	1.50[a]	0.262[b]	0.218[b]	0.3086[b]	1.41[a]
SEM	0.002	0.002	0.002	0.016	0.002	0.005	0.005	0.036	0.006	0.006	0.007	0.023
P-values	0.5	0.2	0.2	0.6	0.2	<0.01	0.04	0.02	<0.01	<0.01	0.05	0.01

[a,b]Means within a column with no common superscript differ ($P \leq 0.05$).

[1]20 birds in each of 7 replicate units used to calculate each treatment mean.

BW1 = d 0 BW; BWG1 = d 0 to 7 BW gain; FI1 = d 0 to 7 feed intake; FCR1 = d 0 to 7 feed conversion ratio; BW2 = d 7 BW; BWG2 = d 7 to 14 BW gain; FI2 = d 7 to 14 feed intake; FCR2 = d 7 to 14 feed conversion ratio; BW3 = d 14 BW; BWG3 = d 0 to 14 BW gain; FI3 = d 0 to 14 feed intake; FCR3 = d 0 to 14 feed conversion ratio.

Table 4.2 Mean coccidia counts by histological examination in the non-injected and diluent-injected control groups, and in the vaccine-injected treatment group at 0, 3, 5, and 7 d of age.[1]

	Duodenum				Midgut				Cecum			
Age of bird in days	d 0	d 3	d 5	d 7	d 0	d 3	d 5	d 7	d 0	d 3	d 5	d 7
Non-injected control	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0[b]	1.0
Diluent-injected control	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0[b]	1.0
Vaccine-Injected	1.0	1.2	1.2	1.1	1.0	1.0	1.3	1.1	1.0	1.0	1.5[a]	1.4
SEM	1	0.08	0.08	0.06	1	1	0.17	0.06	1	1	0.16	0.18
P-value	1	0.13	0.13	0.38	1	1	0.38	0.38	1	1	[illegible]	0.20

[a,b]Means within a parameter with no common superscript differ ($P \leq 0.05$).

[1]1 bird in each of 7 replicate units (7 birds per treatment) was used to calculate each treatment mean. Each intestinal section was scored using the following matrix 1: no coccidia observed; 2: 1 to 30 coccidia observed; 3: 31 to 100 coccidia observed; 4: more than 100 coccidia observed.

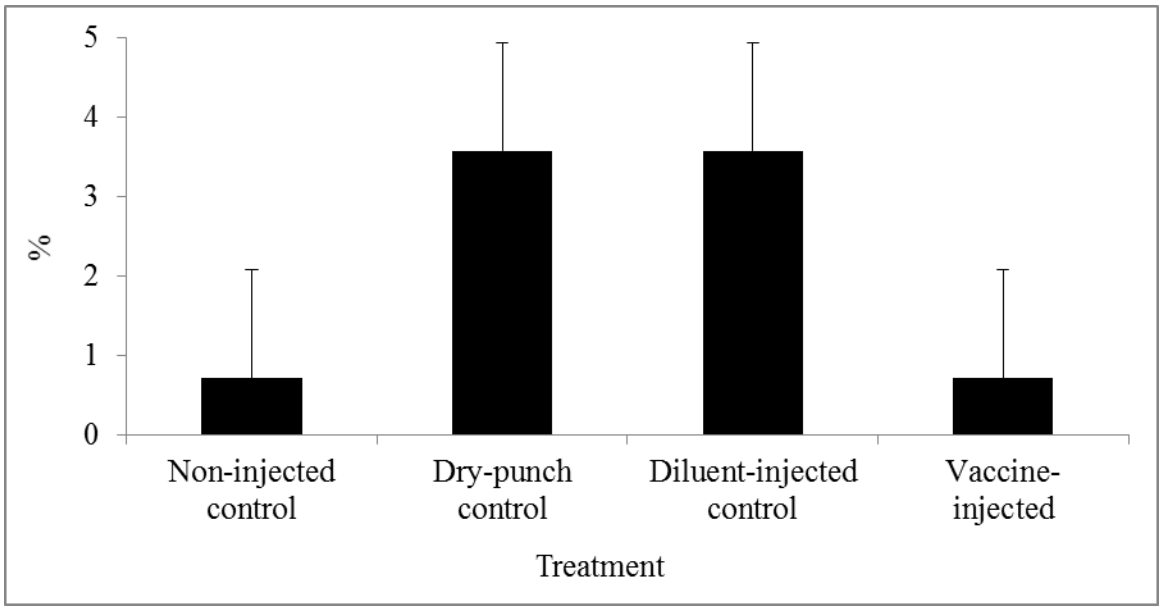

Figure 4.1 Cumulative (d 0 to 14 posthatch) percentage mortality (PM) in birds

In the non-injected, dry-punch and diluent-injected (50 µL) control groups, and in the vaccine-injected treatment group.[1]
[1]No significant difference among treatment groups for percentage mortality ($P = 0.25$).

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CHAPTER V

EFFECTS OF IN OVO INJECTION OF DIFFERENT DOSAGES OF INOVOCOX EM1 VACCINE AND TURN-OUT TIMES ON BROILER PERFORMANCE

Abstract

The in ovo injection of Inovocox EM1 vaccine (EM1) at the recommended dose of 50 µL per egg on 18.5 d of incubation (doi) has been shown to have no detrimental effect on hatchability and the performance characteristics of broilers through 14 d posthatch (poh). The effects of in ovo injection of EM1 at 1× or 10× dosage levels and 2 turn-out times on the chick quality and poh performance of Ross × Ross 708 broilers were determined in this current study. All 48 treatment-replicate groups (6 treatments on each of 8 replicate tray levels) each containing 60 eggs, were randomly arranged in a single-stage Jamesway incubator. On 19 doi, eggs were subjected to 1 of 3 treatments using a commercial Inovoject system. Treatments included a noninjected control, and 1× and 10× dosages of EM1. These 3 treatments groups were then partitioned into 2 turn-out time groups on 21 doi (day of hatch). The subsequent treatment combination designation were as follows: treatment 1- noninjected control with d 7 poh turn-out, treatment 2- noninjected control with d 10 poh turn-out, treatment 3 - 1× dose of EM1 with d 7 poh turn-out, treatment 4 - 1× dose of EM1 with d 10 poh turn-out, treatment 5 - 10× dose of EM1 with d 7 poh turn-out, and treatment 6 - 10× dose of EM1 with d 10 poh turn-out. On 21 doi, hatchability of injected eggs (HI), embryonic mortality, hatchling BW

(HBW), organ weights, yolk sac weight (YSW) and yolk-free BW (YFBW) were determined. Similarly, chicks from each treatment replicate group were placed in corresponding floor pens which were previously sub-divided in order to obtain the desired turn-out times. Feed intake, BW gain, and feed conversion ratio were determined for the weekly and cumulative 0 to 35 poh d of age (doa) intervals. There was no treatment effect on HI, HBW, and YFBW on 21 doi. However there was significant treatment effect on BW, YSW, and RBSW. Body weight on d 28 poh, BW gain and FCR in the 21 to 28 doa interval, and BWG in the 0 to 35 doa interval, were all affected by treatment. There was no significant difference among all the vaccine-injected treatment groups irrespective of dose and turn-out time. In conclusion, the in ovo injection of EM1 vaccine up to 10× the recommended dosage and turn-out times at 7 or 10 d poh, had no detrimental effect on the chick quality and overall posthatch performance of broilers.

Key words: chicks, Inovocox EM1 vaccine, in ovo injection, performance, posthatch

Introduction

Avian coccidiosis caused by a protozoan parasite of the genus *Eimeria,* continues to be one of the most common diseases of poultry. Avian coccidiosis in poultry increases their susceptibility to secondary diseases such as necrotic enteritis (Williams, 2005), and the subclinical form can negatively impact their performance. This parasite develops in the intestinal tract of birds, causing morbidity, mortality, and poor feed efficiency and weight gain. Coccidia oocysts are ubiquitous to commercial chicken houses and, therefore, complete eradication of coccidiosis is impossible (Chapman, 2000; Tewari and Maharana, 2011). The control of coccidiosis is achieved by use of in-feed anticoccidials

and coccidiosis vaccines in different types of grow-out programs, such as a rotation control program (Chapman et al., 2002; Chapman, 2009). In addition, coccidiosis vaccines are used throughout the year for the control of coccidiosis in antibiotics-free or organic commercial operations. Coccidiosis vaccines do not leave any residue in meat and are therefore, safe for poultry meat consumption (Van Immerseel et al., 2009). Efficacy of the coccidiosis vaccine, when used either exclusively year round or in a rotation program with in-feed anticoccidials, requires the establishment of immunological competence by the cycling of oocysts. Exposing birds to multiple coccidial life cycles (oocyst cycling) initiates an immune response necessary for the control of coccidiosis (Chapman et al., 2002; Tewari and Maharana, 2011). Furthermore, the use of partial house brooding management following administration of the coccidiosis vaccine, Coccivac-B by spray at day of hatch, has been recommended by the vaccine manufacturer (Schering-Plough Animal Health, 2007). Partial house brooding over a period of 7 to 14 days allows birds to be repeatedly exposed to high numbers of oocysts produced by the build-up of vaccinal oocysts. This fecal-oral repeated exposure initiates the development of immune competence against coccidiosis (Mathis, 2001). However, unlike use of the spray form of the coccidiosis vaccine, the duration of exposure of birds to built-up coccidial oocysts under partial house brooding conditions and its subsequent effects on performance following the in ovo administration of Inovocox EM1 vaccine has not been documented in the scientific literature. Therefore, the objective of this study was to determine an optimal turn-out time in the presence of high and low doses of Inovocox EM1 vaccine (**EM1**) for the optimization of oocyst re-cycling without negatively impacting grow-out performance.

Materials and Methods

General

All experimental procedures were approved by the Mississippi State University Institutional Animal Care and Use Committee. Ross × Ross 708 broiler hatching eggs (2,160) were obtained from a 45 wk old commercial breeder flock, and held for 2 d under standard storage conditions prior to setting. On 0 d of incubation (**doi**), eggs were weighed, and eggs that had normal appearance (Zhai et al.,2011b; Bello et al., 2014) were labeled and randomly assigned to each of 8 incubator tray levels in a Jamesway model PS 500 single stage incubator (Jamesway Incubator Co. Inc., Cambridge, Ontario, Canada). Each tray level served as a replicate unit. A total of 1,440 eggs were incubated under standard conditions (Peebles and Brake, 1987; Zhai et al., 2011a). There were 6 treatment groups, each containing 30 eggs that were represented on each of 8 replicate tray levels (blocks) in the setter (a total of 48 treatment-replicate groups). Eggs were candled on 18 doi, and all unfertilized eggs or eggs that contained dead embryos were removed (Ernst et al., 2004). After candling, a total of 1,310 embryonated eggs were retained in all 48 treatment-replicate groups, each containing approximately 27 embryonated eggs. Incubator dry and wet bulb temperatures were set at 37.5 ± 0.1 and 28.9 ± 0.1°C, respectively, and monitored twice daily for the entire incubation period. On 19 doi, eggs were either not injected or were subjected to one of the 2 treatments described below.

Injection and experimental layout

Injection of eggs was performed on 19.0 doi using an Embrex Inovoject injector system (Zoetis Animal Health, Research Triangle Park, NC), as described by Sokale et al.

(2015a). In order to achieve the manufacturer's recommendation of a 1× dose of EM1, 3 vials (8,000 doses each) of EM1 were reconstituted in 1,200 mL of sterile commercial MD vaccine diluent (Merial Co., Duluth, GA). Similarly, to achieve a 10× dose of EM1, 30 vials (8,000 doses each) of EM1 vaccine were reconstituted in 1,200 mL of sterile commercial MD vaccine diluent (Merial Co., Duluth, GA). The vaccines were injected through the air cell with 50 µL of solution per egg for both the 1× dose and 10× dose. On 19 doi, the site of injection (**SOI**) was confirmed using coomassie brilliant blue G-250 (colloidal) dye that was concurrently injected in a separate delivery than EM1 (Sokale et al., 2015a). During the injection process, eggs belonging to a particular treatment group were injected together before changing to another treatment group to avoid cross-contamination between treatment groups. Eggs in the non-injected control group were subjected to the same process as the injected treatment groups, except that they were not injected with EM1. Once the entire injection process was completed, eggs were transferred to the hatcher unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada) on their corresponding replicate tray levels. Eggs in the hatcher baskets were arranged in a way to prevent cross-contamination between the injected and non-injected hatched chicks. All eggs were held outside the incubator for an approximate 5 min time interval between injection and transfer. Approximately 60 embryonated eggs per treatment group were randomly arranged on each of 8 replicate tray levels and were subjected to one of the following treatments: treatment 1 - noninjected control; treatment 2 - 1× dose of EM in 50 µL of diluent; or treatment 3 - 10× dose of EM1 in 50 µL of diluent. However, each of the 3 treatment groups were subsequently divided into each of 2 turn-out times during the grow-out phase, so that 6 treatment groups (3 injections × 2

turn-out times treatments) were ultimately formed. Therefore, at the beginning of the grow-out phase, chicks were allotted to one of the following 6 treatment groups: treatment 1- noninjected control with d 7 turn-out (**NIC7**); treatment 2 - noninjected control with d 10 turn-out (**NIC10**); treatment 3 - 1× dose of EM1 with d 7 turn-out (**1× VI7**); treatment 4 - 1× dose of EM1 with d 10 turn-out (**1× VI10**); treatment 5 - 10× dose of EM1 with d 7 turn-out (**10× VI7**); and treatment 6 - 10× dose of EM1 with d 10 turn-out (**10× VI10**).

On 21 doi, a total of 20 straight-run chicks from each of the 48 treatment-replicate groups were randomly selected, wing-banded, weighed, and placed in each of 48 miniature floor pens measuring 1.1 m^2/pen, within a temperature controlled research facility. In order to achieve the specified turn-out times, chicks were initially placed in a 3/4 (0.84 m^2) portion of each pen, with a stocking density of 0.04 m^2 /bird. The pens were divided using plastic wire mesh that prevented birds from crossing over to the unused side of the pen without interfering with air flow. Birds were turned-out on d 7 and 10 posthatch (**poh**). A total of 24 treatment-replicate pens were randomly selected and turned-out at each time period. Turning-out involved the removal of the plastic wire mesh used to divide each pen, so that birds are allowed the entire 1.1 m^2 space in each pen, with a stocking density of 0.06 m^2 /bird up to 35 d poh. A total of 960 chicks (20 chicks × 48 pens) were placed on previously used litter (had been used for 2 previous grow-out cycles). Ad-libitum feed and water were provided to birds in each pen during grow-out. Birds were fed standard Mississippi State University broiler diets, which were formulated to meet or exceed NRC (1994) recommendations, through d 35 poh. Birds were provided crumbled starter diet from d 0 to 14, pelletized grower diet from d 15 to

28, and pelletized finisher diet from d 29 to 35. House temperature conditions were monitored and recorded twice daily for the entire duration of the grow-out period.

Data collection

Set egg weight (SEW) was recorded on 0 doi. Incubation temperature was monitored and recorded twice daily during the incubation period. On day of injection (19.0 doi), SOI and embryo staging (ES) were determined. On 21.0 doi, 16 chicks from each treatment group were wing-banded, euthanized, weighed, and necropsied for determination of chick BW, YSW, liver (**LW**), whole intestine (**IW**), and heart weight (**HW**). The hatchability of injected fertilized eggs **(HI)** and hatching chick BW (**HBW**) were also determined on 21.0 doi. The cumulative percentage mortality of un-hatched embryos at late incubation stage (**PLD**; 15.0 to 18.5 doi) was determined at 21.0 doi. Furthermore, the following hatching chick quality parameters were determined: BW, yolk-free BW (**YFBW**), yolk-sac weight (**YSW**), yolk-sac relative to BW (**RYBW**), IW relative to BW (**RIBW**), IW relative to YFBW (**RIYFW**), LW relative to BW (**RLBW**), LW relative to YFBW (**RLYFW**), HW relative to BW (**RHBW**), HW relative to YFBW (**RHYFW**), BW relative to SEW (**RBSW**), YFBW relative to SEW (**RYFWSW**), and yolk free body mass (**YFBM)**. The YFBM which measures the development of chicks during incubation is calculated by dividing YFBW by BW (Sokale et al., 2015a). On d 28 poh, 2 chicks from each treatment-replicate group (16 total birds per treatment) were euthanized, weighed, and necropsied for determination of BW, fresh IW, and RIBW. Bird numbers, BW, and feed weights on a pen basis were determined weekly for each treatment-replicate group from d 0 to 35 poh. Furthermore, BWG, feed intake **(FI)**, and feed conversion ratio (**FCR**) were determined for the 0 to 7, 7 to 14, 14 to 21, 21 to 28,

28 to 35, and 0 to 35 d of age (**doa**) intervals. Percentage cumulative mortality **(CPM)** for the cumulative 0 to 35 doa interval was also determined.

Statistical description

A randomized complete block design was used in this study in both the incubation and grow-out phase of the study. Each tray level represented a block, and all treatments were equally and randomly represented in each block. The chick quality data on 21.0 doi were analyzed using the 3 injection dose groups, while the performance data were analyzed using the 3 injection dose and the 2 turn-out times. All variables were analyzed using the MIXED procedure of SAS software 9.3 (SAS institute, 2012). Treatment was viewed as a fixed effect and block as a random effect to analyze for the effects of treatments on the chick quality variables, d 28 poh variables, CPM in the 0 to 35 doa interval, and weekly BW gain, FI, and FCR using a one-way ANOVA. A split-plot analysis of absolute weekly BW was performed with treatment, age, and their interaction designated as a fixed effect and block as a random effect. Least-square means were compared in the event of significant global effects (Steel and Torrie, 1980). Global and least-square means differences were considered significant at $P \leq 0.05$.

Results

There was no significant difference in SEW between the noninjected control (**NIC**), 1× dose of EM1 (**1×EM1**), and 10× dose of EM1 (**10×EM1**) treatments at 0 doi. The SEW means for NIC, 1×EM1, and 10×EM1 groups were 64.8, 64.6, and 64.5 g (Pooled SEM = 0.18 g), respectively. The chick quality results in response to the NIC, 1×EM1, and 10×EM1 treatments were also compared. There was no significant treatment

effect on HI or HBW at 21.0 doi (Figs. 5.1 and 5.2). The HI means for the NIC, 1×EM1, and 10×EM1 treatment groups were 96.1, 93.1, and 95.1 % (Pooled SEM = 1.17 %), respectively. The HBW means for the NIC, 1×EM1, and 10×EM1 treatment groups were 45.2, 44.8, and 44.7 g (Pooled SEM = 0.22 g), respectively. The treatment means for each of the hatching chick quality variables evaluated are provided in Table 5.1. There was a significant treatment effect on BW ($P = 0.01$) and YSW ($P = 0.04$) on 21.0 doi. The BW and YSW were highest in the NIC group and lowest in the 10×EM1 group, with the 1×EM1 group being intermediate. However, there was no significant treatment effect on YFBW. There was a significant treatment effect on RBSW ($P = 0.01$). The RBSW was significantly highest in the NIC group and lowest in the 10×EM1 treatment group, with the 1×EM1 group being intermediate. The RBSW means in the NIC, 1×EM1, and 10×EM1 treatment groups were 70.7, 68.5, and 66.7 % (Pooled SEM = 0.92 %), respectively. There was no significant treatment effect on mean BW at d 0, 7, 14, 21, and 35 poh. However, there was significant treatment effect on BW at d 28 poh ($P = 0.003$). The BW on d 28 poh was significantly higher in the NIC7 group in comparison to all the other 4 vaccine-injected and turn-out time combination treatment groups (1×VI7, 1×VI10, 10×VI7, and 10×VI10). Nevertheless, there was no significant difference between the NIC10 group and the NIC7 or 1×VI7 groups. Treatment did not significantly affect mean BWG in the 0 to 7, 7 to 14, 14 to 21, and 28 to 35 doa intervals. However, there was a significant treatment effect on BWG in the 21 to 28 ($P = 0.003$), and the 0 to 35 ($P = 0.05$) doa intervals. The BWG of the birds in the 21 to 28 doa interval was significantly higher in the NIC7 and NIC10 groups in comparison to all of the other 4 vaccine-injected treatment groups. However, there was no significant

difference between all of the 4 vaccine-injected and turn-out time combination treatment groups, irrespective of the dose and turn-out time. The BWG of the birds in the 0 to 35 doa interval was significantly higher in the NIC7 group in comparison to all of the 4 vaccine-injected and turn-out time combination treatment groups. There was no significant treatment effect on FI in the 0 to 7, 7 to 14, 14 to 21, 21 to 28, 28 to 35 or 0 to 35 doa intervals. There was also no significant treatment effect on FCR in the 0 to 7, 7 to 14, 14 to 21, 28 to 35, and 0 to 35 doa intervals. However, there was a significant treatment effect on FCR in the 21 to 28 doa interval ($P = 0.03$). The FCR of the birds was highest in the 1×VI7 and 10×VI10 groups, and lowest in the NIC10 group. There was a significant treatment effect on d 28 poh RIBW ($P = 0.04$). The RIBW of the birds was highest in the 1×VI7 group, and lowest in the NIC10 group. For BW on d 28 poh, BWG in the 0 to 35 doa interval, FCR in the 21 to 28 doa interval (Table 5.3), and for d 28 poh RIBW (Fig. 5.6), there was no significant difference among the 4 vaccine-injected and turn-out time combination treatment groups irrespective of dose and turn-out time. For reference, treatment means for BW at each age period, and for BWG, FI, and FCR in each age interval are provided in Tables 5.2 and 5.3. There was no significant difference among treatment groups for cumulative late dead embryos (PLD) and the CPM (0 to 35 doa) of the birds. The PLD of embryos and the CPM of the poh broilers in the control and treatment groups are presented in Figs. 5.3 and 5.4, respectively. The SOI and ES were evaluated in this study, using approximately 7 % of the in ovo injected embryonated eggs. The mean ES at 19.0 doi was 4.60 ± 0.99. The SOI evaluation of those same eggs at 19.0 doi indicated that 6.8 and 93.2 % of the eggs received vaccine in the AM and EM respectively, with the EM injection being 81.5 % i.m and 11.7 % s.c.

Discussion

In a previous study in which EM1 vaccine was injected in ovo at 50 µL per egg on 18.5 doi, it was shown that EM1 did not have a negative effect on embryogenesis through 21.0 doi (Sokale et al., 2015a) or performance through 14 d poh (Sokale et al., 2015b). In this current study, the effects of 2 types of EM1 doses on embryogenesis, and 2 types of EM1 doses with 2 turn-out times on d 0 to 35 poh performance were evaluated. The evaluation of the results of hatching chick quality variables on 21.0 doi shows that the in ovo injection of Ross × Ross 708 broiler hatching eggs with either the 1× dose or 10× dose of EM1 vaccine in 50 µL of commercial diluent does not affect embryogenesis. In comparison to the NIC, injection of the 10× dose resulted in an increase in BW and RBSW on 21.0 doi. However, there was no difference in YFBW among treatment groups. The difference in BW may have resulted from the difference in YSW which was higher in the 10× dose treatment group in comparison to the NIC group. This result is consistent with a previous study conducted by Zhai et al., (2011a). In that study, it was found that in comparison to noninjected controls, the injection of carbohydrates resulted in an increase in chick BW and YSW on day of hatch without causing any subsequent difference in YFBW. However, in comparison to noninjected controls, Bello et al., (2013) did not show any difference in BW, YSW, RBSW, or YFBW on day of hatch, when embryos were injected with 25 (OH) D_3. It can be said that the in ovo injection process in itself, or the in ovo injection of any substance, may affect some hatching chick quality variables. Therefore, when considering the effects of in ovo injected substances on embryonic development, variables such as BW and YFBW should be evaluated together (Zhai et al., 2011a). In addition, there were no differences among treatment

means for HI, HBW, and PLD on 21.0 doi in the current study. This showed that Ross × Ross 708 hatching chicks can withstand up to 10× the recommended dose of EM1 vaccine without any obvious detrimental effect on embryogenesis. A further evaluation the SOI showed that at 19.0 doi, 81.5 % of the sampled embryos were injected i.m (in the right breast muscle), and, therefore, may have been able to withstand the in ovo injection of EM1 up to 10× the recommended dose. The effective control of coccidiosis by use of vaccines either alone or as part of rotation programs requires 2 major factors; firstly, the uniformity of vaccine application is important, and secondly, a recycling of oocysts for development of immunity is crucial. The former, which typically occurs in the hatchery, has demonstrated tremendous success, primarily due to improvements in vaccine application techniques (Chapman et al., 2002; Chapman, 2009; Tewari and Maharana, 2011), and the ability to determine the stage of embryo development at the time of in ovo injection (Williams and Zadek, 2010). For example, in the current study, the ES and SOI results suggest that the developmental stage of the embryos when eggs were in ovo-injected, corresponded to that between 19.0 and 19.5 doi, with vaccine deposition primarily in the right breast muscle. The EM1 vaccine is recommended for vaccinating 18 to 19 doi embryos, and although the preferred SOI is within the AM, studies have shown as embryos approach hatch, the volume of amniotic fluid available for injection becomes reduced, making it less possible to deposit the vaccine within the amniotic cavity (Jochemsen and Jeurissen, 2002). Therefore, as the broiler embryo developmentally approaches hatch, the probability of injecting in the intra-cranial, intra-orbital, or intra-abdominal regions of the embryo can increase (Williams and Zedek, 2010). For successful vaccination to occur in ED 19.0 embryo using the Inovoject®

system, the EM1vaccine must be delivered into the AM or EM (i.m or s.c) regions of the embryo. Therefore, the success of in ovo vaccination in this current study was 100 % (6.8 % AM; 81.5 % i.m; 11.7 % s.c); which is consistent with the expected outcome for embryos that are injected in ovo between 19.0 and 19.5 doi (unpublished data).

The recycling of oocysts for the development of immunocompetence, which typically occurs in the chicken house, continues to pose a challenge to producers. This is due in part to the interplay of numerous factors present within the chicken house (e.g. light, temperature, ventilation, feed, flock density, litter moisture, etc.). In practice, the recycling of oocysts is achieved through partial house brooding, in which birds are confined to a section of the house, usually a 1/3 or 1/2 portion of the house for a limited period of time. The limited period of time is usually between 7 and 14 d. Thereafter, birds are turned-out to the entire house for the remainder of the grow-out period (Hix, 2013). During partial house brooding, birds are exposed to multiple coccidial life cycles to initiate and establish development of immunity against coccidiosis. The selection of a turn-out time during partial house brooding ensures that optimal oocyst recycling is achieved without the risk of reversion to clinical coccidiosis and a subsequent negative impact on grow-out performance. Unlike the spray type of coccidiosis vaccine (Coccivac-B), an ideal turn-out time following the in ovo administration of EM1 vaccine has not yet been documented in scientific literature. Therefore, an objective in this study was to determine an ideal turn-out time in conjunction with the administration of the EM1 vaccine for achievement of optimal oocyst recycling without having a negative impact on performance. Bird performance was evaluated against 2 turn-out times (d 7 and 10) and 2 doses (1× and 10 × doses) of the EM1 vaccine. The performance of birds administered

the 1× dose that were subsequently turned out at d 7 or 10, was the same in comparison to the performance of birds injected with the 10 × dose and that were also turned out on either day. Although BW at d 28 poh, and BWG and feed efficiency in the 21 to 28 d poh intervals were lower in all the vaccine injected treatment groups in comparison to the control group, these time points corresponded to the peak period of oocyst cycling (Broomhead, 2012). In addition, the RIBW at d 28 poh in all the vaccine injected treatment groups, was similar in comparison to the control group, which further indicated that the peak in coccidial life cycle development and oocyst cycling occurred in the gastrointestinal tract at this period. This is consistent with a study by Küçükyilmaz et al, (2012), that showed a significantly high cecal weight and overall intestine length in coccidial infected birds compared with uninfected birds. Further, the increase in RIBW at d 28 poh may have resulted in decrease BW of the birds belonging to the vaccine group when compared to birds in the control group, at the same time period. However, there was no difference among treatment groups for BW and feed efficiency at d 35 poh. This was due to a compensatory gain in BW that following its reduction at the peak of oocyst cycling (Broomhead, 2012; Mathis, 2001; Williams and Gobbi, 2002).

In conclusion, this study confirms that the in ovo injection of the EM1 vaccine on 19.0 doi, at a either 1× or 10× dose in 50 µL of commercial diluent has no detrimental effect on the hatchability of injected eggs, or on hatchling BW, RBSW, YFBW, embryo survivability, and other hatchling quality variables that were examined in this study. In addition, birds administered either the 1× or 10× dose of EM1 vaccine and that were turned out on either d 7 or 10 poh showed similar grow-out performance outcomes. Therefore, it is recommended that under ideal house conditions, that a 1× dose of EM1

vaccine in 50 µL of commercial diluent in conjunction with partial house brooding up to 10 d poh will ensure adequate oocyst cycling without negatively affecting grow-out performance.

Table 5.1 Somatic and yolk parameter means in the noninjected control group and in the 1× dose and 10× dose EM1 vaccine treatment groups.[1,2]

Treat-ments	BW (g)	YFBW (g)	SEW[3] (g)	RBSW (%)	RYFWSW (%)	IW (g)	RIBW (g)	RIYFW (%)	LW (g)	RLBW (%)	RLYFW (%)	HW (g)	RHBW (%)	RHYFW (%)	YSW (g)	RYBW (%)	YFBM (%)
Non-injected control	45.86[a]	40.92	64.82	70.75[a]	63.11	2.19	4.76	5.33	1.22	2.66	2.98	0.35	0.76	0.85	4.94[a]	10.76	89.24
1× dose vaccine	44.21[ab]	39.52	64.55	68.50[ab]	61.22	2.20	4.98	5.57	1.16	2.63	2.94	0.34	0.77	0.86	4.70[ab]	10.60	89.40
10× dose vaccine	43.01[b]	39.07	64.50	66.69[b]	60.57	2.21	5.14	5.66	1.21	2.81	3.09	0.33	0.78	0.86	3.95[b]	9.15	90.85
SEM	0.61	0.56	0.18	0.92	0.83	0.06	0.13	0.14	0.03	0.06	0.07	0.01	0.02	0.02	0.28	0.60	0.60
P-value	0.01	0.06	0.39	0.01	0.09	0.96	0.15	0.24	0.42	0.12	0.26	0.49	0.91	0.97	0.04	0.13	0.13

[a-b] Means within a variable with no common superscript differ ($P \leq 0.05$).

1Body weight (BW), yolk-free BW (YFBW), set egg weight (SEW), BW as a percentage of set egg weight (RBSW), yolk-free BW as a percentage of set egg weight (RYFWSW), intestine weight (IW), intestine weight as a percentage of BW (RIBW), intestine weight as a percentage of yolk-free BW (RIYFW), liver weight (LW), liver weight as a percentage of BW (RLBW), liver weight as a percentage of yolk-free BW (RLYFW), heart weight (HW), heart weight as a percentage of BW (RHBW), heart weight as a percentage of yolk-free BW (RHYFW), yolk sac weight (YSW), yolk sac weight as a percentage of BW (RYBW), and yolk-free body mass (YFBM).

[2]Two birds in each of 8 replicate units per treatment (16 birds per treatment group) were used to calculate each treatment mean.

[3]Thirty eggs in each of 8 replicate units per treatment (240 eggs per treatment group) were used to calculate each treatment mean.

Table 5.2 Performance parameter means in the noninjected control group and in the 1× dose and 10× dose of EM1 vaccine treatment groups.[1]

	BW1 (kg)	BWG1 (kg)	FI1 (kg)	FCR1	BW2 (kg)	BWG2 (kg)	FI2 (kg)	FCR2	BW3 (kg)	BWG3 (kg)	FI3 (kg)	FCR3
Non-injected control + d 7 turn-out	0.046	0.123	0.159	1.295	0.168	0.294	0.376	1.279	0.463	0.424	0.603	1.421
Non-injected control + d 10 turn-out	0.045	0.116	0.156	1.348	0.161	0.277	0.359	1.296	0.444	0.414	0.596	1.444
1× dose vaccine + d 7 turn-out	0.045	0.117	0.156	1.335	0.162	0.279	0.351	1.260	0.442	0.410	0.597	1.456
1× dose vaccine + d 10 turn-out	0.045	0.121	0.158	1.311	0.166	0.269	0.347	1.296	0.437	0.407	0.584	1.438
10× dose vaccine + d 7 turn-out	0.045	0.116	0.154	1.326	0.161	0.276	0.356	1.290	0.438	0.399	0.582	1.460
10× dose vaccine + d 10 turn-out	0.045	0.115	0.154	1.336	0.160	0.273	0.355	1.299	0.436	0.407	0.590	1.450
SEM	0.0003	0.0029	0.0040	0.0327	0.0029	0.0066	0.0070	0.0140	0.0085	0.0081	0.0099	0.0178
P-values	0.210	0.392	0.907	0.885	0.318	0.151	0.099	0.352	0.249	0.404	0.668	0.676

[1]20 birds in each of 8 replicate units used to calculate each treatment mean.
[2]BW1 = d 0 body weight (BW); BWG1 = d 0 to 7 BW gain; FI1 = d 0 to 7 feed intake; FCR1 = d 0 to 7 feed conversion ratio; BW2 = d 7 BW; BWG2 = d 7 to 14 BW gain; FI2 = d 7 to 14 feed intake; FCR2 = d 7 to 14 feed conversion ratio; BW3 = d 14 BW; BWG3 = d 14 to 21 BW gain; FI3 = d 14 to 21 feed intake; FCR3 = d 14 to 21 feed conversion ratio.

Table 5.3 Performance parameter means in the noninjected control group and in the 1× dose and 10× dose of EM1 vaccine treatment groups.[1]

	BW4 (kg)	BWG4 (kg)	FI4(kg)	FCR4	BW5 (kg)	BWG5 (kg)	FI5(kg)	FCR5	BW6 (kg)	BWG6 (kg)	FI6(kg)	FCR6
Non-injected control + d 7 turn-out	0.888	0.694^{a}	1.089	1.569^{bc}	1.592^{a}	0.547	1.194	2.191	2.159	2.082^{a}	3.421	1.551
Non-injected control + d 10 turn-out	0.863	0.688^{a}	1.077	1.566^{c}	1.561^{ab}	0.564	1.192	2.124	2.139	2.059^{ab}	3.379	1.556
1× dose vaccine + d 7 turn-out	0.852	0.641^{b}	1.056	1.651^{a}	1.512^{bc}	0.543	1.177	2.168	2.076	1.990^{bc}	3.337	1.574
1× dose vaccine + d 10 turn-out	0.844	0.643^{b}	1.038	1.619^{abc}	1.495^{c}	0.545	1.178	2.184	2.056	1.985^{bc}	3.306	1.569
10× dose vaccine + d 7 turn-out	0.839	0.639^{b}	1.047	1.643^{ab}	1.490^{c}	0.532	1.165	2.191	2.046	1.962^{c}	3.304	1.583
10× dose vaccine + d 10 turn-out	0.843	0.640^{b}	1.073	1.676^{a}	1.492^{c}	0.554	1.225	2.216	2.066	1.990^{bc}	3.396	1.596
SEM	0.0144	0.0127	0.0142	0.0267	0.0207	0.0170	0.0263	0.0569	0.0316	0.0302	0.0482	0.0140
P-values	0.176	0.003	0.116	0.026	0.003	0.848	0.672	0.909	0.071	0.047	0.411	0.235

[a-c]Means within a column with no common superscript differ ($P \leq 0.05$).

[1]20 birds in each of 8 replicate units used to calculate each treatment mean.

[2]BW4 = d 21 body weight (BW); BWG4 = d 21 to 28 BW gain; FI4 = d 21 to 28 feed intake; FCR4 = d 21 to 28 feed conversion ratio; BW5 = d 28 BW; BWG5 = d 28 to 35 BW gain; FI5 = d 28 to 35 feed intake; FCR5 = d 28 to 35 feed conversion ratio; BW6 = d 35 BW; BWG6 = d 0 to 35 BW gain; FI6 = d 0 to 35 feed intake; FCR6 = d 0 to 35 feed conversion ratio.

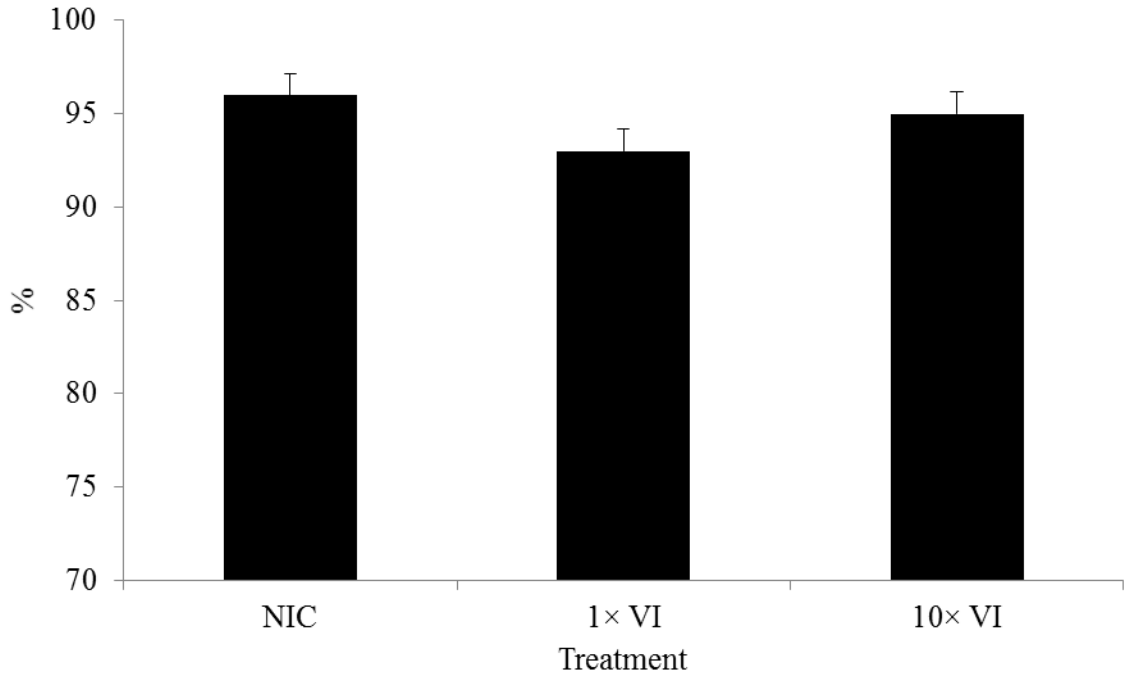

Figure 5.1 Hatchability on d 21.0 of incubation as a percentage of fertilized injected eggs (HI)

In noninjected control group (NIC) and in eggs injected with 1× dose of EM1vaccine (1× VI), or 10× dose of EM1vaccine (10× VI) in 50 µL of diluent.[1,2]
[1]Data from 8 replicate units was used for calculation of means for each treatment group.
[2]No significant difference among treatment groups for hatchability ($P = 0.22$).

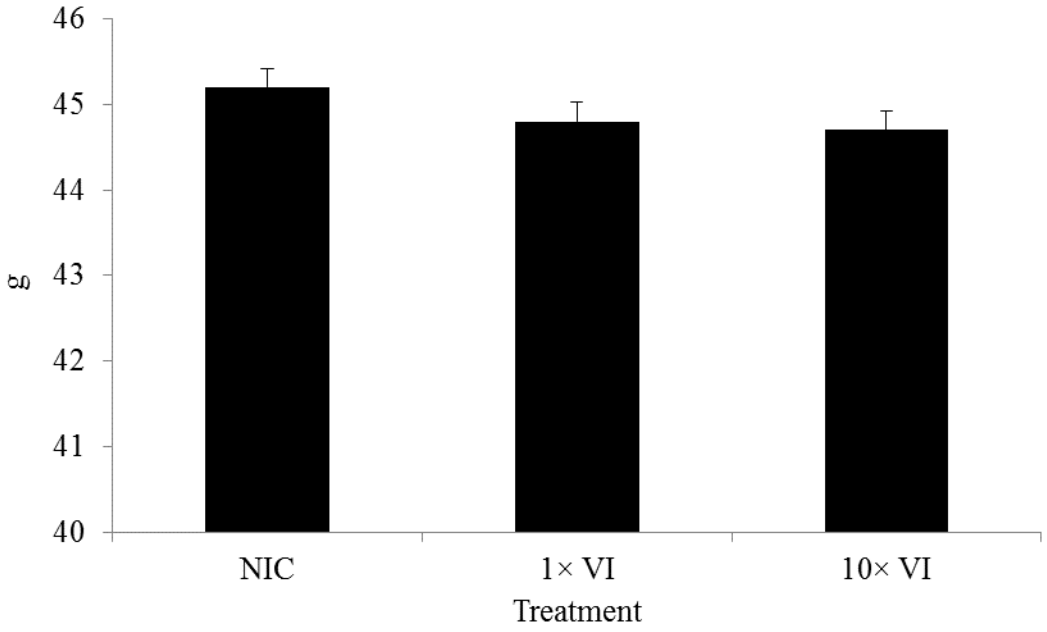

Figure 5.2 Hatching BW on d 21.0 of incubation

In noninjected control group (NIC) and in eggs injected with 1× dose of EM1vaccine (1× VI), or 10× dose of EM1vaccine (10× VI) in 50 µL of diluent.[1,2]
[1]Data from 8 replicate units was used for calculation of means for each treatment group.
[2]No significant difference among treatment groups for hatching BW ($P = 0.32$).

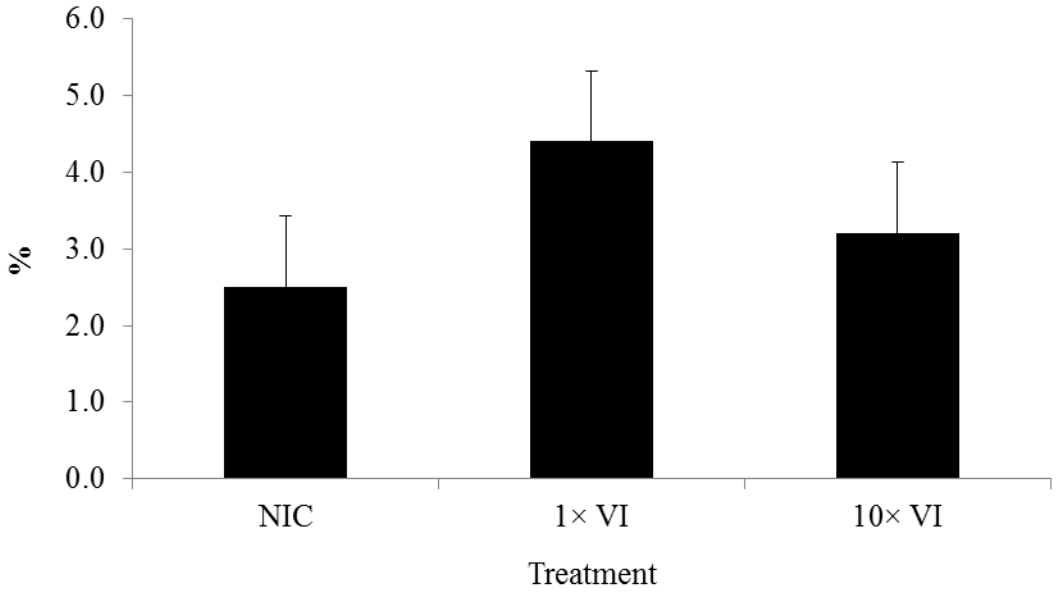

Figure 5.3 Late dead chicks on d 21.0 of incubation as a percentage of fertilized injected eggs

In noninjected control group (NIC) and in eggs injected with 1× dose of EM1 vaccine (1× VI), or 10× dose (10× VI) of EM1vaccine (10× VI).[1,2]
[1]Data from 8 replicate units was used for calculation of means for each treatment group.
[2]No significant difference among treatment groups for late dead chicks ($P = 0.36$).

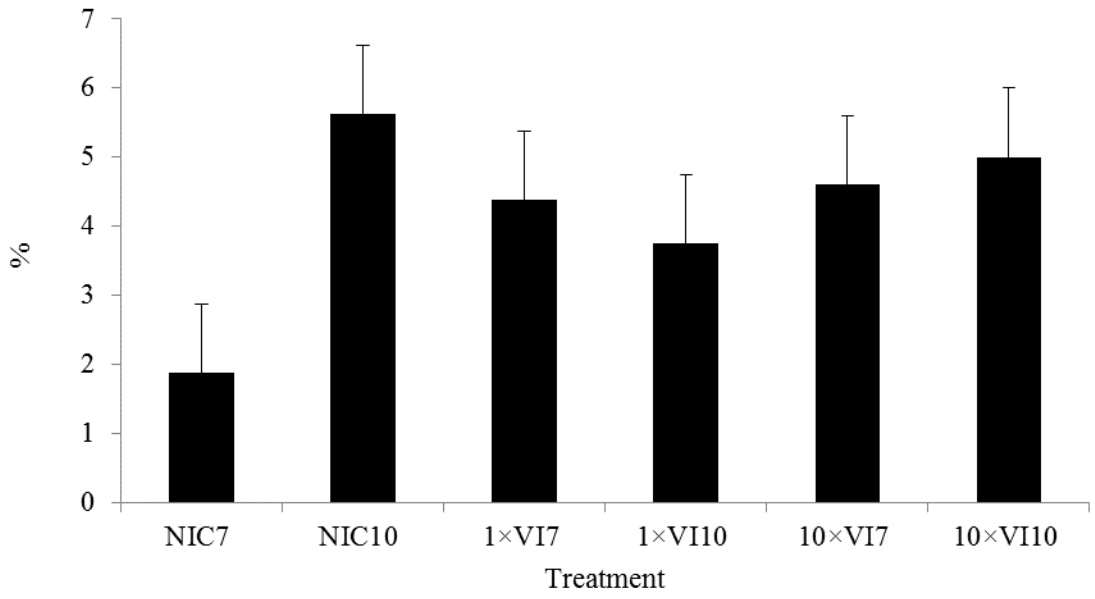

Figure 5.4 Grow-out cumulative percentage mortality

In the noninjected control + d 7 turn-out (NIC7) and noninjected control + d 10 turn-out (NIC10) groups, and the 1× dose + d 7 turn-out (1×VI7) , 1× dose + d 10 turn-out (1×VI10), 10× dose + d 7 turn-out (10×VI7), and 10× dose + d 10 turn-out (10×VI10) of EM1vaccine treatment groups.[1]

[1] No significant difference among treatment groups for percentage mortality ($P = 0.09$).

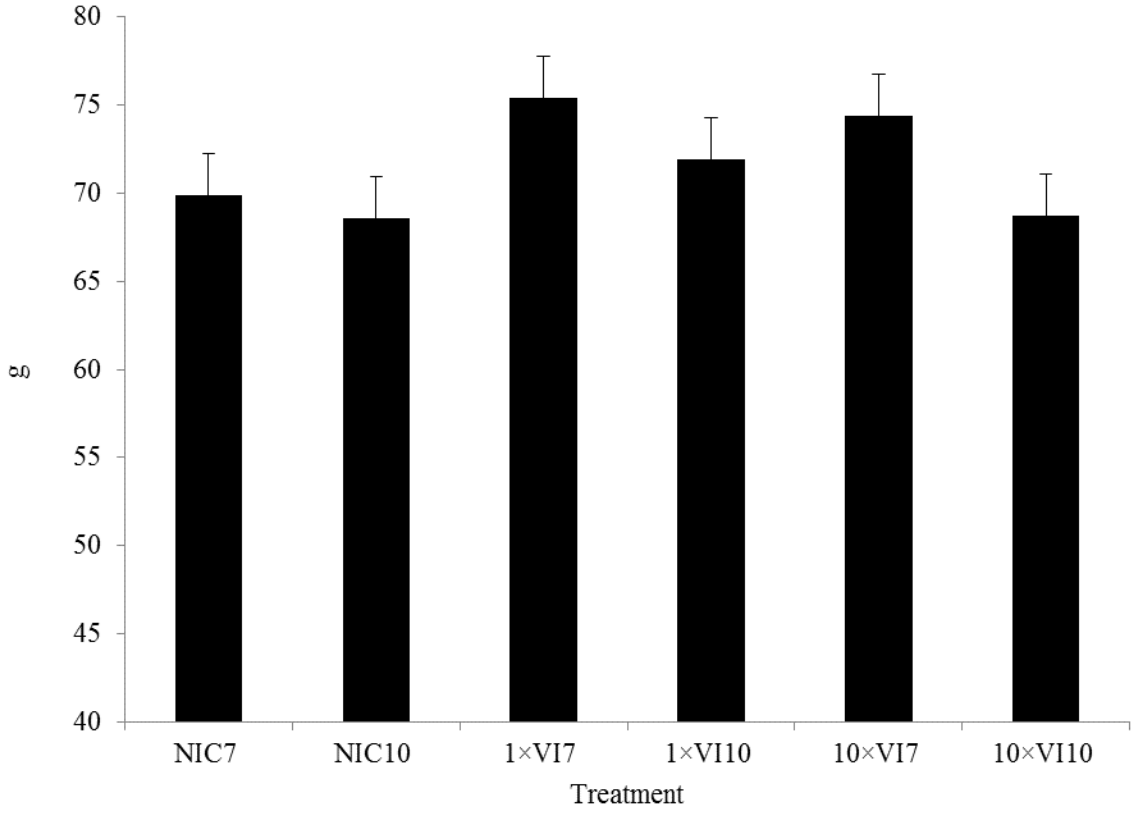

Figure 5.5 Absolute intestine weight on d 28 post-hatch

In the noninjected control + d 7 turn-out (NIC7) and noninjected control + d 10 turn-out (NIC10) groups, and the 1× dose + d 7 turn-out (1×VI7) , 1× dose + d 10 turn-out (1×VI10), 10× dose + d 7 turn-out (10×VI7), and 10× dose + d 10 turn-out (10×VI10) of EM1vaccine treatment groups.[1,2]
[1]Data from 8 replicate units was used for calculation of means for each treatment group.
[2]No significant difference among treatment groups for intestine weight ($P = 0.20$).

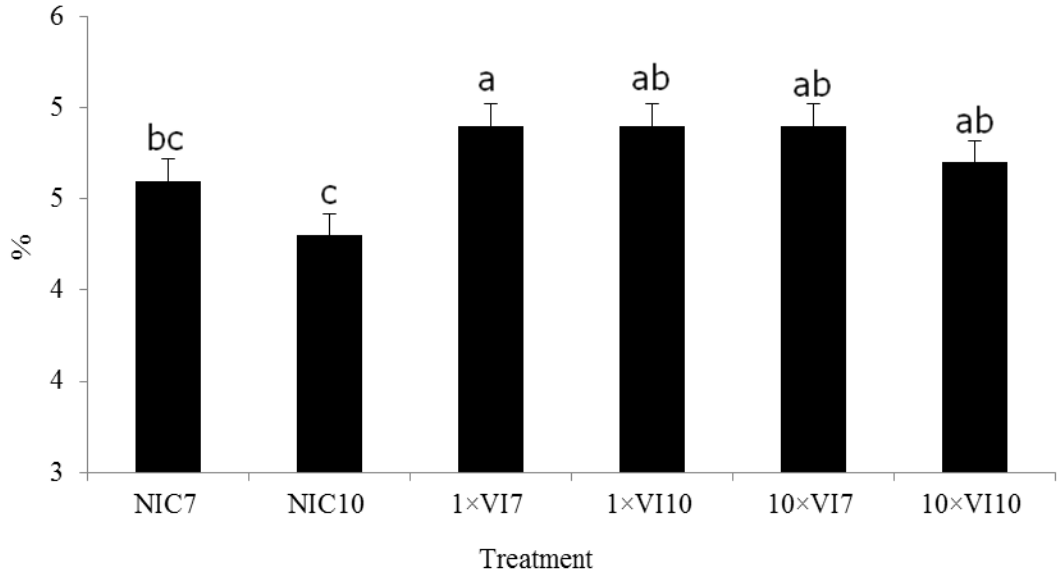

Figure 5.6 Relative intestine weight on d 28 post-hatch as a percentage of BW

In the noninjected control + d 7 turn-out (NIC7) and noninjected control + d 10 turn-out (NIC10) groups, and the 1× dose + d 7 turn-out (1×VI7) , 1× dose + d 10 turn-out (1×VI10), 10× dose + d 7 turn-out (10×VI7), and10× dose + d 10 turn-out (10×VI10) of EM1vaccine treatment groups.[1,2]

[a-c]Means among treatment groups with no common superscript differ ($P \leq 0.05$).

[1]Data from 8 replicate units was used for calculation of means for each treatment group.

[2]Significant difference among treatment groups for relative intestine weight ($P = 0.04$).

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CHAPTER VI

DIFFERENTIAL EFFECTS OF BROILER EMBRYO AGE ON THE QUANTITATIVE PATHOGENICITY OF THE INOVOCOX EM1 VACCINE

Abstract

Control of coccidiosis continues to pose a challenge to commercial poultry producers and as a result, coccidia vaccines are being used in a rotation program to achieve effective coccidiosis control. Inovocox EM1 vaccine (**EM1**) allows early vaccine oocyst cycling and the development of immunity to reduce the effects of wild-type coccidia present within broiler houses. This immunocompetence results in a reduction of intestinal lesions and a subsequent improvement in performance. The EM1 is administered to healthy broiler embryos at 18- to 19- d of incubation (**doi**) as an aid in the prevention of coccidiosis caused by 3 spp. of *Eimeria*. Based on this recommendation, several commercial hatcheries vaccinate broiler embryos during transfer at either 18.5 or 19 doi. However, it is unclear whether a difference in the age of the embryos at the time of in ovo injection can impact the cycling of the vaccine oocysts and subsequent posthatch broiler performance. Therefore, the objective of our study was to evaluate effects of administering the EM1 at 18.5 and 19.0 d of embryo age (**EDOA**) on hatching chick quality, oocyst output per gram of litter, the presence of intestinal lesions, and grow-out performance. Chicks were hatched on 21.0 doi and placed on floor pens that had been covered with fresh new shavings from 0 to 35 posthatch (**poh**). In general,

treatments were comprised of 3 injection-types (**IT**) and 2 EDOA that were arranged in a factorial design. Main effects of EDOA and IT, and their interactive effects were observed on various hatching chick quality variables. Furthermore, main effects of EDOA on grow-out performance were observed up to d 35 poh. Peak oocyst shedding and intestinal lesion scores were also observed at d 21 and 28 poh, respectively. In conclusion, IT had no detrimental effects on hatching chick quality. However, difference in embryonic age affected performances.

Key words: chicks, embryo, Inovocox EM1 vaccine, in ovo injection, performance

Introduction

Coccidia are obligate intracellular parasites that develop and multiply in the intestinal epithelium, causing damage to the structure of the intestine, impaired nutrient absorption, poor feed utilization, poor growth, high morbidity and mortality (McDougald et al., 2008), and susceptibility to other diseases (William et al. 2003; Li et al., 2010). Inovocox EM1 vaccine (**EM1**) is used for the in ovo vaccination of embryonated chicken eggs at 18 to 19 d of incubation (**doi**) for the prevention of coccidiosis caused by *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella*. The control of coccidiosis by a live non-attenuated EM1 vaccine involves the in ovo injection of a controlled dose of *Eimeria* oocysts that stimulates a localized immune response in the bird. This stimulation results more specifically through a replication of the vaccine oocysts within the mucosal lining of the intestine, thereby inducing some degree of pathogenicity within the gut (McDougald et al., 2008; Tewari, 2011). The degree of pathogenicity of coccidiosis can be measured by parameters such as performance, levels of mortality and morbidity,

extent of intestinal gross and microscopic lesions, and amount of oocyst shedding (Johnson and Reid, 1970; Idris et al., 1997). Although successful immuno-protection by EM1 against a coccidiosis challenge has been reported in several studies, no study has reported effects of the in ovo administration of EM1 at 18.5 or 19.0 doi on the development of pathogenicity in broilers and the subsequent effects on their grow-out performance. It is unclear whether the difference in embryonic age at the time of injection has any effect on the efficacy of EM1 against a coccidiosis infection.

Therefore, the objective in this study was to determine effects of the EM1 vaccine administered to Ross × Ross 708 broiler chicken embryos at 18.5 or 19.0 d of age on subsequent chick quality, posthatch performance, coccidiosis pathogenicity, and pattern of oocyst cycling. An additional objective was to determine whether or not effects of EM1 vaccination are influenced by physiological differences in embryos at 18.5 and 19.0 d of embryo age (**EDOA**).

Materials and Methods

General

All experimental procedures involving animals were approved by the Mississippi State University Institutional Animal Care and Use Committee. A total of 2,880 Ross × Ross 708 broiler hatching eggs obtained from a single commercial breeder flock at 45 wk of age, were held for approximately 2 d under standard storage conditions before setting. Prior to set on 0 doi, eggs were individually weighed, and only eggs that had normal appearance (Zhai et al., 2011b; Bello et al., 2014) and that were within ± 10 % of the mean weight of all set eggs, were randomly assigned to each of 10 incubator tray levels, with each tray level representing a replicate unit in a Jamesway model PS 500 single

stage incubator (Jamesway Incubator Co. Inc., Cambridge, Ontario, Canada). At 0 doi, a total of 2,400 eggs were incubated under standard conditions (Peebles and Brake, 1987; Zhai et al., 2011a). In order to achieve 18.5 and 19.0 EDOA, eggs were set approximately 12 h apart. A 3 × 2 factorial arrangement of treatments was utilized in this study. This arrangement was comprised of 3 injection-types (**IT**) and 2 EDOA (18.5 and 19.0). There were a total of 3 treatment groups as follows: treatment 1 - noninjected control (**NIC**) on 18.5 and 19.0 EDOA; treatment 2 - diluent injected control (**DIC**; 50 µL of diluent injected) on 18.5 and 19.0 EDOA; and treatment 3 - EM1 in 50 µL of diluent (**VI**) on 18.5 and 19.0 EDOA. A total of 1,200 eggs were randomly assigned to each of the 3 treatment groups on each of 10 replicate tray levels at each of the 18.5 and 19.0 EDOA (2,400 eggs total). Each of the 3 treatment groups contained 80 eggs that were represented on each of the 10 replicate setter tray levels. Eggs were candled on 18 doi, and any egg containing dead embryo or unfertilized eggs were removed (Ernst et al., 2004). Overall, approximately 2,100 embryonated eggs were retained after candling, and were randomly arranged in all of the 30 treatment-replicate groups for each of 18.5 and 19.0 EDOA (60 total IT × EDOA treatment-replicate groups), with each containing approximately 35 embryonated eggs. Incubator dry and wet bulb temperatures were set at 37.5 ± 0.1 and 28.9 ± 0.1°C, respectively, and monitored twice daily for the entire incubation period.

Vaccination and experimental layout

In ovo injections were given when the embryos attained 18.5 or 19.0 d of age. An Embrex Inovoject injector system (Zoetis Animal Health, Research Triangle Park, NC) was used to deliver the injections, as described by Sokale et al. (2015a). Eggs were

injected through the air cell with a blunt tip injector needle [18.4 cm length and 1.27 mm bore width] to target the amnion. The needle provide approximately 2.49 cm injection depth from the top of the large end of the egg. The EM1 vaccine was reconstituted with sterile commercial Marek's Disease vaccine diluent (Merial Co., Duluth, GA) and administered at a volume of 50 µL per egg. On d of injection, 2 embryonated eggs from each flat were concurrently injected with coomassie brilliant blue G-250 (colloidal) dye for subsequent evaluation of site of injection (**SOI)** and embryo stage score **(ES),** as described by Sokale et al., (2015a). During the injection process, eggs were injected based on IT treatment group, starting with the NIC group, then the DIC and VI groups, in that order. The IT treatment were administered in this particular order to ensure that there was no cross-contamination between treatment groups. Although eggs in the NIC group were not injected with EM1, these eggs were subjected to the same injection process as were the injected treatment groups. Eggs were transferred to the hatcher unit (Jamesway Incubator Company Inc., Cambridge, Ontario, Canada) following the injection process. All eggs remained outside the incubator at room temperature for a maximum of 5 min, during the injection and transfer processes. The hatcher baskets (containing the injected embryonated eggs) were arranged in a manner that prevented cross-contamination between the injected and non-injected hatched chicks.

Data collection

Chick quality data

Individual set egg weights (**SEW**) were recorded on 0 doi. On d of injection (18.5 and 19.0 EDOA), SOI and ES were determined. On d of hatch (21.0 doi), the hatchability of injected embryonated eggs **(HI**) and hatching chick BW (**HBW**) were determined.

Approximately 30 chicks from each of the 60 treatment-replicate groups were used to determine mean HBW. In addition, 2 chicks from each of those treatment-replicate groups were wing-banded, euthanized, weighed, and necropsied for determination of chick BW (**BW**), yolk sac weight (**YSW**), yolk-free BW (**YFBW**), and intestine weight (**IW**). The following hatching chick quality parameters were also subsequently determined: yolk-sac weight relative to BW (**RYBW**), IW relative to BW (**RIBW**), IW relative to YFBW (**RIYFW**), and yolk free body mass (**YFBM;** which is a proportion of YFBW to BW).

Performance data

On d of hatch, 17 straight-run chicks were randomly selected from each of the 60 treatment-replicate groups and were wing-banded, weighed, and placed in each of 60 corresponding miniature floor pens, measuring 0.91 m × 1.22 m, within a temperature- and light-controlled research facility. Chicks were placed in the pens using the same experimental design that was utilized for the arrangement of embryonated eggs in the hatcher unit. Birds were placed on fresh wood-shavings litter, and house temperature conditions were monitored and recorded twice daily throughout the entire grow-out period. The entire grow-out phase was conducted in a manner that ensured close conformity to commercial broiler production standards. All birds were provided ad-libitum feed and water. Feed was formulated according to that for standard Mississippi State University broiler basal diets (diets contained no in-feed anti-coccidial), and met or exceeded NRC (1994) recommendations through d 35 posthatch (**poh**). Birds were provided starter (crumbled), grower (pelletized), and finisher (pelletized) diets on d 0 to 14, 14 to 28, and 29 to 35 poh, respectively. Bird numbers, BW, and feed weights on a

pen basis were determined weekly from d 0 to 35 poh. Body weight gain, feed intake **(FI)**, and feed conversion ratio (**FCR**) were determined for the 0 to 7, 7 to 14, 14 to 21, 21 to 28, 28 to 35, and 0 to 35 d of age (**doa**) intervals. On d 14, 21, 28 and 35 poh, litter samples were collected from each individual pen for the determination of oocyst output per gram of litter (**OPGL**) by a method previously described by Sokale et al., (2015a).

Microscopic pathology data

On each of d 14, 21, 28, and 35 poh, 5 birds were randomly selected from the DIC-18.5 EDOA; and the VI-18.5 and 19.0 EDOA treatment groups, for histopathological examination. The selected birds were individually weighed and euthanized, and their intestinal tracts (duodenum, mid-intestine, and cecum) were collected and fixed in 10 % buffered neutral formalin solution. The formalin-fixed intestine tissues were routinely processed and examined by a histopathology support method used in poultry production, as described by Wilson et al. (2015) and Menconi et al. (2015). The pathological evaluation conducted on the intestinal samples include coccidia counts, coccidial lesions, villus height and crypts measurements, presence of inflammatory cells, the presence of bacteria and other protozoa, and signs of inflammatory reactions in the intestinal mucosa. In general, the evaluations obtained were categorized into coccidia lesion mean scores, and mean total gut lesion scores (inflammation, repair, and coccidia lesions). Based on the extent of the lesions, the intestinal samples were assigned one of the following observational scores: 1: within normal limits; 2: mild lesion; 3: moderate lesion; and 4: marked to severe lesion. Similarly, based on the number of *E. acervulina* present in the entire gut, the samples were assigned one of the following quantitative scores: score 1: no coccidia; score 2: 1 to

2 clusters; score 3: 3 to 5 clusters; score 4: more than 5 clusters. Further, based on the number of *E. maxima* and *E. tenella* present in the entire gut, samples were assigned one of the following scores: score 1: 0 to 2 coccidia; score 2: 3 to 30 coccidia; score 3: 31 to 100 coccidia; score 4: more than 100 coccidia.

Statistical description

A randomized complete block design was utilized in both the incubation and grow-out phases of the study. IT, EDOA, and their interaction were viewed as fixed effects and block as a random effect. A one-way ANOVA was used to analyze the following parameters: HI, HBW, YFBW, YSW, RYBW, YFBM, IW, RIBW, and RIYBW on d of hatch; and BWG, FI, and FCR at separate weeks. A one-way ANOVA was also used to test for EDOA related differences for the SOI and ES. A split-plot analysis was used to test for the main effects of IT and EDOA, and the interactive effects of IT and EDOA on weekly BW from d 0 to 35 poh. All parameters were analyzed using the MIXED procedure of SAS software 9.3 (SAS institute, 2012). The microscopic lesion scores (**MLS**) were analyzed by the Kruskal-Wallis test for non-parametric data. The main effects of treatment (DIC- and VI-18.5 EDOA, and VI-19.0 EDOA) and poh (d 14, 21, 28 and 35) on coccidial and total gut lesion mean scores, were evaluated. Least-square means were compared in the event of significant global effects (Steel and Torrie, 1980). Global and least-square means differences were considered significant at $P \leq 0.05$.

Results

Hatchability and chick quality

Mean SEW was 64.5 g (Pooled SEM = 0. 34 g). There were no main or interactive effects involving IT or EDOA on HI at d of hatch (Table 6.1). There were significant main effects due to EDOA on HBW ($P = 0.002$), YSW ($P = 0.001$), RYBW ($P = 0.001$), and IW ($P < 0.001$) at d of hatch (Table 6.1). The HBW, YSW, and RYBW of birds in the 18.5 EDOA group were higher compared with those of birds in the 19.0 EDOA group. However, the IW of birds in the 19.0 EDOA group was higher compared with that of birds in the 18.5 EDOA group. There were significant main effects of IT ($P = 0.045$), and EDOA ($P < 0.001$) on RIBW (Table 6.1). The RIBW of birds in the DIC group was higher compared with the NIC group, with the VI group being intermediate. In addition, the RIBW of birds in the 19.0 EDOA group was higher compared to birds in the 18.5 EDOA group. There was a significant IT × EDOA interaction effect on RIYFW ($P = 0.05$; Table 6.1). In the 18.5 EDOA group, the RIYFW of birds in the VI group was higher compared with that in the NIC group, with the DIC group being intermediate. Whereas, in the 19.0 EDOA group, the RIYFW of birds in the DIC group was higher compared with that in the NIC and VI groups. There was no main effect due to IT or an IT × EDOA interactive effect on YFBW or YFBM. However, there was a significant ($P = 0.001$) main effect due to EDOA on YFBM (Table 6.1). The YFBM of birds in the 19.0 EDOA group was higher compared with that of birds in the 18.5 EDOA group. For reference, the means for all of the hatching chick quality variables evaluated are presented in Table 6.1.

Embryo Stage Score (ES) and Site of Injection (SOI)

A proportion of embryonated eggs in this study were injected in real time with coomassie brilliant blue dye along with all the eggs in the various IT groups. The dye-injected eggs were evaluated for ES and SOI in other to estimate the ES and SOI of all the treatment eggs in this study. Mean ES in the 18.5 EDOA group was significantly (P = 0.01) different compared with the mean ES of the 19.0 EDOA group. Mean ES on 18.5 and 19.0 EDOA were 2.44 and 3.24, respectively. Furthermore, the SOI result showed that there was a significant difference in the dye deposited in the amnion (**AM;** P = 0.03), subcutaneous (**s.c;** P = 0.01), and intramuscular (**i.m;** P = 0.02) regions in embryos. Dye deposition in the AM was significantly higher in the 18.5 EDOA group compared with the 19.0 EDOA group; whereas, dye deposition in the s.c and i.m were significantly higher in the 19.0 EDOA group compared to the18.5 EDOA group. Dye deposition in the AM in the 18.5 and 19.0 EDOA groups were 88.2 and 73.2 %, respectively. Dye deposition in the s.c in the 18.5 and 19.0 EDOA groups were 2.9 and 4.9 %, respectively, and dye deposition in the i.m in the 18.5 and 19.0 EDOA groups were 8.8 and 21.9 %, respectively.

Live Performance

There was an IT × EDOA interactive effect for mean BW of the birds at d 0 poh (P = 0.005), of FI (P = 0.036) and BWG (P = 0.014) in the d 14 to 21 interval; of FI (P = 0.051) in the d 28 to 35 interval; and of FI (P = 0.018) and FCR (P = 0.009) in the d 0 to 35 poh interval. In the 18.5 EDOA group, the BW of the birds at d 0 poh in the DIC and NIC groups were higher compared with those in the VI group. Whereas, in the 19.0 EDOA group, the BW of the birds at d 0 poh was not significantly different among the

treatment groups. In addition, BW at d 0 poh was higher in birds belonging to the 18.5 EDOA group in comparison to those in the 19.0 EDOA group. However, there was no main effect due to IT on BW at d 0 poh. The BWG and FI of birds in the 19.0 EDOA group in the d 14 to 21 poh interval was higher in the NIC and DIC groups in comparison to those in the VI group. Whereas, there was no significant treatment effect on d 14 to 21 poh BWG and FI in the 18.5 EDOA group. There was a significant main effect due to EDOA on the BW of birds at d 7 ($P < 0.001$), 14 ($P < 0.001$), 21 ($P < 0.001$), and 35 ($P = 0.004$) poh; and 0 to 7 ($P = 0.003$), 7 to 14 ($P = 0.009$), 14 to 21 ($P < 0.001$), 28 to 35 ($P = 0.015$), and 0 to 35 ($P = 0.001$) d poh FI. There was also a significant main effect due to EDOA on BWG ($P < 0.001$) and FCR ($P < 0.001$) in the d 0 to 7 interval; BWG in the d 7 to 14 ($P = 0.023$) and d 14 to 21 ($P = 0.002$) interval; and BWG ($P = 0.003$) and FCR ($P = 0.048$) in the 0 to 35 d poh interval. The BW and BWG at these time periods were higher in birds belonging to the 19.0 EDOA group compared to birds belonging to the 18.5 EDOA group. There was a significant main effect due to IT on BW at d 28 poh ($P = 0.032$); on BWG in the 14 to 21 d interval ($P = 0.040$); on FI in the d 21 to 28 interval ($P = 0.002$); on BWG ($P = 0.052$) and FI ($P = 0.045$) in the d 28 to 35 interval; and on FI ($P = 0.03$) in the d 0 to 35 poh interval. For all poh d intervals, the BW, BWG and FI of birds in the VI group were lower in comparison to those in the control group. For reference, the means for all the performance parameters in each of the treatment groups are presented in Tables 6.2 and 6.3.

Oocysts counts and microscopic lesion score

Coccidial oocyst shedding from litter samples collected on week 2, 3, 4, and 5 of this study was maximal at d 21 poh. There was a significant ($P < 0.05$) treatment effect

on total coccidia counts and on mean total gut lesion scores on d 14, 21, 28 and 35 poh. The total coccidia counts and mean total gut lesion scores of birds in the 19.0 EDOA-VI treatment group was higher compared to birds in the 18.5 EDOA-DIC treatment group. However, those for the 19.0 EDOA-VI were not significantly different from those of birds in the 18.5 EDOA-VI treatment group. Furthermore, there were no significant differences among the treatment groups for coccidia counts and mean total gut lesion scores at d 14, 21, 28 and 35 poh. Total coccidia count and mean total gut lesion scores of the birds were highest at d 28 poh. For reference, coccidia lesion mean scores and mean total gut lesion scores in each of the treatment groups are presented in Figs. 6.1 and 6.3, respectively. The mean coccidia lesion scores and total mean gut lesion scores in each of the treatment group on each day are presented in Figs. 6.2 and 6.4, respectively. The OPGL for birds belonging to the VI treatment group on d 0, 14, 21, 28, and 35 poh are presented in Fig. 6.5.

Discussion

In ovo injection of broiler chickens with live oocysts vaccine is an accepted method of coccidiosis disease control. Several methods of administering live oocyst vaccines to bird have been developed, since the first anticoccidial vaccine (Coccivac®) was made available in 1952 (Williams, 2002). The in ovo injection of broiler hatching eggs has now become widely accepted worldwide in the commercial poultry industry (Williams, 2007). Earlier developed methods of vaccine administration have included feed or drinking water application (Williams, 1994), eye-spray application to 1-day-old chicks (Chapman, et al., 2002), and gel application (Danforth et al., 1998). Weber and Evans (2003) have demonstrated the possibility of immunizing broiler chickens via in

ovo injection for the different life cycle stages of *Eimeria tenella*. The live non-attenuated EM1 vaccine is widely used among commercial broiler chicken producers for the vaccination of healthy broilers between 18 and 19 doi, for the prevention of coccidiosis caused by *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella*. The effects of EM1 administered on d 18 (Weber and Evans, 2003; Weber et al., 2004) and d 19 (Mathis et al., 2014) of incubation on subsequent poh live performance have been studied. However, effects of 18.5 and 19.0 EDOA EM1 injections on embryogenesis, and on vaccination efficacy and poh live performance have not been previously studied. As in previous studies, the in ovo injection of EM1 vaccine administered to Ross × Ross 708 broiler hatching eggs did not adversely affect embryogenesis. Upon examination of the hatching chick quality parameters evaluated in this study, it is apparent that there were developmental differences in the embryos from the Ross × Ross 708 broiler hatching eggs at 18.5 and 19.0 EDOA. This observation is based on differences in the HBW, YSW, RYBW, IW, RIBW, RIYFW, and YFBM of the 18.5 and 19.0 EDOA embryos. The HBW, YSW, and RYBW parameters were greater in birds belonging to the 18.5 EDOA group in comparison to the 19.0 EDOA group. However, the IW, RIBW, RIYFW and YFBM of birds belonging to 19.0 EDOA group were greater in comparison to those belonging to the 18.5 EDOA group. The lack of IT and IT × EDOA effects on HBW, YSW, RYBW, IW, and YFBM, suggests that the observed effects were due entirely to EDOA. Although HBW, YSW, and RYBW were significantly higher in birds belonging to the 18.5 EDOA group in comparison to the 19.0 EDOA group, there were no treatment-related differences in YFBW. The YFBW of birds belonging to 18.5 EDOA group was not different from those belonging to the 19.0 EDOA group. This suggests that

the increase in HBW was associated with an increase in YSW. The greater amount of residual yolk sac was in turn due to a higher retention of moisture in the yolk sac and body tissues of birds in the 18.5 EDOA group. This effect is similar to previous studies, in which it was reported that there was an increase in total hatching chick BW following the in ovo injection of saline or carbohydrates (Zhai et al., 2011a,b), digestible carbohydrates (Uni et al., 2005), or 25 (OH) D_3 (Bello et al., 2013). Yolk sac-free BW is a more accurate indicator of actual embryo growth, because it eliminates the additional effects of changes in yolk sac weight in response to yolk sac absorption by the hatching chick (Zhai et al., 2011a, b). Further, differences in the hatching chick quality parameters of 18.5 and 19.0 EDOA may have resulted from differences in the various incubational factors that optimize the incubation process. Previous studies have described various incubational factors that influence broiler embryonic physiology and subsequent poh growth characteristics. Such factors include temperature and relative humidity (Molenaar et al., 2011a; Pulikanti et al., 2012), egg composition and subsequent yolk sac nutrient utilization (Murakami et al., 1992; Moran, 2007), egg weight loss (Peebles et al., 2005; Pulikanti et al., 2012), and incubation length (van de Ven et al., 2011). The embryonic developmental process of chicken requires a balance between these multiple factors in order to achieve optimum hatchability and chick quality. The 12 h incubational difference between 18.5 and 19.0 EDOA may have resulted in the chick quality differences observed at d of hatch as well as the subsequent treatment-related differences observed in poh live performances. ES and SOI results in this study further indicate that there are developmental differences between the 18.5 and 19.0 EDOA embryos. An ES of 3.24 in the 19.0 EDOA group compared with that of a 2.44 in the 18.5 EDOA group, indicate

that at the time of in ovo injection, the embryos belonging to the 19.0 EDOA group were more advanced in development than those in the 18.5 EDOA group. In addition, the SOI results indicate that degree of embryonic development affected the percentage of AM injections. Dye deposition occurred more often in the AM of embryos belonging to the 18.5 EDOA group in comparison to those belonging to the 19.0 EDOA group. Further, YFBM was greater in birds belonging to 19.0 EDOA group compared to those belonging to the 18.5 EDOA group. This may be due to a higher internal temperature, an increased water loss, lower moisture concentration, and more rapid embryonic metabolism (Zhai et al., 2011b; Pulikanti et al., 2012) in the eggs belonging to the 19.0 EDOA group. Yolk free body mass (YFBW divided by total BW), which is a measure of percentage of yolk-free BW, has been positively correlated with the birds' subsequent poh performance. A higher YFBM is indicative of a more advanced stage of embryonic development (Molenaar, 2011).

Poultry producers utilize performance (BWG and FCR) data as criteria for evaluating the effects of coccidiosis on a broiler flock. In a previous study, in which the EM1 vaccine was administered at 18.5 doi, it was shown that EM1 did not affect mean BW at 0 d poh (Sokale et al., 2015b). This finding was different from results obtained in this current study. In the current study, the 0 d poh BW of the birds were decreased when they were injected at18.5 doi, with EM1 vaccine (Table 6.2). The contrasting results of the two studies may be attributed to differences in the incubation processes used in each. In the current study, embryos of two different EDOA were incubated together, whereas, embryos of like EDOA were incubated together in the contrasting study. Therefore, the total heat production of the eggs at a particular doi (that was based on the 18.5 EDOA

eggs) was greater in the study examining the two different EDOA. This difference in the two studies may have caused the EM1 vaccine to have different effects. An EDOA effect was also observed on BW at d 7, 14, 21, and 35 poh, and on BWG and FI at all the poh age intervals (except for the d 28 to 35 poh d interval), and for FCR in the d 0 to 7 and d 0 to 35 poh age intervals. These effects of EDOA on poh performance suggest that chick quality differences between the 18.5 and 19.0 EDOA groups are extended up to d 35 poh. This finding is consistent with a previous study conducted by Pulikanti et al. (2013), in which embryo temperature during incubation influenced the BW and relative organ weights of broilers, up to d 48 poh. The effect of IT on BW and BWG were observed at d 28 poh and in the d 28 to 35 poh age interval, respectively. It is possible that equilibrium for poh performance in the 18.5 and 19.0 EDOA groups was reached in the d 28 and 35 poh age interval, and may be due to the fact that only IT-related effects on BW, BWG, and FI were observed by d 28 poh.

The BW at d 28 poh, and the BWG and FI that were observed in the 28 to 35 d poh age interval were higher in birds belonging to the control groups compared with those in the EM1 group. Furthermore, litter oocysts counts, coccidia lesion scores, and total gut lesion scores in the EM1 injected birds were highest at d 21 and 28 poh. These findings are consistent with findings in the report by Mathis et al. (2014), in which the recovery of coccidial oocysts from litter was highest from birds in the EM1-vaccinated group at 21 d poh, with oocyst shedding continuing up to d 35 poh. In addition, similar to an earlier finding, the peak of oocyst shedding at d 21 poh and coccidial cycling at d 28 poh may have resulted in a lower BW, BWG, and FI that was observed in the d 28 to 35 poh interval. Previous studies have shown that a depression in performance may occur

during peak oocyst shedding, with a compensatory improvement in performance occurring later during grow-out (Mathis, 1999; Williams and Gobbi, 2002; Broomhead, 2012; Mathis, 2014). Mathis (1999) stated that birds exhibiting peak intestinal coccidial lesions at d 21 poh, still have adequate time for compensatory weight gain. However, birds exhibiting intestinal lesions at d 35 poh are not afforded the adequate time for compensatory weight gain. In this current study, peak intestinal lesion occurred at d 28 poh. However, by d 35 poh, there was no difference in the BW of birds among the treatment groups, indicating that compensatory weight gain had occurred within the flock. The delay in peak coccidial cycling can be attributed to a lack of early moisture build up in the new litter that was utilized in this study. It has been shown that a minimum litter moisture content of approximately 25 % is needed for maximum coccidial cycling (Gingerich, 2012).

In conclusion, chick quality was optimized in chicks that had an additional 12 h incubational time advantage (19.0 EDOA group). Noted improvements in the quality characteristics of the chicks were likewise observed in their poh grow-out performance parameters up to d 35 poh. In addition, the performance (BW, BWG, and FI) of the EM1-vaccinated group was reduced during peak coccidia oocyst cycling and shedding. Peak oocyst shedding occurred at d 21 poh, with coccidia cycling occurring up to d 35 poh. This resulted in a reduction in performance in the d 28 to 35 poh age interval. The results obtained in this study also indicated that improvements in embryonic development during incubation are able to likewise influence grow-out live performance. However, differences in chick quality parameters that are associated with differences in embryo age at the time of in ovo injection do not influence EM1 vaccine efficacy.

Table 6.1 Hatching chick quality parameter means in the embryonic day of age (EDOA) 18.5 and 19.0, with injection-type (IT) of noninjected and diluent-control groups, and vaccine-injected group.[1, 2]

	Treatments	EDOA	HI[3] (%)	HBW[3] (g)	BW[2] (g)	YSW[2] (g)	YFBW[2] (g)	RYBW[2] (%)	IW[2] (g)	RIBW[2] (%)	RIYFW[2] (%)	YFBM[2] (%)
	Noninjected control	18.5	86.99	47.51	48.54	7.44	41.10	15.26	1.80	3.71	4.37^{c}	84.74
		19.0	87.14	46.42	46.95	5.81	41.14	12.27	2.08	4.44	5.05^{b}	87.73
	Diluent-injected control	18.5	84.80	47.02	46.49	6.88	39.61	14.81	1.87	4.02	4.72^{bc}	85.19
		19.0	88.93	46.38	46.13	6.10	40.03	13.13	2.20	4.77	5.49^{a}	86.87
	Vaccine-injected	18.5	83.71	47.09	46.54	6.93	39.60	14.89	1.90	4.09	4.81^{b}	85.11
		19.0	84.83	46.35	46.61	5.89	40.73	12.49	2.02	4.37	4.99^{b}	87.51
	SEM		1.809	0.310	0.65	0.40	0.56	0.78	0.05	0.12	0.13	0.78
IT	Non-injected control		87.06	46.96	47.74	6.62	41.12	13.77	1.94	4.07^{b}	4.71^{b}	86.23
	Diluent-injected control		86.86	46.70	46.31	6.49	39.82	13.97	2.04	4.39^{a}	5.10^{a}	86.03
	Vaccine Injected		84.27	46.72	46.57	6.41	40.17	13.69	1.96	4.23^{ab}	4.90^{ab}	86.31
	SEM		1.35	0.22	0.46	0.28	0.39	0.55	0.03	0.09	0.09	0.55
EDOA	18.5		85.17	47.21^{a}	47.19	7.08^{a}	40.10	14.99^{a}	1.85^{b}	3.94^{b}	4.63^{b}	85.01^{b}
	19		86.97	46.38^{b}	46.57	5.93^{b}	40.63	12.63^{b}	2.10^{a}	4.53^{a}	5.18^{a}	87.37^{a}
	SEM		1.15	0.18	0.37	0.23	0.32	0.45	0.03	0.07	0.07	0.45
P-values	IT		0.200	0.649	0.074	0.862	0.064	0.937	0.123	0.045	0.013	0.937
	EDOA		0.203	0.002	0.247	0.001	0.250	0.001	<0.001	<0.001	<0.001	0.001
	IT× EDOA		0.482	0.744	0.422	0.553	0.622	0.704	0.099	0.110	0.050	0.704

[a-b] Means within a variable with no common superscript differ ($P \leq 0.05$).

[1] Hatchability of injected eggs (HI), Hatchling BW (HBW), Chick BW (BW), yolk sac weight (YSW), yolk-free BW (YFBW), yolk sac weight as a percentage of BW (RYBW), intestine weight (IW), intestine weight as a percentage of BW (RIBW), intestine weight as a percentage of yolk-free BW (RIYFW), yolk-free body mass (YFBM).

[2] 4 birds in each of 10 replicate units per treatment (40 birds per treatment group) were used to calculate each treatment mean.

3 Approximately 60 eggs in each of 10 replicate units per treatment group were used to calculate each treatment mean.

Table 6.2 Performance parameter means in the embryonic day of age (EDOA) 18.5 and 19.0, with injection-type (IT) of noninjected and diluent-control groups, and vaccine-injected group.[1, 2]

Treatments	EDOA	BW1 (kg)	BWG1 (kg)	FI1 (kg)	FCR1	BW2 (kg)	BWG2 (kg)	FI2 (kg)	FCR2	BW3 (kg)	BWG3 (kg)	FI3 (kg)	FCR3
Noninjected control	18.5	0.048[a]	0.124	0.170	1.38	0.173	0.284	0.384	1.35	0.459	0.468[b]	0.619[b]	1.32
	19.0	0.046[c]	0.135	0.157	1.16	0.185	0.290	0.392	1.35	0.480	0.513[a]	0.662[a]	1.29
Diluent-injected control	18.5	0.048[a]	0.123	0.164	1.35	0.176	0.276	0.377	1.36	0.458	0.470[b]	0.612[b]	1.31
	19.0	0.046[c]	0.140	0.161	1.17	0.187	0.295	0.400	1.36	0.485	0.499[a]	0.665[a]	1.33
Vaccine-injected	18.5	0.047[b]	0.125	0.165	1.32	0.177	0.275	0.379	1.38	0.463	0.472[b]	0.617[b]	1.31
	19.0	0.047[bc]	0.138	0.157	1.15	0.188	0.285	0.396	1.40	0.478	0.472[b]	0.628[b]	1.33
	SEM	0.0002	0.0034	0.0031	0.0339	0.0031	0.0061	0.0072	0.0214	0.0057	0.0074	0.0093	0.0144
IT	Noninjected control	0.047	0.129	0.164	1.27	0.179	0.287	0.388	1.35	0.469	0.491[a]	0.641	1.31
	Diluent-injected control	0.047	0.131	0.163	1.26	0.181	0.286	0.389	1.36	0.472	0.484[ab]	0.639	1.32
	Vaccine-injected	0.047	0.132	0.161	1.24	0.183	0.280	0.388	1.39	0.471	0.472[b]	0.623	1.32
	SEM	0.0002	0.0024	0.0022	0.0239	0.0022	0.0045	0.0051	0.0159	0.0041	0.0052	0.0073	0.0102
EDOA	18.5	0.048[a]	0.124[b]	0.166[a]	1.35[a]	0.175[b]	0.279[b]	0.380[b]	1.36	0.460[b]	0.470[b]	0.616[b]	1.31
	19	0.046[b]	0.138[a]	0.158[b]	1.16[b]	0.187[a]	0.290[a]	0.392[a]	1.37	0.481[a]	0.495[a]	0.652[a]	1.32
	SEM	0.0002	0.0019	0.0018	0.02	0.0018	0.0037	0.0042	0.0135	0.0033	0.0043	0.0065	0.0083
P-values	IT	0.4924	0.7435	0.7215	0.6024	0.4810	0.4851	0.9904	0.1666	0.9314	0.0395	0.0660	0.7374
	EDOA	<0.0001	<0.0001	0.0028	<0.0001	<0.0001	0.0232	0.0091	0.8109	<0.0001	0.0002	<0.0001	0.6121
	IT × EDOA	0.0051	0.6640	0.2831	0.8468	0.9808	0.5078	0.5803	0.8614	0.6122	0.0135	0.0364	0.0947

[a-b]Means within a column with no common superscript differ ($P \leq 0.05$).

[1]34 birds in each of 10 replicate units used to calculate each treatment mean.

[2]BW1 = d 0 BW; BWG1 = d 0 to 7 BW gain; FI1 = d 0 to 7 feed intake; FCR1 = d 0 to 7 feed conversion ratio; BW2 = d 7 BW; BWG2 = d 7 to 14 BW gain; FI2 = d 7 to 14 feed intake; FCR2 = d 7 to 14 feed conversion ratio; BW3 = d 14 BW; BWG3 = d 14 to 21 BW gain; FI3 = d 14 to 21 feed intake; FCR3 = d 14 to 21 feed conversion ratio.

Table 6.3 Performance parameter means in the embryonic day of age (EDOA) 18.5 and 19.0, with injection-types (IT) of noninjected and diluent-control groups, and the vaccine-injected group.[1, 2]

Treatments	EDOA	BW4 (kg)	BWG4 (kg)	FI4 (kg)	FCR4	BW5 (kg)	BWG5 (kg)	FI5 (kg)	FCR5	BW6 (kg)	BWG6 (kg)	FI6 (kg)	FCR6
Noninjected control	18.5	0.946	0.434	0.642	1.53	2.154	0.411	1.239^{ab}	2.83	2.242	2.195	2.915^{b}	1.33^{bc}
	19.0	0.995	0.464	0.680	1.54	1.974	0.457	1.319^{a}	2.84	2.303	2.259	3.101^{a}	1.38^{ab}
Diluent-injected control	18.5	0.946	0.396	0.610	1.60	2.091	0.398	1.012^{c}	2.52	2.213	2.164	2.819^{b}	1.30^{c}
	19.0	0.988	0.474	0.649	1.38	2.019	0.461	1.380^{a}	2.93	2.295	2.249	3.108^{a}	1.38^{a}
Vaccine-injected	18.5	0.953	0.403	0.589	1.51	1.534	0.356	1.080^{bc}	2.76	2.179	2.131	2.873^{b}	1.35^{ab}
	19.0	0.973	0.412	0.573	1.45	1.571	0.384	1.094^{bc}	2.85	2.261	2.214	2.914^{b}	1.32^{c}
	SEM	0.0103	0.0261	0.0207	0.1039	0.2157	0.0298	0.0802	0.196	0.0303	0.0299	0.042	0.0184
IT	Noninjected control	0.971	0.449	0.661^{a}	1.53	2.064^{a}	0.434^{a}	1.279^{a}	2.83	2.273	2.227	3.008^{a}	1.35
	Diluent-injected control	0.967	0.435	0.630^{a}	1.49	2.055^{a}	0.430^{a}	1.196^{ab}	2.73	2.254	2.207	2.964^{ab}	1.34
	Vaccine Injected	0.963	0.408	0.581^{b}	1.48	1.552^{b}	0.370^{b}	1.087^{b}	2.96	2.220	2.173	2.894^{b}	1.33
	SEM	0.0073	0.0187	0.0147	0.0734	0.1534	0.0219	0.0603	0.1387	0.0216	0.0214	0.0297	0.0133
EDOA	18.5	0.949^{a}	0.411	0.614	1.54	1.927	0.388	1.110^{b}	2.81	2.211^{b}	2.163^{b}	2.869^{b}	1.33^{b}
	19	0.985^{b}	0.450	0.634	1.45	1.855	0.434	1.264^{a}	2.87	2.287^{a}	2.241^{a}	3.041^{a}	1.36^{a}
	SEM	0.006	0.0154	0.012	0.06	0.126	0.0186	0.0521	0.1132	0.0177	0.0176	0.0242	0.011
P-values	IT	0.7822	0.266	0.002	0.857	0.032	0.052	0.045	0.492	0.219	0.190	0.030	0.562
	EDOA	<0.0001	0.074	0.236	0.289	0.683	0.057	0.015	0.682	0.004	0.003	<0.001	0.048
	IT × EDOA	0.3446	0.3969	0.3249	0.5229	0.8798	0.827	0.051	0.272	0.925	0.927	0.018	0.009

[a-b]Means within a column with no common superscript differ ($P \leq 0.05$).

[1]34 birds in each of 10 replicate units used to calculate each treatment mean.

[2]BW4 = d 21 BW; BWG4 = d 21 to 28 BW gain; FI4 = d 21 to 28 feed intake; FCR4 = d 21 to 28 feed conversion ratio; BW5 = d 28 BW; BWG5 = d 28 to 35 BW gain; FI5 = d 28 to 35 feed intake; FCR5 = d 28 to 35 feed conversion ratio; BW6 = d 35 BW; BWG6 = d 0 to 35 BW gain; FI6 = d 0 to 35 feed intake; FCR6 = d 0 to 35 feed conversion ratio.

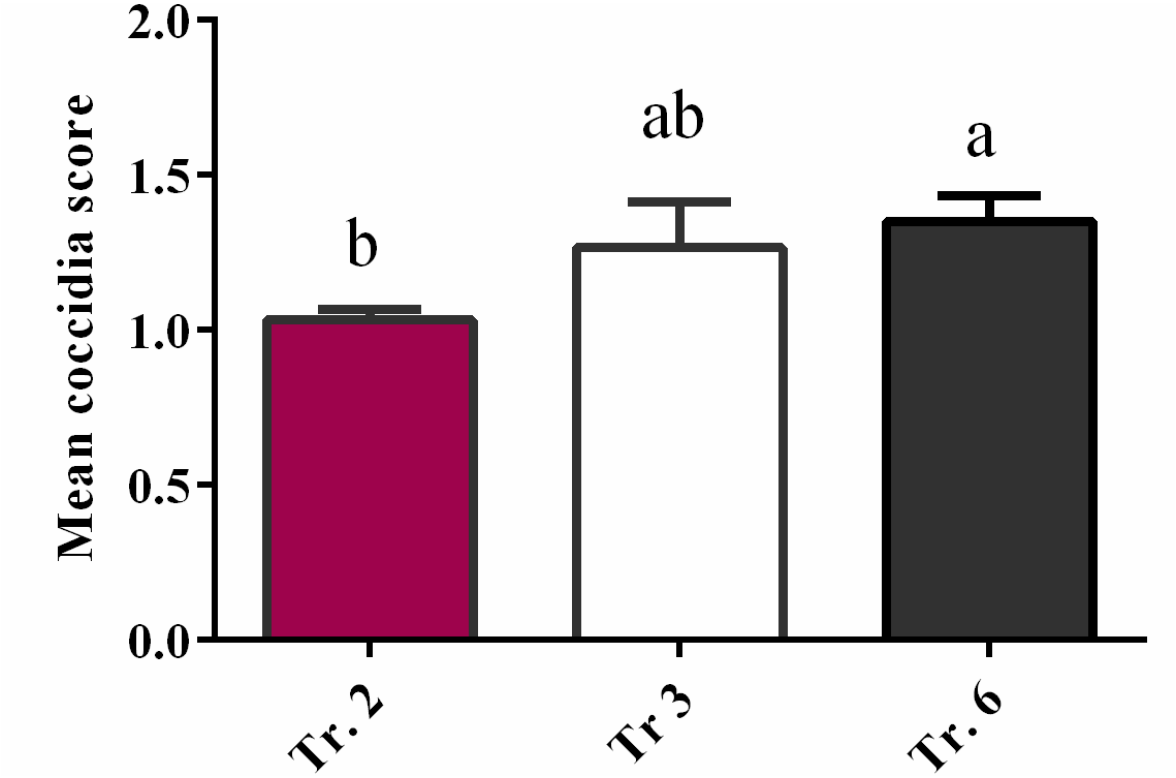

Figure 6.1 Mean coccidia score by treatment for *Eimeria acervulina*, *E. maxima*, and *E. tenella*.

Trt. 2 = Diluent-injected control group; Trt. 3 = EDOA 18.5 EM1 vaccine-injected group, and Trt.6 = EDOA 19.0 EM1 vaccine-injected group on d 14, 21, 28 and 35 posthatch[1].
[1]Data from 20 birds was used for calculation of means for each treatment group.
[a-b] Means within a column with no common superscript differ ($P = 0.007$).

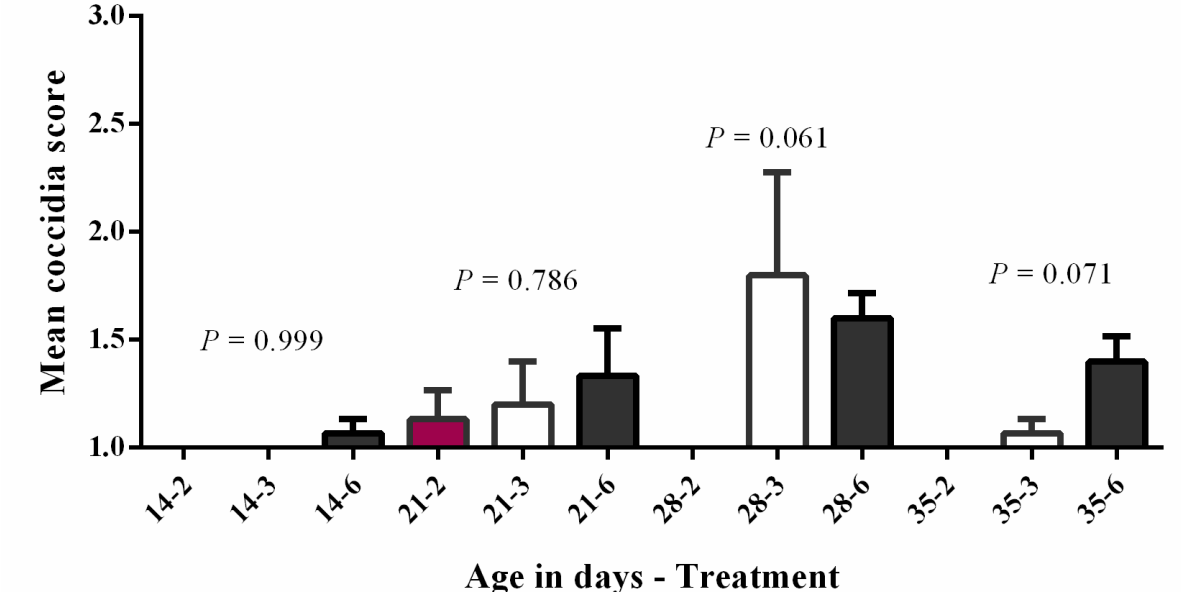

Figure 6.2 Mean coccidia score by treatment and day posthatch for *Eimeria acervulina*, *E. maxima*, and *E. tenella*

14-2 = day 14 diluent-injected control group; 14-3 = day 14 EDOA 18.5 EM1 vaccine-injected group; 14-6 = day 14 EDOA 19.0 EM1 vaccine-injected group; 21-2 = day 21 diluent-injected control group; 21-3 = day 21 EDOA 18.5 EM1 vaccine-injected group; 21-6 = day 21 EDOA 19.0 EM1 vaccine-injected group; 28-2 = day 28 diluent-injected control group; 28-3 = day 28 EDOA 18.5 EM1 vaccine-injected group; 28-6 = day 28 EDOA 19.0 EM1 vaccine-injected group; 35-2 = day 35 diluent-injected control group; 35-3 = day 35 EDOA 18.5 EM1 vaccine-injected group; 35-6 = day 35 EDOA 19.0 EM1 vaccine-injected group.[1,2]

[1]Data from 5 birds was used for calculation of means for each treatment group.

[2]No significant difference was observed among treatment groups within each day of age.

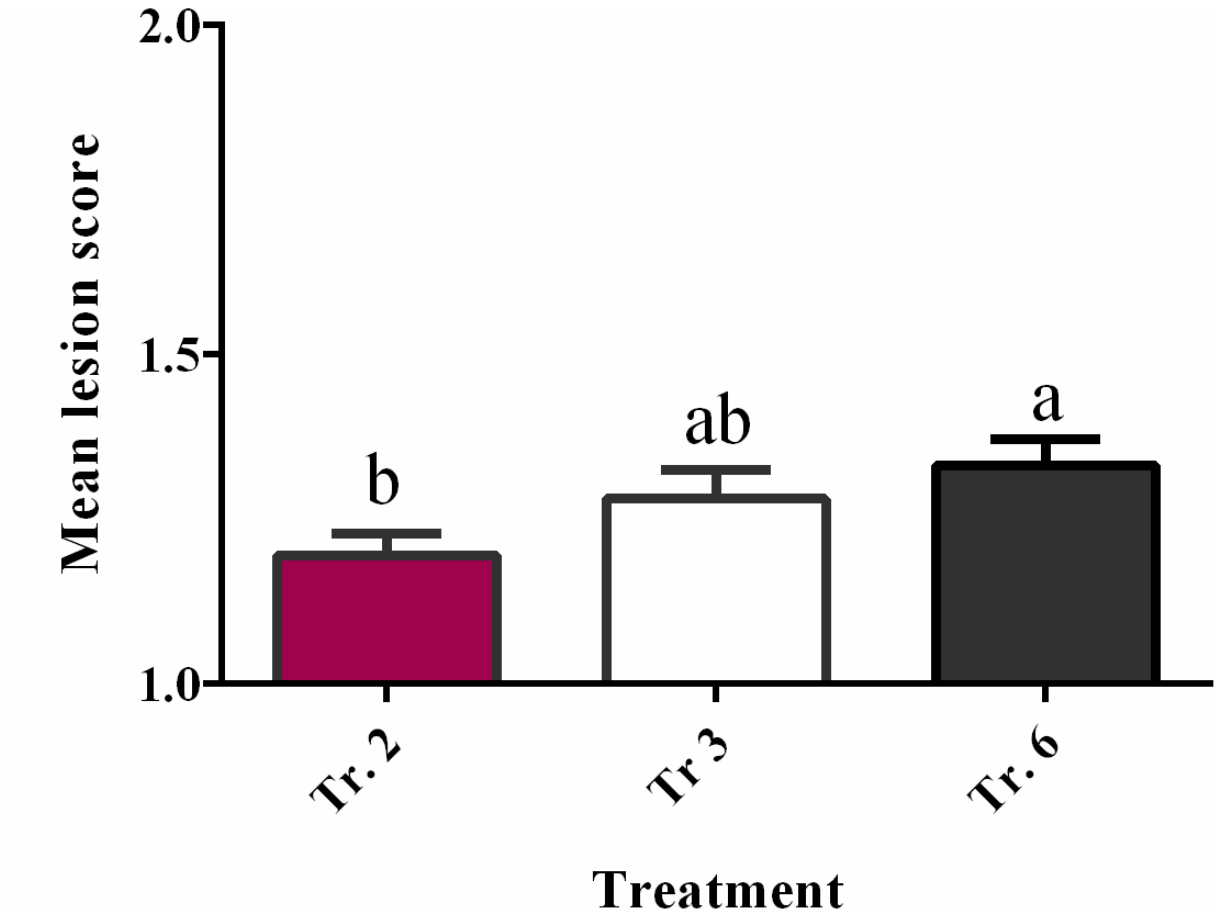

Figure 6.3 Mean total gut lesion score (inflammation, repair, and coccidia) by treatment for *Eimeria acervulina*, *E. maxima*, and *E. tenella*

Trt. 2 = Diluent-injected control group; Trt. 3 = EDOA 18.5 EM1 vaccine-injected group; Trt.6 = EDOA 19.0 EM1 vaccine-injected group on d 14, 21, 28 and 35 posthatch.[1]
[1]Data from 20 birds was used for calculation of means for each treatment group.
[a-b] Means within a column with no common superscript differ ($P = 0.015$).

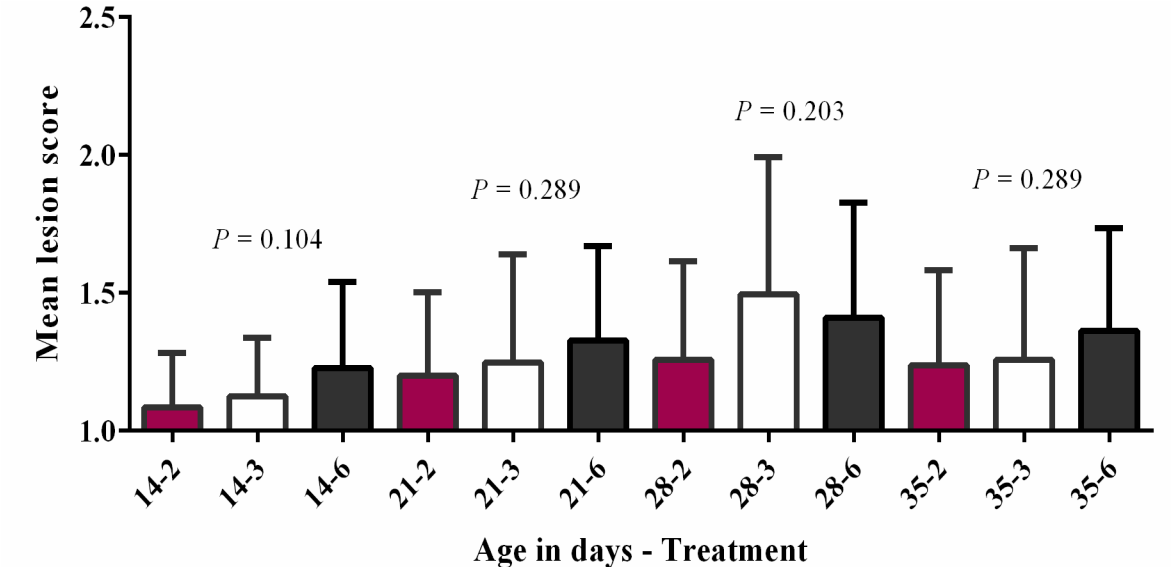

Figure 6.4 Mean total gut lesion score (inflammation, repair, and coccidia) by treatment and day posthatch for *Eimeria acervulina*, *E. maxima*, and *E. tenella*

14-2 = day 14 diluent-injected control group; 14-3 = day 14 EDOA 18.5 EM1 vaccine-injected group; 14-6 = day 14 EDOA 19.0 EM1 vaccine-injected group; 21-2 = day 21 diluent-injected control group; 21-3 = day 21 EDOA 18.5 EM1 vaccine-injected group; 21-6 = day 21 EDOA 19.0 EM1 vaccine-injected group; 28-2 = day 28 diluent-injected control group; 28-3 = day 28 EDOA 18.5 EM1 vaccine-injected group; 28-6 = day 28 EDOA 19.0 EM1 vaccine-injected group; 35-2 = day 35 diluent-injected control group; 35-3 = day 35 EDOA 18.5 EM1 vaccine-injected group; 35-6 = day 35 EDOA 19.0 EM1 vaccine-injected group.[1,2]

[1]Data from 5 birds was used for calculation of means for each treatment group.

[2]No significant difference was observed among treatment groups within each day of age.

Figure 6.5 Oocysts per gram of litter on Days 0, 14, 21, 28, and 35 posthatch, for birds that were vaccinated with the EM1 vaccine

Peak oocyst shedding in the litter was on d 21 posthatch.[1]
[1]Data from 10 replicate pens was used for calculation of means for each day.

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CHAPTER VII

CONCLUSIONS AND APPLICATIONS

Today, in ovo application of the Inovocox EM1 vaccine for the control of coccidiosis occurs routinely in many commercial broiler hatcheries in the USA. The overall purpose of in ovo vaccination is to vaccinate every viable broiler embryo safely and uniformly in order to allow them to achieve immunocompetence prior to potential coccidiosis field challenges. However, several factors can affect the development of an adequate immune response before a field challenge occurs. This current research reveals some of the physiological components of this process that when discerned, can help maximize the outcome and efficacy of the EM1 vaccine. The following are components of this process that are demonstrated in this study:

1. Accurate vaccine deposition into the amnion (AM) is influenced by the physiological stage of the embryo at the time of injection. In ovo vaccination of late-stage embryos (D18.5) with an embryo stage score of 2 to 3 resulted in accurate vaccine deposition in over 90 % of embryos. Site of injection (SOI) accuracy was higher in embryos injected on 18.5 doi, than in those injected on 19.0 doi. On the other hand, embryonic stage score (ES) was higher in embryos injected on 19.0 doi when compared to those injected on 18.5 doi. A higher ES indicates a more developed embryo. As the embryos develop towards the hatching stage, the accuracy of vaccine deposition decreases because of a reduction in the amount of AM within the embryonated egg. The preferred SOI for the EM1vaccine is the AM. Other SOI such as intramuscular (preferably at the right breast muscle area) and subcutaneous sites, are also accepted as viable injection sites.

2. The in ovo administration of EM1 vaccine at 18.5 or 19.0 doi is safe, with no detrimental effects on hatchability, chick quality parameters, and embryo survivability. In addition, EM1 administered up to 10 × the recommended dose showed no detrimental effects on embryonic and chick quality parameters.

3. The kinetics of oocyst shedding showed that fecal oocyst shedding began at day 4 posthatch (6 d post injection), and peaked at 7 d posthatch (10 d post injection), with a smaller peak at day 10 posthatch.

4. Coccidia oocyst output and cycling were highest between day 21 and 28 posthatch, which resulted in a decrease in BWG during this period in the EM1 vaccinated birds. However, a compensatory BWG occurred by day 35 posthatch.

5. Chicks that were produced from the embryos injected on 19.0 doi had better chick quality characteristics in comparison with those produced from embryos injected on 18.5 doi. This differential effect was discovered on day of hatch and subsequently affected grow-out performance up to 35 days posthatch. However, grow-out performance and the pattern of coccidia cycling were similar in embryos injected with the EM1 vaccine on either day 18.5 or 19.0 of incubation.

6. This study showed that turn-out times on day 7 or 10, in the presence of a low dose (1×) or high dose (10×) EM1 vaccine, did not negatively affect performance. Therefore, under ideal conditions, partial house brooding up to day10 posthatch at the recommended EM1 dose, will ensure adequate oocyst cycling without negatively affecting grow-out performance.