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Assessment of the Level of Protection against Coccidiosis in Broiler Breeders Conferred by a
Live Anticoccidial Vaccine, and its Influence on Early Growth and Development.

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Poultry Science

by

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ABSTRACT

Coccidiosis continues to be a great challenge to the poultry industry all over the world and in broiler breeders vaccination with live oocysts of *Eimeria* has been widely adopted as a control measure. Broiler breeders are usually subjected to feed restriction during their early growth however no studies have been undertaken on the effects of vaccination in birds whose feed has been restricted in this manner. The objective of this study was to assess the level of protection acquired by broiler breeder chickens when immunized at day-old with live eimerian oocysts and given a restricted diet. Effects upon growth and development were measured. In the first experiment, 220 vaccinated and unvaccinated broiler breeder chickens were raised in separate pens on new litter. At 4 weeks of age, and every week thereafter until 8 weeks of age, 40 of the vaccinated and unvaccinated birds were assigned to 4 treatment groups in cages and challenged with 100,000 oocysts of *Eimeria tenella*. The 4 groups were 1) vaccinated and challenged (VC), 2) unvaccinated and challenged (UC), 3) vaccinated and unchallenged (VU), and 4) unvaccinated and unchallenged (UU). The level of protection acquired was assessed by the presence of lesions in the ceca and dropping pan scores 6 days after challenge. In the second experiment one group of birds was infected orally with 500 oocysts each of *E. acervulina*, *E. maxima* and *E. tenella* at day old and a second group was kept as uninfected controls. Doses were intended to simulate those provided in commercial coccidiosis vaccines. Body weight, chest girth, shank length, and keel length were used as criteria to judge the effect of infection on growth and development. In the first experiment, lesions and dropping pan scores were significantly reduced in the VC ($P < 0.05$) birds compared to UC birds at 4, 5, 6, 7, and 8 weeks of age indicating that birds have developed sufficient immunity to protect them from the pathology caused by *E. tenella*. In the second experiment, infected birds showed significantly reduced ($P < 0.05$) body weights and chest girths compared to the control birds. This indicates that infection affects body growth and

development of these birds. Results were inconclusive with shank length and keel length measurements.

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION.....	1
References	3
CHAPTER 2: LITERATURE REVIEW	4
Poultry coccidiosis – An overview	4
Life cycle of a typical <i>Eimeria</i> spp.	5
Epidemiology of poultry coccidiosis	6
Pathogenesis and pathology	7
Diagnosis of avian coccidiosis	9
Immune response to <i>Eimeria</i> infection.....	10
Control of coccidiosis.....	11
Control by vaccination	13
Justification for the current study	15
References	17
CHAPTER 3: ASSESSMENT OF THE LEVEL OF PROTECTION AGAINST COCCIDIOSIS IN BROILER BREEDERS CONFERRED BY A LIVE ANTICOCCIDIAL VACCINE, AND ITS INFLUENCE ON EARLY GROWTH AND DEVELOPMENT.	26
Introduction.....	26
Materials and methods	27
Birds and husbandry:	27
Feeding:.....	27
Parasite for challenge study	28
Infective material	28
Criteria for assessing the level of protection against the challenge infection	28
Criteria for assessing the effect of vaccine on growth and development of birds	29
Experimental design:	29
Oocyst count	30
Statistical analysis	30
Results	30
Acquisition of protection against <i>Eimeria</i> challenge	30
Effect of vaccination on body growth and development	31

Discussion.....	31
Figures:	34
Tables:	44
References	46
CHAPTER 4: CONCLUSION.....	49
CHAPTER 5: APPENDICES	50
Appendix 1: Procedure for fumigation with ammonia.	50
Appendix 2: Feed restriction of birds.	51
Appendix 3: Procedure for preparation of oocyst inoculum for vaccination and challenge study.	52
Appendix 4: Procedure for propagation of oocysts for the study.	53
Appendix 5: Method of lesion scoring for <i>E. tenella</i>.	55
Appendix 6: Method of dropping pan scoring for <i>E. tenella</i>.	56
Appendix 7: Procedure for infecting individual birds.....	57
Appendix 8: Procedure for counting oocysts from the litter.	58

LIST OF FIGURES

- Figure 1.** Design for experiment 1. Birds were vaccinated at the hatchery with a commercial vaccine and raised in pens with new litter. The challenge study was done in cages to assess the level of protection when birds were challenged with 100,000 oocysts of *E. tenella*..... 34
- Figure 2.** Design for experiment 2. Birds were infected with 500 oocysts each of *E. acervulina*, *E. maxima* and *E. tenella* at day zero, and were raised on new litter until 12 weeks. Feed restriction was practiced. Birds were given a starter feed (0-4 weeks) and grower feed (5-12 weeks). 35
- Figure 3.** Bar diagram showing mean cecal lesion scores of birds of different treatments (UU, UC, VU, & VC), 6 days after an oral challenge with 100,000 oocysts of *E. tenella*. Birds were challenged at 4, 5, 6, 7, & 8 weeks of age. For each week, error bars with different lower case letters are significantly different ($p < 0.05$). 36
- Figure 4.** Bar diagram showing mean dropping pan scores of different treatments (UU, UC, VU, & VC), 6 days after an oral challenge with 100,000 oocysts of *E. tenella*. Birds were challenged at 4, 5, 6, 7, & 8 weeks of age. For each week, bars with different lower case letters are significantly different ($p < 0.05$). 37
- Figure 5.** Bar diagram showing body weights of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and uninfected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$). 38
- Figure 6.** Bar diagram showing chest girths of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and un-infected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$). 39
- Figure 7.** Bar diagram showing shank lengths of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and uninfected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$). 40
- Figure 8.** Bar diagram showing keel lengths of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and uninfected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$). 41

Figure 9. Average number of oocysts counted in the litter from the pens containing birds that had been vaccinated with a commercial live vaccine at the hatchery (Experiment 1). 42

Figure 10. Average number of oocysts counted in the litter from the pens containing birds that had been infected with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella* at day zero (Experiment 2). 43

LIST OF TABLES

Table 1. Feed composition of the breeder starter diet used in both experiments that was fed to birds from 0 to 4 weeks..... 44

Table 2. Feed allowance and average body weight of restricted feed birds in the infected and uninfected group from week 1 through 12. Birds in both the groups were fed only 4 days in a week to follow '4-3' feed restriction. 45

CHAPTER 1: INTRODUCTION

Coccidiosis is an economically important poultry disease caused by several species of microscopic eukaryotic protozoan parasites of the genus *Eimeria* which belong to the phylum Apicomplexa. The parasites develop in the gastrointestinal tract following ingestion of infective sporulated oocysts. The organism undergoes several developmental changes in cells of the intestinal epithelium and causes extensive damage to the gut epithelium. This affects the absorption of various nutrients across the gut wall leading to poor growth and reduced performance of the infected flock. Coccidiosis is highly linked to intensive animal production systems. In modern poultry rearing, high stocking densities of susceptible young birds provide an ideal environment for the development and reproduction of coccidia. Coccidiosis is the most commonly reported poultry disease all over the world (Xie et al, 2001). According to Williams, (1999) a commercial poultry production unit without the presence of these parasites is extremely rare. The infection may be subclinical (causing reduced weight gain and a drop in egg production without obvious signs of disease) or clinical (characterized by mortality and morbidity) (Williams, 1999).

Substantial efforts have been made over the past few decades to control this disease through improved sanitation, better flock management, development of various chemotherapeutic agents, and by the use of vaccines. Eradication of the parasites has been proven to be impossible. According to Dalloul and Lillehoj (2006) coccidiosis causes an estimated loss of \$3 billion to the poultry industry every year. Subclinical infection and its impact on feed conversion and weight gain accounts for 80% of this loss (Williams, 1999). Moreover the difficulty in diagnosis of subclinical infection makes it difficult to evaluate the efficacy of appropriate strategies to control the parasite (De Gussem, 2007).

Control of coccidiosis in broilers has relied greatly on anticoccidial drugs, and the poultry industry would not have developed to its present magnitude in the absence of these drugs. More than 30 anticoccidial drugs of different classes have been introduced. The rapidity with which drug resistant organisms has developed has been problematic and currently, no new drugs are under development. Vaccines are considered an alternative means to control this disease and have especially been employed by the broiler breeder industry. Problems with vaccination include a lack of uniformity in vaccine delivery systems, and the risk of subclinical disease and subsequent growth impairment associated with vaccination (Chapman et al, 2002). In broiler breeders and replacement layer stocks vaccines are used as a long term measure of control. Most research has been carried out in broilers, and little information is available concerning the effect of the use of vaccines in layers or breeders. An important consideration which has received little attention is assessment of vaccination on birds when they are subjected to feed restriction. Studies are needed to better understand the various aspects of vaccination in restricted fed birds and this may help the development of better procedures for optimizing bird performance. The objectives of this study were to evaluate the efficacy of live vaccination in preventing coccidiosis in broiler breeders under feed restriction and determine vaccination effects upon early growth and development.

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CHAPTER 2: LITERATURE REVIEW

Poultry coccidiosis – An overview

Coccidiosis is a widely studied poultry disease principally due to its distribution, frequency of occurrence, and importance in causing economic losses in poultry operations (McDougald & Reid, 1997). Unlike other poultry diseases, coccidia are almost universally present wherever poultry are raised and the disease affects all types of poultry including chickens, turkeys and waterfowl. Live birds host the organism - transport its various stages and sometimes remain carriers for a long time. Not much was known about various species of the coccidian family until the end of 19th century. H. B. Fantham (1876 – 1937), who studied coccidiosis in wild grouse, believed that coccidiosis in birds is caused by only one species of coccidia, and he named it as *Eimeria avium* (Fantham, 1910). Later on, various researchers established the existence of different species of *Eimeria* that affect different species of birds and that occur in different parts of the intestine (Ruff, 1999). The species of *Eimeria* that infect the chicken are highly host specific. The 7 species of coccidia currently recognized as infecting chickens, are *E. acervulina*, *E. maxima*, *E. mitis* (described by Tyzzer, 1929) *E. tenella*, (by Railliet & Lucet, 1891), *E. necatrix*, *E. praecox* (Johnson, 1930), and *E. brunetti* (Levine, 1942). These species differ significantly in prevalence, pathogenicity and immunogenicity (Rose and Long, 1980). Except for *E. mitis* and *E. praecox*, all other species can cause considerable harm to the gut mucosa leading to visible mucosal lesions on postmortem examination.

The occurrence of the disease in chickens varies from severe outbreaks to mild infections. It is quite possible that mild infection is overlooked due to the lack of any obvious signs or symptoms of disease. A mild or subclinical infection is difficult to diagnose and the birds may appear normal. However in an acute infection, symptoms include sudden drop in feed

consumption, huddling, general droopiness, emaciation, diarrhea and possibly death. The birds may have roughled feathers, and a pale comb and wattle. Generally, in a flock, birds can be found infected with more than one species of *Eimeria*. *E. acervulina*, *E. maxima*, and *E. tenella* are frequently observed together in litter samples obtained from poultry houses in the United States (Schwarz, Jenkins, Klopp, & Miska, 2009). Microscopic examination of fecal or litter samples from the infected house and a postmortem examination of dead birds by an expert will give an indication about the species of coccidia that are involved in an infection.

Life cycle of a typical *Eimeria* spp.

Eimeria has a short life cycle, which, depending on the species, takes 4 to 6 days. The life cycle is direct without the involvement of an intermediate host. The infective stage of the organism is a thick double walled oocyst which on release from the host can persist in the environment for a long time (Williams, 1995). A typical *Eimeria* life cycle has 3 stages: sporogony, schizogony or merogony, and gametogony. Sporogony is the process by which the oocysts contained zygote in the environment undergo a reduction division to form four haploid sporoblasts. Sporoblasts develop to form sporocysts each with a distinct cell wall. Each sporoblast divides mitotically to produce two sporozoites. Hence the eimerian oocyst at this stage has eight sporozoites (four sporocysts with two sporozoites each) and is said to be sporulated (Hammond, 1946). The process of sporulation is affected by temperature, relative humidity and the level of oxygen which usually is optimally in the poultry house (Chapman et al., 2013). For example about 95% of *E. acervulina* oocysts are found to be sporulated in the normal poultry house environment within 5 days of shedding (Williams, 1995). Upon ingestion of the oocysts the sporocysts are released in the gizzard due to the mechanical crushing action of the gizzard musculature (Doran & Farr, 1962). The sporocysts subsequently release motile sporozoites

which can infect intestinal epithelium. It is found that trypsin, chymotrypsin and bile salts in the intestine are involved in the excystation process (Chapman, 1978). The asexual phase of the life cycle (merogony) starts when the released sporozoites invade the gut epithelial cells to form trophozoites. Glycosylphosphatidylinositol is a glycolipid which helps sporozoites attach to the individual host enterocytes (Chapman et al., 2013). The trophozoite enlarges and then undergoes repeated, mitotic nuclear division to form an immature multinucleated stage known as a meront. Cytoplasmic differentiation occurs around the nuclei to produce large number of merozoites which burst out from the meront killing the host cell in the process. The merozoites penetrate other healthy cells and then undergo one or more generations of merogony depending on the species of *Eimeria* (McDougald, 2013). Gametogony follows merogony and begins when the last generation of merozoites develop into either a macrogamont or a microgamont. The macrogamont becomes a macrogamete and the microgamont undergoes multiple division to form many flagellated microgametes which are released upon rupture of the cell. A zygote is formed when the macrogamete is fertilized by a microgamete which then develops a double layered oocyst wall. The fertilized zygote, now known as an oocyst is released and passes via the feces into the litter. Oocysts become infective under conditions that favor sporulation (Soulsby, 1968). All the species of *Eimeria* infecting chickens vary in the size and dimension of their oocysts which is an important microscopic identification criteria.

Epidemiology of poultry coccidiosis

Birds acquire infection through contaminated feed, water and litter that contains oocysts. It has been observed that coccidiosis is less frequent when the number of attendants in the poultry house, and their movement from one farm to another is limited, thereby reducing the chance of spread through fomites (Vermonten & Kouwenhoven, 1993). Since birds are reared in

contact with feces on built-up-litter, the disease will continue to occur even though a control measure is adopted. Continued oocyst production is the key in establishing the infection. When birds are exposed to daily doses of sporulated oocysts, they would excrete oocyst continually for up to 25 days, in the absence of protective immunity (Stiff and Bafundo, 1993). This means that the infection will persist in the house for a long duration. Internal climate of the house will affect the rate of accumulation of oocysts in the litter. Increased initial oocyst numbers, increased chick stocking density, and greater innate susceptibility of birds to the disease increase the likelihood of infection. However high ammonia content of the litter and lower oxygen levels are unfavorable for oocysts and will decrease their number (Chapman et al., 2002). Light schedule in the poultry house is another important factor for oocyst accumulation. Light and dark cycles in the poultry house, stimulates the birds to actively manipulate the litter periodically that may favor oocyst sporulation. It is proposed that during dark hours the accessibility of anticoccidials through feed would be reduced because of the reduced feed intake at this time and hence may lead to infection (Vermonten & Kouwenhoven, 1993).

Pathogenesis and pathology

The intracellular sexual and asexual reproductive stages of the eimerian life cycle is responsible for the entire pathology associated with the disease. The site of infection within the gut varies depending on the species of *Eimeria* involved; *E. acervulina* and *E. praecox* affect the duodenum, *E. maxima*, *E. mitis* and *E. necatrix* infect the mid intestine that may extend to the posterior intestine, *E. brunetti* infects the rectum and *E. tenella* develops in the ceca (Joyner, 1978). Pathogenicity also varies between species, and it is the magnitude of infection along with pathogenic potential, that determines the level of damage caused to the intestine (Tyzzer, Theiler, & Jones, 1932). Less pathogenic *E. mitis* and *E. acervulina* result in mild enteritis whereas

highly pathogenic *E. tenella* and *E. necatrix* cause the destruction of intestinal villi leading to hemorrhage and death (Chapman, 2014). Due to the likelihood of the presence of more than one species in an outbreak, competition within and between species for a particular region of the intestine would lead to what is known as the ‘crowding effect’ (Williams, 2001). For example, competition for enterocytes between *E. praecox* and *E. maxima* or *E. acervulina*, due to its close proximity of area of preference in the gut could lead to crowding, a state where all the available cells are infected (Jenkins, Allen, Wilkins, Klopp, & Miska, 2008).

The sporozoites of *E. tenella* and *E. necatrix*, after penetrating the surface epithelium are carried by macrophages in the lamina propria, deep into the glands of Lieberkühn, where they undergo further development. Deeply penetrated second generation schizonts of these species are responsible for extensive hemorrhage (Soulsby, 1968). *E. tenella* causes the ceca to be dilated, thickened and often filled with unclotted or partly clotted blood. *E. necatrix* infection in the middle third of the small intestine produces a swollen intestine with white opaque foci surrounded by a zone of hemorrhage (Soulsby, 1968). *E. maxima* is moderately pathogenic and most of the pathology is due to its sexual stages. In severe infection the intestinal wall will be thickened with many petechial hemorrhages and excess production of mucus.

E. acervulina is considered a less pathogenic species. More than a million oocysts are needed to cause mortality in birds experimentally infected (Horton-Smith, & Long 1959). Most of the research on the effect of *Eimeria* infection on absorption of nutrients has been focused on *E. acervulina* (Chapman, 2014) because it infects the duodenum, where most nutrient absorption occurs. Infection with this species were found to cause impaired absorption of glucose (Preston-Mafham, & Sykes, 1970), fats (Sharma & Fernando, 1975), calcium (Turk, 1973), zinc (Southern, & Baker, 1983), vitamin A and carotenoids (Kouwenhoven, & Horst, 1972). Brush

border enzyme activity of the intestine is also reported to be decreased due to *E. acervulina* and *E. mitis* infection (Allen, 1987). Infection with *Eimeria* can cause a reduced absorption of amino acids; the absorption of L-Histidine (Preston-Mafham, & Sykes, 1970), L-Methionine (Ruff, & Wilkins, 1980) were found to be reduced. Ileal amino acid digestibility studies in vaccinated broilers subsequently challenged with high dose of a mixed infection of *E. acervulina*, *E. maxima* and *E. tenella* revealed an overall reduction in the digestibility of amino acids with greatest reduction for branched chain amino acids (Leu, Ile, Val), Thr and Cys (Parker et al., 2007). In another study Ala, Cys, Ile, and Thr were found to be most affected (Amerah & Ravindran, 2015). The precise mechanism of the reduction of nutrients from the gut due to the infection is still unclear. However recent molecular and genetics works have found a reduced expression of genes responsible for various amino acid transporters in the gut (Paris & Wong, 2013; Su, Miska, Fetterer, Jenkins, & Wong, 2014) and a down regulation of brush border aminopeptidase enzyme (Su, Miska, Fetterer, Jenkins, & Wong, 2014).

Diagnosis of avian coccidiosis

Generally the pathogenic species of *Eimeria* can be differentiated on the basis of clinical signs, typical lesions at particular sites of the intestine, morphology and size of the oocysts and other intracellular stages. Molecular biological techniques are also available as a diagnostic aid. Earlier techniques of identification of different species were based on the isoenzyme patterns of oocysts (Shirley, 1975) and rRNA and rDNA probes (Ellis & Bumstead, 1990). Assay based on PCR is considered to be a rapid and accurate method of identification (Schnitzler, Thebo, Mattsson, Tomley, & Shirley, 1998). This technique amplifies species-specific DNA sequence to a hundreds of millions, in just a few hours and the new copies that are produced can be separated by electrophoresis to visualize it under UV light by a fluorescent dye. PCR method of

identification to determine the incidence and occurrence of the parasite species and its proper documentation will help in future control regimes.

Immune response to *Eimeria* infection

The development of immunity against *Eimeria* infection involves various mechanisms including innate resistance, acquired and maternal immunity. Rose and Long (1962) observed that birds infected with small doses of *Eimeria* oocysts showed resistance to reinfection and this resistance has increased following a second infection. Further studies revealed several aspects of immunity against coccidiosis. Immunity is directed against the development of both asexual (Pierce, Long, & Horton-Smith, 1962) and sexual stages (Rose, 1963) of the parasite.

Immunization with one species of *Eimeria* will not protect the bird from infection by other species (Long & Pierce, 1963). The role of the innate immune system against a primary infection has been demonstrated by an increased number of granulocytes (Ovington, Smith, & Joysey, 1990), NK cell activity (Smith, Rose, & Wakelin, 1994), and production of cytokines (Ovington, Alleva, & Kerr, 1995; Wakelin, Rose, Hesketh, Else, & Grecis, 1993) along with faster T-cell response (Rose, Wakelin, & Hesketh, 1990; Wakelin, Rose, Hesketh, Else, & Grecis, 1993) and an elevated levels of oxygen free radicals (Ovington, et al., 1995). B cells have a minor role in the development of resistance against coccidia (Smith & Hayday, 1998; Long & Pierce, 1963; Rose & Hesketh, 1979). CD4⁺ cells, a subset of T cells is considered to be important in innate immunity which acts via INF- γ secretion to enhance NK cell activity, increase iNOS activity, increase antigen presentation and lysosome activity of macrophages.

Gut Associated Lymphoid Tissue (GALT) plays a critical role in host immunity by processing and presentation of antigens, production of intestinal secretory antibodies, and activation of cell mediated immunity (CMI). CMI involves antigen specific or nonspecific

activation of T cells. Even though the production of antibodies (IgA, IgM, & IgY) are observed in *Eimeria* infection, their precise role in immunity is yet to be established (Dalloul & Lillehoj, 2005). Cytokines and proinflammatory molecules secreted by CMI directs appropriate immune responses to the invading parasite. Several cytokines found to be involved in CMI are tumor necrosis factor, transforming growth factor, a variety of interleukins and granulocyte macrophage colony stimulating factor (Dalloul & Lillehoj, 2005).

The transfer of maternal antibodies from an immune hen to the hatchling has been demonstrated for *E. tenella* (Rose & Long, 1971) and *E. maxima* (Rose, 1972) and this could protect the chicks at least during the first week post hatching (Smith, Hunt, Ellenrieder, Eckert, & Shirley, 1994). Experiments by Rose, (1972) and Lee et al., (2009) confirm that *Eimeria* specific antibodies purified from egg yolks of immunized hens following injection to a susceptible chick can confer protective immunity against a challenge infection. Hence maternal immunization is a potential means of control of infection with coccidia (Chapman et al., 2013).

Control of coccidiosis.

Due to the ubiquitous nature of the disease, rapidly spreading between chicken houses, and owing to the large reproductive potential of the parasite, it is very difficult to prevent birds from infection, especially under current intensive rearing systems that involve raising birds on litter in contact with their feces. Use of anticoccidial drugs and vaccination, have been the two major control measures employed against poultry coccidiosis. However these measures require adoption of good poultry house management practices. A coccidiosis outbreak can be prevented by minimizing the infection pressure on the flocks. Maintaining good litter quality by use of efficient drinkers to prevent wet litter, cleaning feeders and drinkers between flocks, strict hygiene and enforcement of biosecurity, maximizing downtime between flocks, regular

monitoring of bird health and timely removal of dead birds if any from the poultry house etc. are considered important in reducing oocyst load in the poultry house (Suls, 1999).

Inclusion of drugs in the feed to control the disease demonstrated by Grumbles & co-workers (1948), was a landmark discovery in the history of the battle against coccidiosis. Anticoccidial drugs would either inhibit the replication and growth of *Eimeria* (coccidiostat) or kill different developmental stages (coccidiocide). Coccidiocidal drugs are more effective since those with a coccidiostatic mode of action, upon drug withdrawal would allow the development of inhibited *Eimeria* parasites which could then resume the infection (McDougald & Fitz-Coy, 2009). Sulfanilamide was the first anticoccidial drug used against coccidiosis and many drugs have been subsequently introduced. Two classes of drugs are recognized, with different modes of action; synthetic drugs and ionophores. Generally, synthetic drugs act via inhibiting different biochemical pathways of the developing parasite, whereas ionophores interfere with the passage of ions across the parasite cell membrane affecting osmotic balance and thereby causing death (Chapman, Jeffers, & Williams, 2010). Sulfonamides, nicarbazin, clodol, quinolones, halofuginone, amprolium, robenidine etc. are examples of synthetic drugs. Nicarbazin is one of the most successful since it has maintained its efficacy for many years, while most others lost their potency fairly quickly due to drug resistance (Chapman, 1994a). Ionophores, produced by bacterial fermentation are a very successful tools for control of coccidiosis due to their unique mode of action, and the relatively slow development of drug resistance (Chapman, 2014). Drugs like monensin, lasalocid, narasin, salinomycin etc. are examples of ionophores. Unfortunately almost all of the drugs introduced became ineffective due to drug resistance (Chapman, 1997). However drug shuttle and rotation programs proved to delay the onset of resistance. A shuttle program employs two or more drugs with different modes of action in the feed throughout the

life of the flock, whereas a rotation program involves the use of drugs with different modes of action in successive flocks (Chapman, 2014).

Anticoccidial drugs are sometimes used in layer birds and broiler breeder stock. Those that have been used include sulfaquinoxaline (Dickinson 1949), amprolium (Stephens & Barnett, 1970), and toltrazuril (Schmid et al, 1991). The use of nicarbazin is contraindicated as it has adverse effects on the reproductive system of birds (Chapman, 1994).

Control by vaccination

Early studies by Beach and Corl (1925) and subsequently by Johnson (1927), and Joyner and Norton (1973), reported that solid immunity can be developed when birds are repeatedly exposed to live oocysts, and Long et al (1986) showed that protective immunity is developed when day old chicks are given small doses of live oocysts. These findings on the induction of immunity with live oocysts formed the basis of vaccination. There are two types of vaccines available; live attenuated and live non-attenuated vaccines. Non attenuated vaccines contain field isolates or laboratory strains of viable oocysts that are not subjected to any kind of modification (Dalloul & Lillehoj, 2005). Attenuated vaccines (Paracox[®] and Livacox[®]) contain strains of *Eimeria* that are modified to reduce virulence without affecting immunogenicity. Two methods of attenuation used are a) selection for early maturation (precocious lines) and b) selection of egg adapted lines. Selection of oocysts for early maturation by serial passage in susceptible hosts (Jeffers, 1975; Shirley, 1989) yielded precocious lines that complete their life cycle up to 30h faster than the parent strain (Shirley & Bedrník, 1997; McDonald & Shirley, 2009). Also, serial passage of oocysts through the chorio-allantoic membrane of embryonated chicken eggs (egg adapted) is another method of attenuation (Long, 1972). The attenuation is due to the loss of reproductive capacity, failing to produce large second generation schizonts and hence loss of

virulence (Long, 1973). Attenuated vaccines are believed to cause less damage to the gut compared with non-attenuated vaccines (Vermeulen, Schaap, & Schetters, 2001). The number and strains of species in a vaccine depends on the formulation and field of application (Lee, 1987). For example Coccivac-D[®], which is used in broiler breeders, contains low doses of oocysts from all the known pathogenic species of *Eimeria* affecting chicken whereas Coccivac-B, employed in broilers, contains four species (Vermeulen et al, 2001).

A subunit vaccine, conceived by Wallach et al., (1989, 1992), has been developed but has not been successful. This vaccine is based on the finding that when hens are injected with proteins (gam56, gam82) derived from the wall forming bodies of macrogamonts, can induce production of immunoglobulin (IgY) and transferred to chicks via egg yolk that confers protection against *Eimeria*.

Various methods of vaccine administration have been employed including via the drinking water, spraying on the feed, in an edible gel, and hatchery spray. A method of administration involving the intra-ocular route of administration in newly hatched chicks has proven to be very efficient in the uniform delivery of vaccine, but its use in United States is limited due to impracticality (Chapman et al., 2002). In the field, several factors are considered crucial in the success of vaccination including uniform delivery of vaccine over the flock, recycling of oocysts from the litter, inclusion of strains of *Eimeria* in the vaccine and its immunogenic potential, kinetics of oocysts production, number and density of chicks, house management and bird behavior, and interaction between vaccinal strain of *Eimeria* and the strain that are present in the litter. A detailed description of various factors affecting vaccination by live vaccine is provided by Chapman et al., (2002). In broilers, long term control of coccidiosis is mostly achieved by alternating immunization and chemotherapy (Jeffers, 1986). Furthermore

interchanging vaccines with drugs is found to ameliorate drug resistance in the field (Chapman, 1994b).

Justification for the current study

The impact of coccidiosis on egg production has been well documented. Johnson (1931) conducted experiments to assess the impact of coccidiosis in layer flocks, where he observed that infection with *E. acervulina*, *E. maxima* and *E. tenella* caused termination of egg production. *E. mitis* and *E. praecox* caused a decrease in egg yield (Johnson, 1931; 1933). Loss of production was also observed wherein backyard poultry (Peterson, 1949). Hedge and Reid (1969) experienced an 80% decline in egg production due to the experimental infection of coccidia at 2 months of production. Birds did not recover until 5 – 6 weeks post infection. Adverse effects of coccidiosis in layer birds may last for 3 to 6 weeks leading to declined production by 30 -50% (Vezey 1938) and this is due to the malabsorption of nutrients which is critical for egg production (Turk, 1978).

With the advancement in the knowledge of immunity and vaccine development, and due to the issue of drug resistance, vaccination is becoming the key to controlling coccidiosis especially in layer flocks and broiler breeders. These birds are vaccinated at the hatchery at day zero and are raised in a litter system, which allows the recycling of the oocysts to induce solid immunity. It takes 3-4 weeks for the birds to become immune. In broiler breeder production for eggs, pullets are kept under feed restriction in order to avoid being overweight at the time of lay. It is important that a breeder pullet should meet its nutrient intake target at the first 4 weeks because this time is critical for the establishment of a uniform body frame (Griffin et al, 2005). Even though vaccines are a practicable alternative for chemotherapy in broilers, they have the potential to cause mild transient infection that may lead to an impaired performance (Chapman et

al., 2002). This aspect of vaccination has never been studied in broiler breeder birds. It is also hypothesized that vaccination of birds during feed restriction would cause more stress to the flock and may affect its early growth. There is no published information about the influence of vaccination on early growth and development of broiler breeder pullets. This information would be helpful in building better disease management strategies without compromising economic production. Mindful of this information, studies were conducted on vaccinated broiler breeder pullets raised on litter and under feed restriction to assess the protective efficacy of a vaccination program.

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CHAPTER 3: ASSESSMENT OF THE LEVEL OF PROTECTION AGAINST COCCIDIOSIS IN BROILER BREEDERS CONFERRED BY A LIVE ANTICOCCIDIAL VACCINE, AND ITS INFLUENCE ON EARLY GROWTH AND DEVELOPMENT.

Introduction

In broiler breeders, vaccination is the key in coccidiosis control. Unavailability of effective drugs and the cost of long term prophylactic medication in broiler breeders would explain the rationale behind the use of vaccination as an important strategy to prevent the disease in these flocks (Leeson & Summers, 2000). It is assumed that the immunity developed by the birds would be sufficient to protect them from the disease throughout their rearing phase. However there is no published information on the level and duration of protection acquired from vaccination with live oocysts in birds which have a longer life expectancy than commercial broilers.

Genetic selection of broiler breeders, pullet management, and utilizing varying degrees of feed restriction have improved growth and reproductive potential with greater flock uniformity (Hudson, Lien & Hess, 2001). The primary aim of feed restriction is to reduce weight gain and related issues that may affect the reproductive potential of the flocks such as fertility and hatchability of eggs, peak egg lay and timely ovulation (Robinson & Robinson, 1991; Robinson, Robinson, Hardin & Wilson, 1995; Renema, Sikur, Robinson, Korver & Zuidhof, 2008). Hence breeder diets are carefully formulated to deliver all the essential nutrients for proper growth and reproductive efficiency. In these experiments it is hypothesized that the administration of live oocysts of *Eimeria* (vaccination) may have an impact on nutrient absorption and hence affect proper growth and development of the broiler breeder.

The current study has two objectives

- a) To assess the protective efficacy of a live anticoccidial vaccine against challenge with a high dose of *Eimeria*.
- b) To assess the impact of vaccination on the growth of feed restricted broiler breeders

Materials and methods

Birds and husbandry: The study was conducted as two separate experiments. Day old broiler breeder female chicks (Cobb 500 FF) used in both the experiments were obtained from a local Cobb hatchery and were raised on new litter. For the first experiment, the birds were either vaccinated at the hatchery (with a commercial live vaccine) or not vaccinated. The two groups (vaccinated & unvaccinated) with 220 birds in each, were kept in separate rooms in an isolation facility with 110 chicks in each room (2 rooms/group). In the second experiment 154 vaccinated and unvaccinated chickens were raised in new litter pens as separate groups, and the vaccinated group has received a single dose of vaccine prepared in the laboratory, which contained live oocysts of 3 species of *Eimeria*.

All the pens and equipment were thoroughly cleaned, disinfected and fumigated with ammonium hydroxide to kill any existing oocysts (Horton-Smith, Taylor & Turtle, 1940) (appendix 1). Fresh wood shavings were used as litter material and the pens in each group were serviced separately to avoid cross contamination with *Eimeria*. The facility was preheated to a temperature of 85-90°F, (recorded 5 cm from the litter at the brooder edge) 24 hours before the arrival of chicks.

Feeding: The birds were fed a standard broiler breeder starter diet from week 0 to 4 (see Table 1) (CP 19% & 2900 Kcal/kg of energy) and grower diet from week 5 to 12 (CP 15.5% & energy

2750 Kcal/kg). After ad libitum feeding for the first 2 weeks, the birds were fed based on a '4-3 feed restriction program' (see Appendix 2 for details) described in Cobb breeder management guide (Anon, 2013). Feeding and flock management followed guidelines of Cobb breeder management guide (Anon, 2013) with an initial stocking density of 0.36 sq. ft. / bird for first 5 days and later 1.75 sq. ft. per bird until 12 weeks, to simulate commercial practice. All the experimental procedures were approved by the Animal Care and Use Committee of the University of Arkansas (protocol # 14057).

Parasite for challenge study: *E. tenella* was used for the challenge study in the first experiment, because it is one of the least immunogenic and most pathogenic species of chicken coccidia (Rose & Long, 1961; Chapman, 2014). Hence it can be speculated that if the vaccinated birds could resist the challenge with *E. tenella*, it would resist the infection with other species of *Eimeria* as well. Preparation of infective doses is explained in Appendix 3.

Infective material: The infective material that represents a live vaccine used for the second experiment was prepared in the laboratory to contain 500 viable oocysts each of *E. acervulina*, *E. maxima* and *E. tenella* in 0.3ml of distilled water. All the strains of *Eimeria* used in the study had been maintained in the laboratory since their isolation, by periodic propagation in broiler birds. The strains were tested for virulence and pathogenicity before use. The method involved in the propagation of oocysts (Appendix 4) and preparation of vaccine is described (Appendix 3).

Criteria for assessing the level of protection against the challenge infection: The immunity of birds against the challenge infection was assessed by scoring lesions in the ceca, as described by Johnson & Reid (1970), (Appendix 5) and by a dropping pan score (Morehouse & Baron, 1970) (see Appendix 6 for details). Lesion score and dropping score for each bird varied from 0

(normal) to 4 (most severe). The strain of *E. tenella* used for challenge study was previously dose titrated to obtain maximum lesions and dropping scores without inducing bird mortality.

Criteria for assessing the effect of vaccine on growth and development of birds:

Measurements of body weight, chest girth, keel length and shank length were used to compare the difference in growth of vaccinated and unvaccinated birds. Shank length and keel length were measured using a Vernier caliper, chest girth with a measuring tape and body weight was measured with a scale accurate to 1g. All the instruments were calibrated before use.

Experimental design:

Experiment 1: At 4 weeks of age, 40 birds from vaccinated and unvaccinated groups were transferred and randomly allocated to wire floored grower cages to 4 treatment groups; vaccinated challenged (VC), unvaccinated challenged (UC), vaccinated unchallenged (VU), and an unvaccinated unchallenged control (UU). Each treatment group had 4 replicates with 5 birds per replicate (Figure 1). After 2 days to acclimatize to the cages, the birds in UC and VC groups were challenged with 100,000 oocysts of *E. tenella* by individual oral gavage (Appendix 7). Six days after challenge, all the birds were humanely euthanized with CO₂ gas, a method approved by the University IACUC. They were then necropsied, and their intestines removed for visual scoring of the lesions in the ceca. Dropping scores were also recorded from the feces collection pan located underneath every cage. This challenge study was repeated when the birds were 5, 6, 7, and 8 weeks of age. The experiment was terminated at 56 days of age.

Experiment 2: Broiler breeder birds obtained from the hatchery were allocated to two groups - vaccinated and unvaccinated, with 154 birds. Each group contained 7 replicate pens of 22 birds (Figure 2). The vaccinated group was given an oral gavage of 0.3ml of the infective material

(vaccine) prior to placement in the pens. In each treatment group half of the birds in each pen were identified by spraying a few feathers with black paint. These birds were selected for odd numbered (3, 5, 7, 9, and 11) weekly measurements. The unpainted birds were selected for measurements on even weeks (2, 4, 6, 8, 10, and 12). Body weights were measured on weekly basis from 1 to 12 weeks while chest girth, shank length and keel lengths were measured at 2, 6, 9, & 12 weeks of age. The end of the experiment was marked by the completion of week 12 measurements.

Oocyst count: Oocysts numbers were recorded for both vaccinated and unvaccinated group on a weekly basis. The procedure for counting oocysts is described in Appendix 8.

Statistical analysis: Both the experiments involved a randomized statistical design. All the statistical analysis were performed using mixed procedure of SAS, version 9.4 (SAS Institute, Cary, NC, USA) and the data were expressed as mean \pm SEM. The means were separated using least significant difference procedure (LSD) and the statistical significance was declared at $P < 0.05$.

Results

Acquisition of protection against *Eimeria* challenge: Lesion score and dropping pan scores following an oral challenge with 100,000 oocysts of *E. tenella* is shown in Figure 3 & Figure 4 respectively. The VC birds showed a significant reduction in both the scores compared to the UC birds at 4, 5, 6, 7 and 8 weeks of age, indicating that the vaccinated birds are sufficiently protected to prevent the pathology caused by this species of *Eimeria*. A high lesion and dropping score in the UC group indicates severe destruction of the cecal wall as a result of the parasite infection. However when compared to UU a significantly higher lesion score (at week 4 & 8)

and dropping score (at week 4) was observed in VU group. This suggests that, even in the absence of challenge, recycling of vaccine strains might have been sufficient to cause a mild damage to the cecum.

Effect of vaccination on body growth and development: In the second experiment, after feeding equal quantities (see Table 2 for quantity of feed given every week) of feed for all the birds, the vaccinated group showed a significantly lower ($p<0.05$) mean bird weight than the unvaccinated birds (Figure 5) for all weeks of age. The same trend was also seen in chest girth measurement (Figure 6). Reduction in both body weight and chest girth in vaccinated birds indicates a reduced body growth due to vaccination with live oocysts of *Eimeria*. The result was inconclusive for shank length and keel length. At 2 weeks of age shank length was increased in vaccinated birds, where its keel length was decreased. Measurements at week 12 showed an increase in keel length and a decrease in shank length for vaccinated than unvaccinated birds (Figure 7 & Figure 8).

Discussion

Even though vaccination is widely practiced in commercial broiler breeder production, little information has been published regarding the protective efficacy and duration of protection conferred by live oocyst immunization of these birds. The current study was done to evaluate the efficacy of live vaccination and its effect on broiler breeder growth and development in the early stages of pullet life. In the first experiment it was observed that the birds immunized with small doses of live coccidian oocysts at day 1 were protected against a heavy challenge with *E. tenella*. It is important that vaccinated birds reared on litter receive multiple reinfections of oocysts as this will allow development of solid immunity (Chapman et al, 2002; Chapman, Matsler, Muthavarapu & Chapman, 2005). The oocyst shedding pattern in experiment 1 indicated that

reinfection by vaccinal oocysts had occurred as peak oocyst numbers were observed on week 3 (see Figure 9). After peak shedding, the number of oocysts remained low in the litter until the end of the study. Hence it is suggested that after 2-3 cycles of reinfection, the birds have developed solid immunity and are able to resist a challenge infection by 4 weeks of age. This immunity was maintained through 5 to 8 weeks of age and it is likely that this immunity may protect the birds throughout their life.

In the second experiment a side effect of immunization was a lower average weight and decreased chest girth indicating growth retardation in vaccinated birds compared to the uninfected controls. This could be due to re-infection by the vaccinal parasites from the litter. Presence of low numbers of oocysts in the litter after a peak production in both the experiments (see figure 9 and 10) indicates that the shedding and recycling of oocysts were continuing even in the presence of solid immunity. This infection would have manifested as a mild lesions (at week 4 & 8) and dropping scores (week 4) in VU group of birds in the first experiment (see Figure 3 & 4). This observation can be equated to a mild infection that has already been reported in broilers (Chapman et al, 2002). Moreover Yaissle, Morishita & Lilburn (1999), reported a lower gross lesion and microscopic lesion scores in broiler breeders vaccinated with live vaccine. They also reported the presence of oocysts, macrogametes and microgametes of *Eimeria* in the mucosal cells lining the villi.

According to Summers (2008), protein and energy are the two important components of feed needed for broiler breeders at an early age. Provision of adequate energy through feed is critical for its optimum growth. In feed restricted birds an optimum average daily body weight increase of 15g (from 7 to 21 weeks) will require 46.5 Kcal, which represents 33% of total daily energy requirement of the bird (Costa, 1981). Experiments on birds vaccinated with a live

vaccine showed no effect on protein absorption/ retention by the gut wall (Yaissle, Morishita & Lilburn, 1999). But accumulation of large fat globules were observed in duodenal villous epithelial cells infected with *E. acervulina* gamonts indicating reduced fat metabolism by these cells (Sharma & Fernando, 1975) and glucose absorption was also found to be reduced (Preston-Mafham & Sykes, 1970). Recent studies on the expression of digestive enzymes and nutrient transporters in birds infected with *E. acervulina* showed a downregulation of sucrase-isomaltase, sugar transporters GLUT 1, 2 & 5 and zinc transporter ZnT1 (Su, Miska, Fetterer, Jenkins, & Wong, 2014). Hence it is possible that a reduction in the digestion and absorption of carbohydrates and fats which are the main energy sources, might have led to the reduced growth of these birds. The shank length and keel length measurements did not show any consistent results between the infected and uninfected birds. It could be because of its relatively lower growth rate. The reduction in nutrient absorption due to vaccination demands a greater nutrient supply, which may increase the feeding costs.

In conclusion, feed restricted broiler breeder birds, infected with small doses of live eimerian oocysts, are protected against challenge with a dose of *Eimeria* that will cause clinical coccidiosis but this may affect the growth and development of these birds.

Figures:

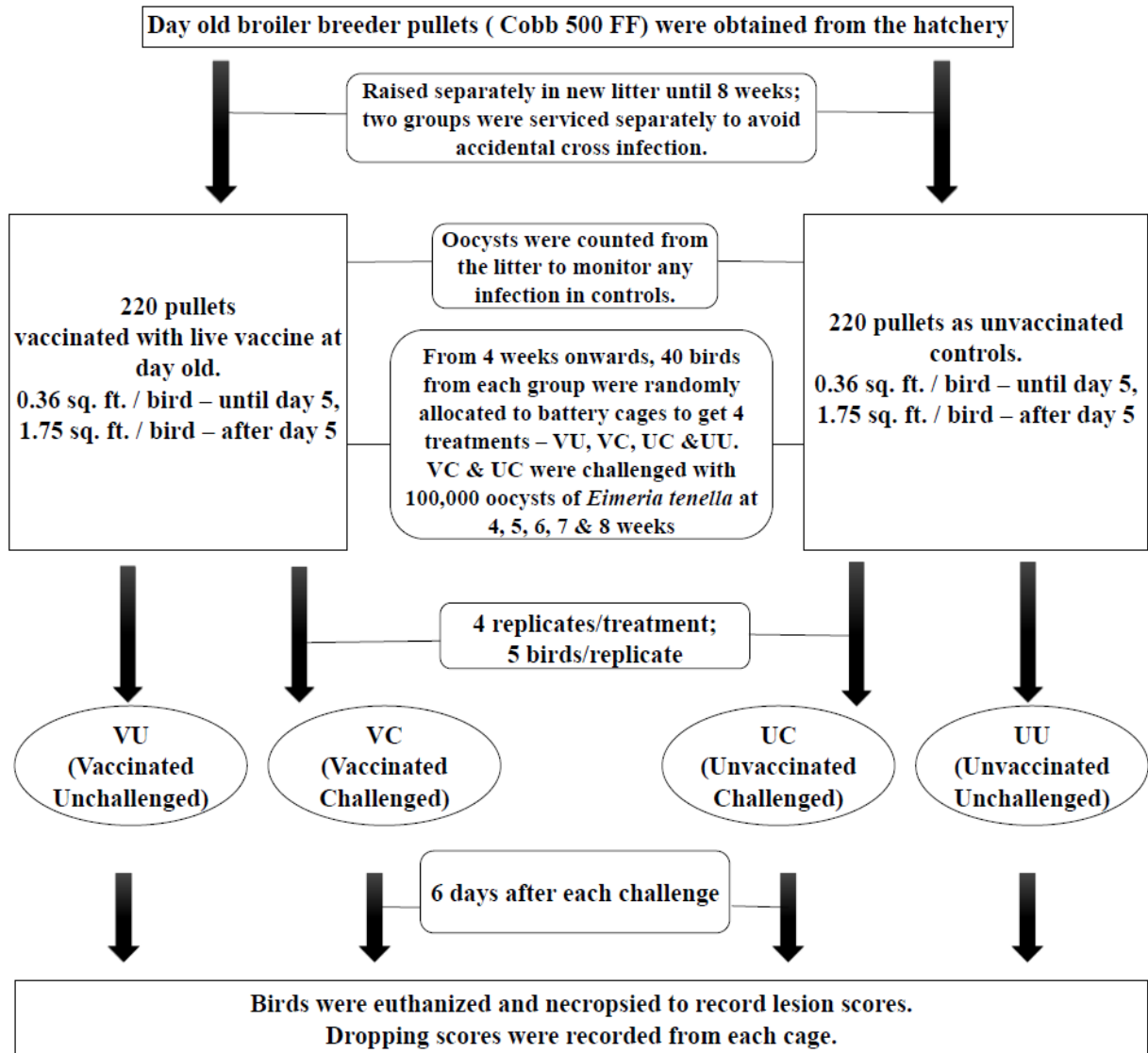


Figure 1. Design for experiment 1. Birds were vaccinated at the hatchery with a commercial vaccine and raised in pens with new litter. The challenge study was done in cages to assess the level of protection when birds were challenged with 100,000 oocysts of *E. tenella*.

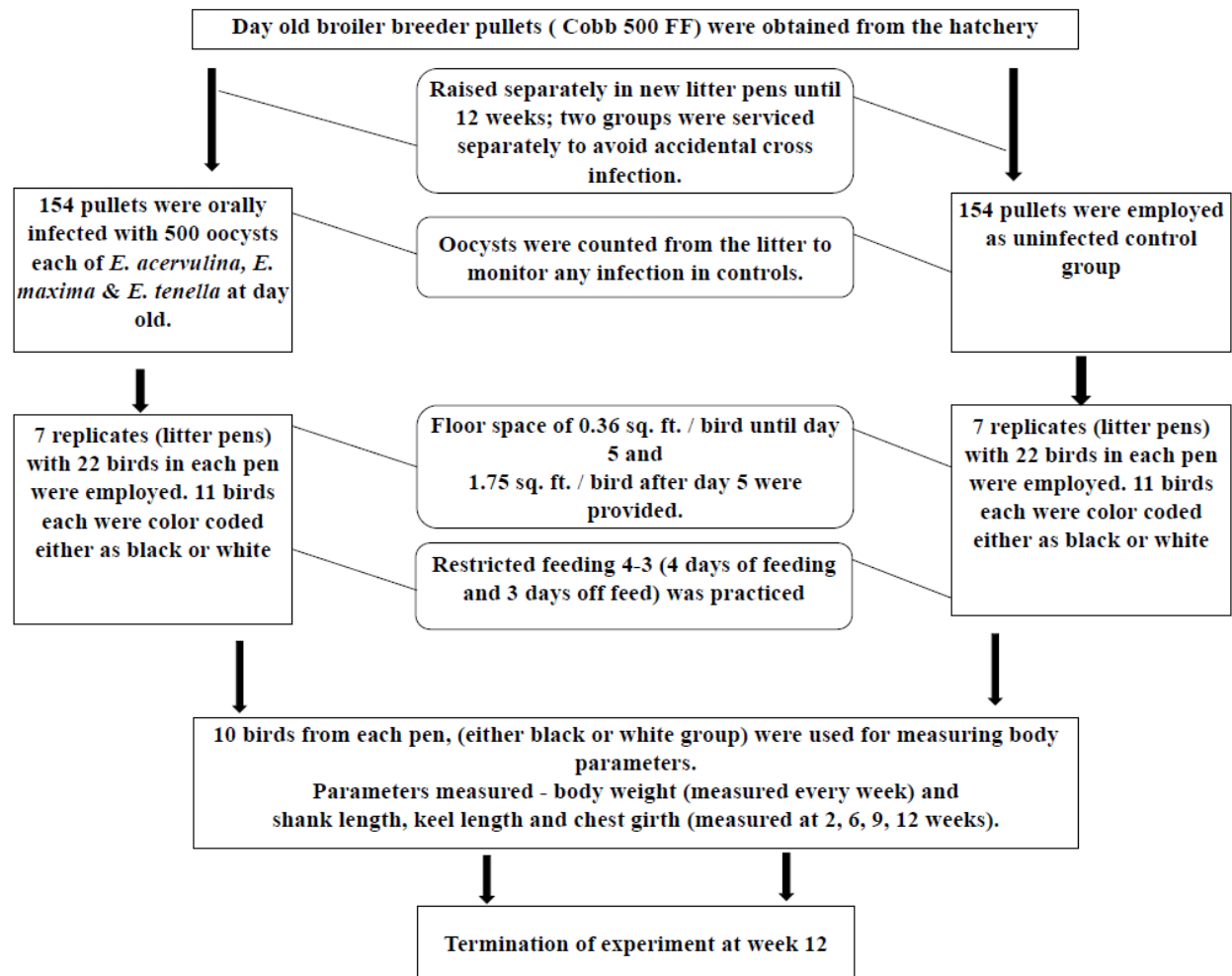


Figure 2. Design for experiment 2. Birds were infected with 500 oocysts each of *E. acervulina*, *E. maxima* and *E. tenella* at day zero, and were raised on new litter until 12 weeks. Feed restriction was practiced. Birds were given a starter feed (0-4 weeks) and grower feed (5-12 weeks).

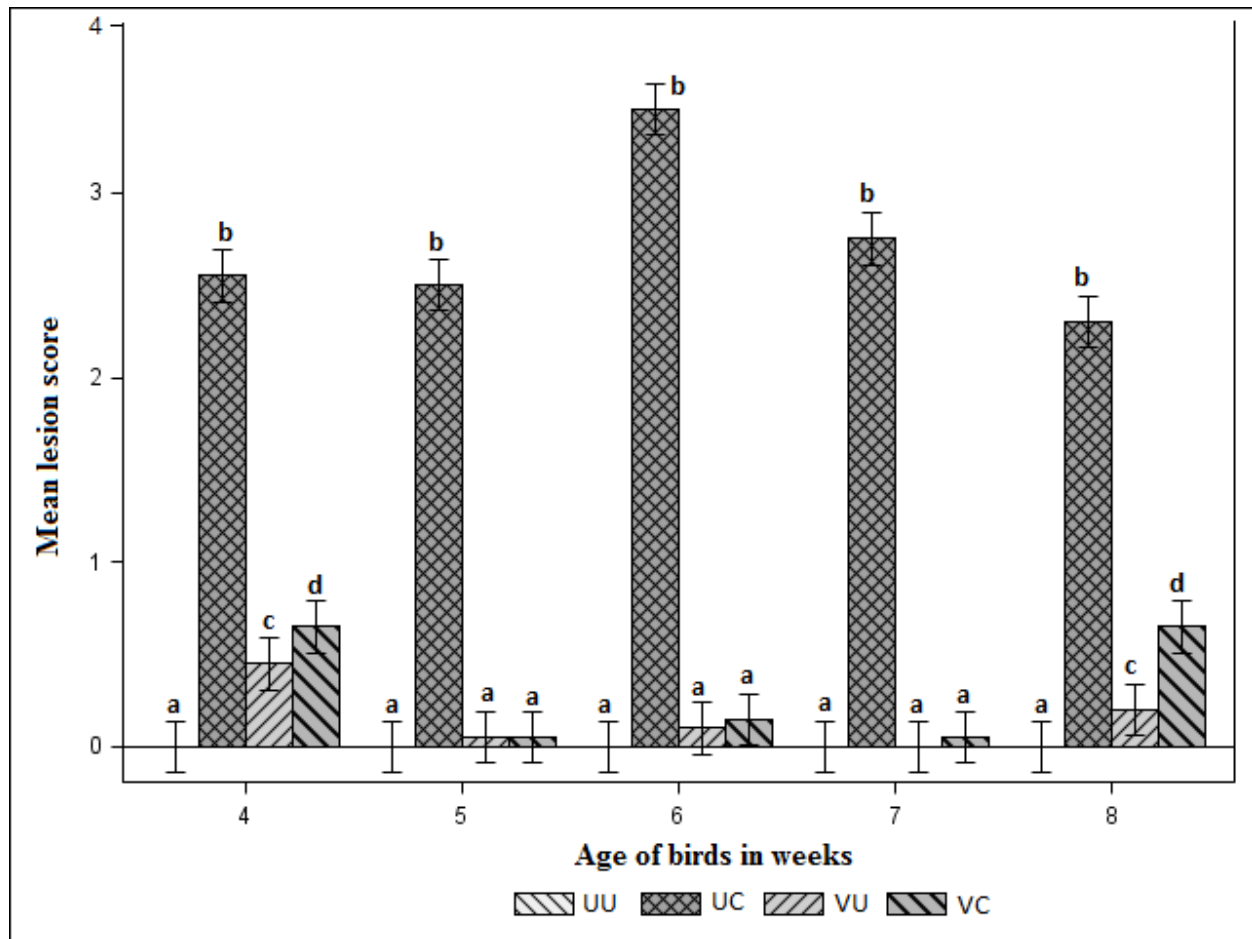


Figure 3. Bar diagram showing mean cecal lesion scores of birds of different treatments (UU, UC, VU, & VC), 6 days after an oral challenge with 100,000 oocysts of *E. tenella*. Birds were challenged at 4, 5, 6, 7, & 8 weeks of age. For each week, error bars with different lower case letters are significantly different ($p < 0.05$).

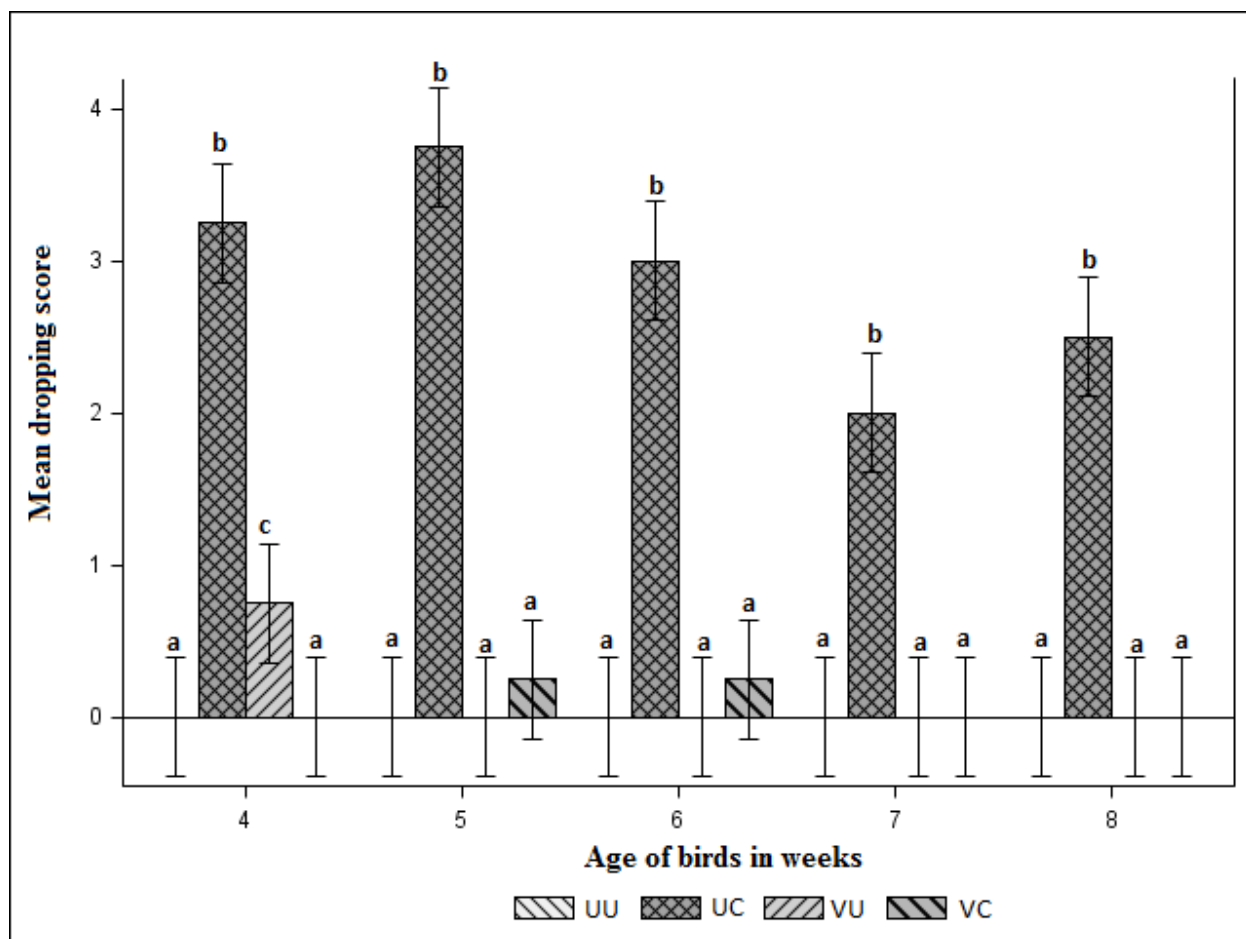


Figure 4. Bar diagram showing mean dropping pan scores of different treatments (UU, UC, VU, & VC), 6 days after an oral challenge with 100,000 oocysts of *E. tenella*. Birds were challenged at 4, 5, 6, 7, & 8 weeks of age. For each week, bars with different lower case letters are significantly different ($p < 0.05$).

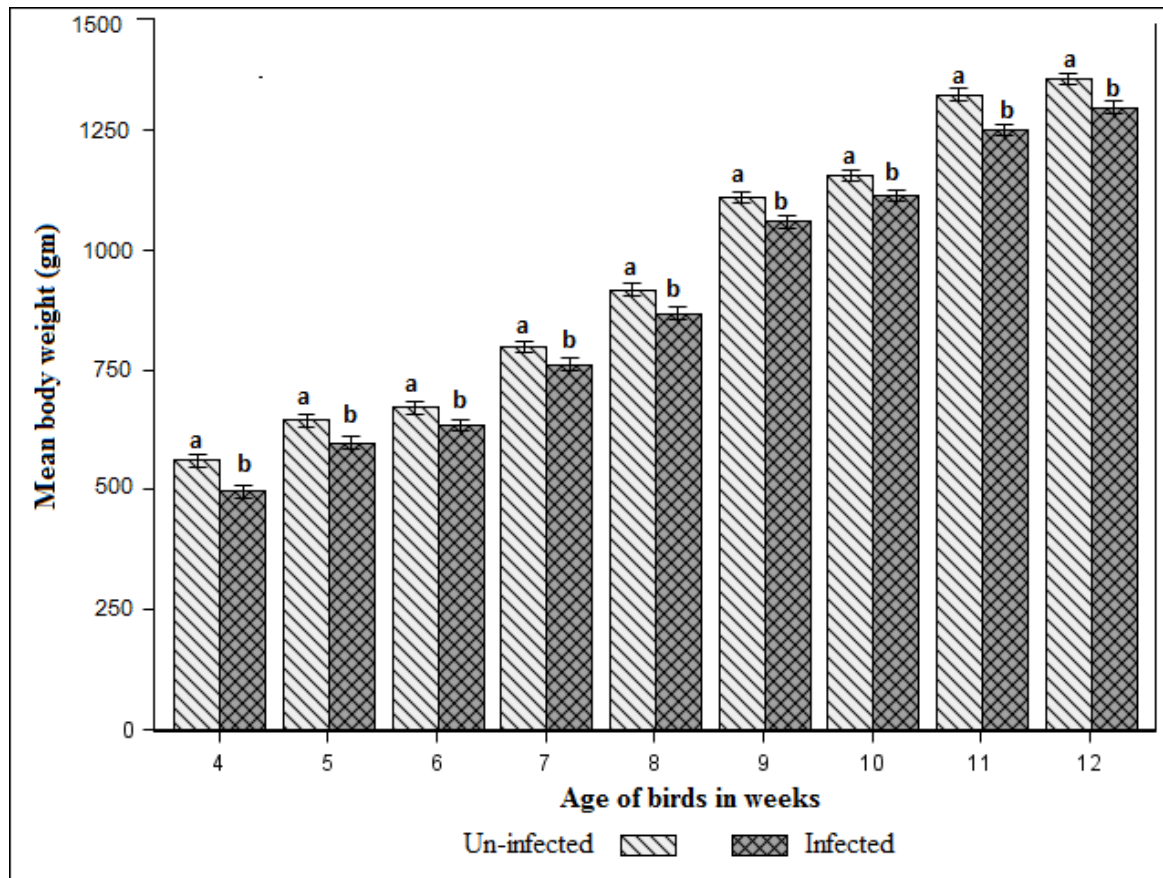


Figure 5. Bar diagram showing body weights of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and uninfected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$).

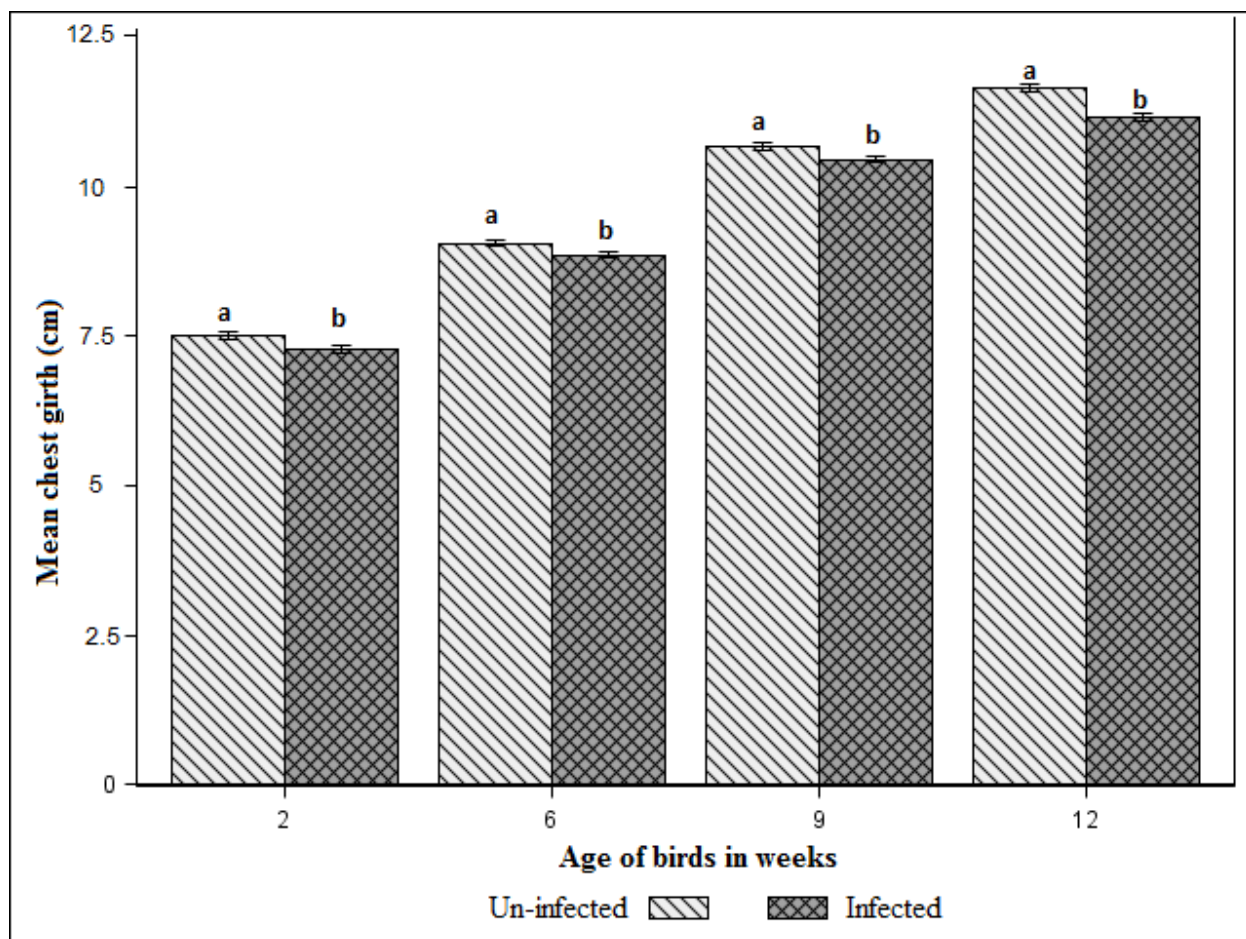


Figure 6. Bar diagram showing chest girths of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and un-infected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$).

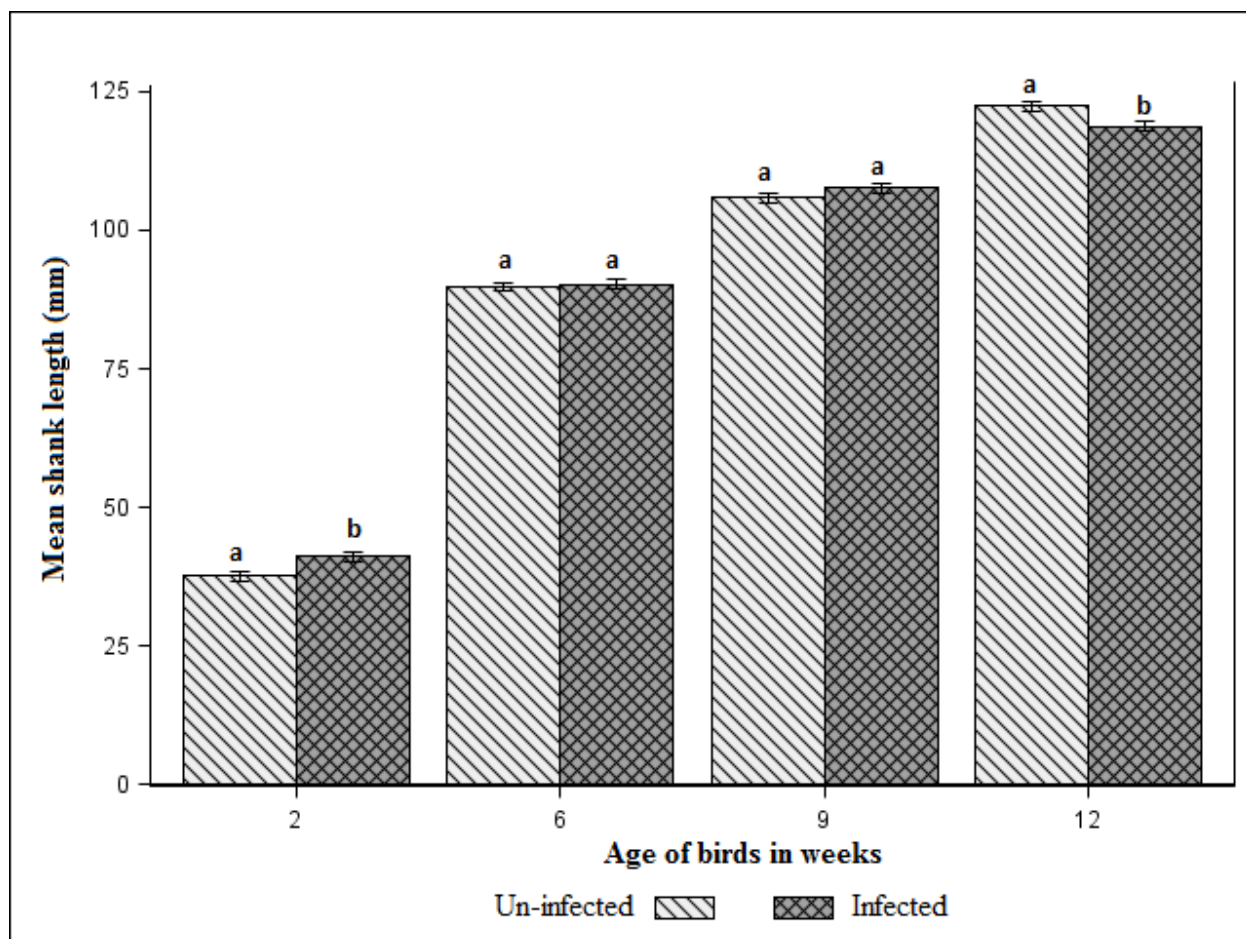


Figure 7. Bar diagram showing shank lengths of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and uninfected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$).

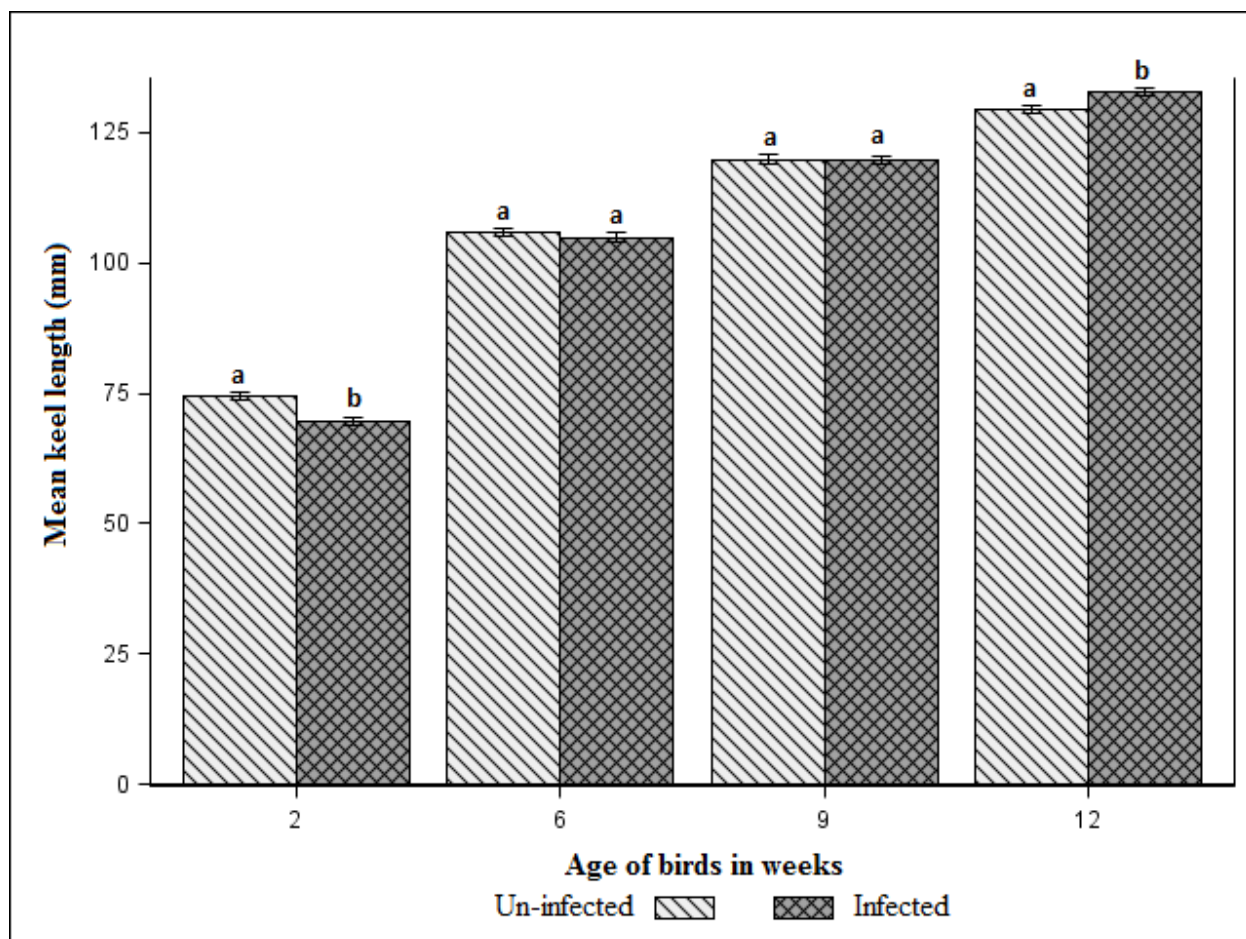


Figure 8. Bar diagram showing keel lengths of infected (with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella*) and uninfected groups of birds raised in floor pens. For each weekly measurement, bars with different lower case letters are significantly different ($p < 0.05$).

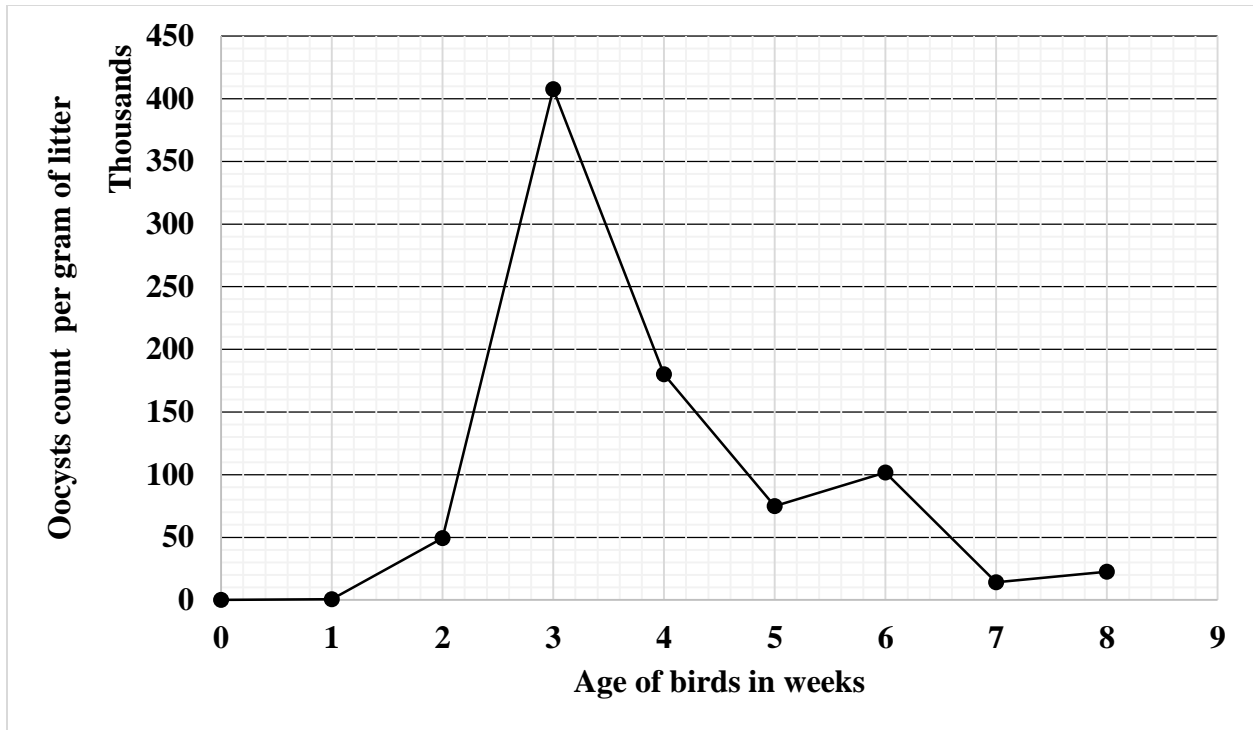


Figure 9. Average number of oocysts counted in the litter from the pens containing birds that had been vaccinated with a commercial live vaccine at the hatchery (Experiment 1).

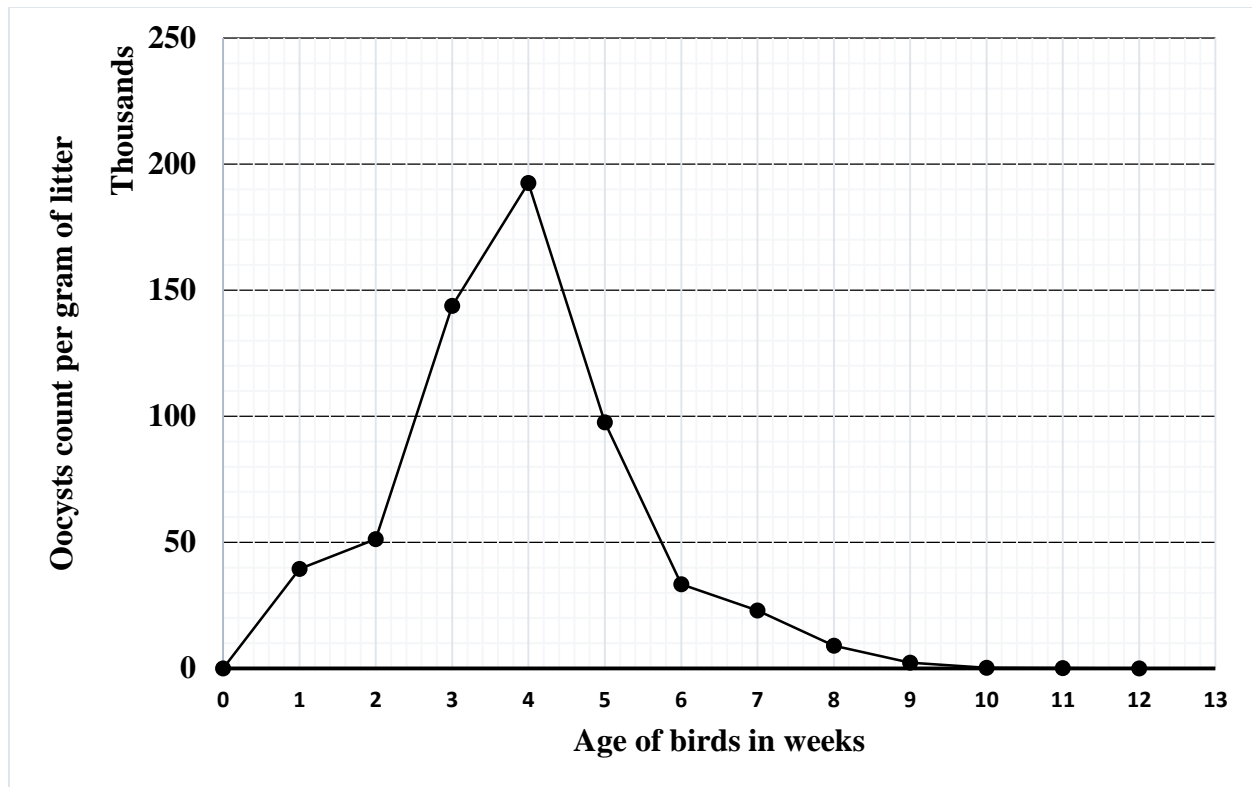


Figure 10. Average number of oocysts counted in the litter from the pens containing birds that had been infected with 500 oocysts each of *E. acervulina*, *E. maxima* & *E. tenella* at day zero (Experiment 2).

Tables:

Ingredients (lbs.)	Starter Broiler Breeder 0 - 4 wks
Corn	619.1
Soybean meal	261.8
Wheat middling	70.0
Poultry fat	5.0
Calcium carbonate	11.8
Dicalcium phosphate	18.6
Salt	3.27
Sodium bicarbonate	1.25
DL-Methionine	1.92
Lysine HCl	1.79
Threonine 98%	1.13
Choline-60%	2.00
Vitamin premix	1.00
Mineral premix	1.00
Antioxidant	0.20
Propionic acid 50%	0.20
Total (lbs.)	1000.0

Table 1. Feed composition of breeder starter diet used in both the experiments that was fed to birds from 0 to 4 weeks.

Age of birds	Vaccinated	Avg. amount of feed consumed /bird for the week (g)	Feed allowance per ration*/bird (g)	Observed Avg. body weight of birds in each treatment (g)	Target body weight** (g)
Wk1	No	141.15	Adlibitum	144.28	160
	Yes	152.1	Adlibitum	137.12	
Wk 2	No	396.9	Adlibitum	384.28	280
	Yes	423.23	Adlibitum	354.85	
Wk 3	No	238	59.5	445.71	400
	Yes	283	59.5	405.55	
Wk 4	No	252	63	560.87	520
	Yes	252	63	497.13	
Wk 5	No	266	66.5	650.69	620
	Yes	266	66.5	604.03	
Wk 6	No	280	70	682.27	720
	Yes	280	70	627.86	
Wk 7	No	315	78.75	807.11	820
	Yes	315	78.75	754.9	
Wk 8	No	336	84	898.69	920
	Yes	336	84	843.73	
Wk 9	No	406	101.5	1108.7	1020
	Yes	406	101.5	1059.19	
Wk 10	No	385	96.25	1188.66	1105
	Yes	385	96.25	1092.56	
Wk 11	No	392	98	1323.49	1190
	Yes	392	98	1249.3	
Wk 12	No	350	87.5	1356.24	1280
	Yes	350	87.5	1296.31	

*single ration represents 1/4th of the total weekly feed/bird. ** recommended target body weight for Cobb. 500 FF; Cobb breeder management guide (Anon, 2013).

Table 2. Feed allowance and average body weight of restricted fed birds in the infected and uninfected group from week 1 through 12. Birds in both the groups were fed only 4 days in a week to follow ‘4-3’ feed restriction.

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CHAPTER 4: CONCLUSION

There is no doubt that coccidiosis is an exasperating disease, damaging the poultry industry at a greater rate than one would expect. Vaccination with live oocysts is widely employed as a long term measure of control of coccidiosis. Vaccination introduces low number of live oocysts in the litter that will be recycled through reinfection, and subsist in the poultry house for long time. This means that vaccinated birds are getting low dose of infection throughout its life. Hence it is expected that this infection might affect bird growth. In this circumstances the study on the effect of vaccination on broiler breeders that are kept for longer duration was highly warranted.

The objective of this study was to investigate the efficacy of vaccination and its effect on broiler breeders' growth. The results of the experiments indicate that vaccination with live oocysts of *Eimeria* at an early stage in the bird's life can provide protection from clinical damage, but may affect early bird growth and development during feed restriction. This suggest that we should reconsider the nutritional and feeding management of these vaccinated birds. This study also emphasizes the need for a better disease control and prevention strategy during feed restriction.

CHAPTER 5: APPENDICES

Appendix 1: Procedure for fumigation with ammonia.

- Poultry house were cleaned thoroughly before fumigation.
- Laboratory grade absorbent cotton was placed in the bottom fourth of a 250 ml glass bottle.
- 150 ml of liquid ammonium hydroxide (27%) were pipetted out quickly into the bottle and the lid was closed immediately.
- 4 - 5 glass bottles were prepared and transported to the poultry house in a Styrofoam box with a lid.
- All the ventilations of the house were shut and air conditioners were turned off.
- The bottles were placed near all four corners and at the center of the room.
- All bottles were opened before placing.
- A sign stating “Do not enter, ammoniation in progress” was hung at all the entrances.
- The room was reopened 2 days later, when only a slight smell of ammonia was left.
- The ammonia bottles were collected back and returned it to the box.
- Then the ventilation was established, A/c turned on and the signs were removed.

Appendix 2: Feed restriction of birds.

- 4 – 3 feed restriction program was employed throughout the study.
- Two diets; starter fed from 0 – 4 weeks, and a grower diet from 5 – 12 weeks were employed.
- Adlibitum feeding were done for first two weeks.
- Feed was weighed each day before adding.
- Feed weigh back was recorded at the end of first and second week (there were no feed left overs for the remaining week).
- Total feed consumed by all the birds after 2 weeks was calculated and average feed consumption per bird was estimated.
- The above calculation was used to assume the total amount of feed that would be given to the entire flock to meet the average target bird weight for week 3 (found in Cobb broiler breeder management guide).
- This represents the total ration for all the birds for 4 feeding days (3 days off feeding in a week). Then the total feed was divided into 4 equal parts. Each part was divided with the number of birds in each pen. This is the restricted fed ration.
- Further adjustments in allocation of feed for each week was done assuming it to meet the target weight for that week.
- Proper record for the feeding schedule was maintained throughout the experiment.

Appendix 3: Procedure for preparation of oocyst inoculum for vaccination and challenge study.

- The stock solution containing sporulated oocysts in (2.5%) potassium dichromate was transferred to a large centrifuge bottle (500ml) and centrifuged for 10 minutes at 1500 RPM. Supernatant was poured off.
- The pellet was resuspended in water after vigorous shaking and was centrifuged again to obtain clear stock without dichromate in it.
- The clear solution obtained after resuspending the final pellet in little tap water was transferred to 100 ml conical flask.
- A drop of this suspension was used to count the number of sporulated oocysts in a Fuchs Rosenthal counting chamber.
- A fixed volume of concentrated suspension is used to dilute the number of oocysts in a flask to achieve the required dose of oocysts per ml.
- The oocysts in the final infective solution was recounted and labelled for use.
- The volume of inoculum should not be more than 0.5 ml for infecting birds at day zero. For older birds the volume would not exceed 1ml.

Illustrated example

- Let the final oocysts count of the suspension after centrifugation be 200,000/ml.
- If one need to infect 20 birds (20 doses) with 20,000 oocysts each; that is, a total of 400,000 oocysts are needed. Since 1 ml of the stock has 200, 000/ml. One can take 2 ml of the stock and can be diluted with water to make 20 ml.
- This would give 20 doses of 20,000 oocysts/ml.

Appendix 4: Procedure for propagation of oocysts for the study.

- Day old broiler chicks free from *Eimeria* infection was obtained from a local hatchery.
- These birds were raised in brooder cages (Petersime brooder) after thorough cleaning and disinfection. They were fed adlibitum with a standard chick starter diet with a free access to water. Strict biosecurity was enforced in the setting.
- At the age of 2 weeks the birds were transferred to cleaned battery cages fitted with feces collection pans, and were inoculated with laboratory maintained strain of sporulated *Eimeria* oocysts. The birds had adlibitum access to feed and water.
- Separate propagation procedures were done for each species of *Eimeria*.
- For *E. acervulina*, the collection trays were cleaned on day 4 and feces were collected at day 6 post inoculation.
- For *E. maxima*, trays were cleaned on day 5 and feces were collected at day 8 post inoculation.
- For *E. tenella*, trays were cleaned on day 4 and feces were collected at day 7 post inoculation.
- The feces collected separately from each cage into sterile plastic Ziploc bags were transferred to the laboratory for harvesting.
- Fecal samples were diluted with tap water to make a number of diluted samples of 2000 ml each.
- Each of these samples were stirred with electric paddler for 10 minutes to make it a homogeneous mixture.
- This homogenate was strained through a muslin cloth into a separate beaker to remove coarse debris.

- The filtrate was then poured to several plastic centrifuge bottles (700ml). After weighing each bottle and adjusting the balance, these were centrifuged at 3000 RPM for 10 minutes.
- The supernatants were discarded and a saturated salt solution were added to it. The level of salt solution was again adjusted to balance the weight in the centrifuge.
- After shaking the contents vigorously, the bottles were centrifuged at 1500 RPM for 5 minutes.
- The oocysts were siphoned from the top layer of the supernatant with a large bore flat ended needle fitted to a 50 ml syringe. The siphoned material were transferred to 50 ml centrifuge tubes to fill its one-third. The tubes were then filled with water and centrifuged at 1500 RPM for 10 minutes.
- The supernatant obtained were discarded and pellets were re-suspended in 2.5% potassium dichromate solution. This suspension was transferred to a conical flask and diluted further.
- The oocysts were counted in a counting chamber and the dilution was adjusted so that it contains not more than 100,000 oocysts/ml.
- The flasks containing harvested oocysts were then placed in a magnetic stirrer that allows natural aeration for 2 days.
- After 2 days the sample was recounted for sporulated oocysts, labelled and stored at 4°C.

Appendix 5: Method of lesion scoring for *E. tenella*.

- The euthanized birds were necropsied to pull out the intestine.
- Paired ceca was separated from the intestine and transferred to a clean white bottomed metal dish along with their respective wing band.
- Ceca was then cut open to visualize the pathological change after the challenge infection.
- Gross lesion score scores were recorded following Johnson & Reid (1970).
- The standard lesion scoring system for *Eimeria tenella* is as follows.
 - 0 = Ceca with no gross lesions.
 - +1 = Very few scattered petechiae on the cecal wall; no thickening of the cecal walls and normal cecal content present
 - +2 = Lesions more numerous with noticeable blood in the cecal contents; cecal wall is somewhat thickened; normal cecal contents present.
 - +3 = Large amounts of blood or cecal cores present; cecal walls greatly thickened; little, if any, fecal contents in the ceca.
 - +4 = cecal wall greatly distended with blood or large caseous cores; fecal debris lacking or included in cores.
- Birds dying of coccidiosis are scored as 4, regardless of the nature and magnitude of lesions that may be present.

Appendix 6: Method of dropping pan scoring for *E. tenella*.

- At the end of each week's challenge study all cages were examined for change in fecal consistency and contents
- The dropping pans were pulled out from all the cages one by one for visual examination of fecal contents.
- Dropping scores for *E. tenella* is as follows;
 - 0 = normal droppings
 - +1 = 10% of the cecal droppings blood stained
 - +2 = some normal cecal droppings, but many are blood stained
 - +3 = formed blood droppings, most cecal droppings blood stained
 - +4 = pools of blood in pan, no visible cecal droppings

Appendix 7: Procedure for infecting individual birds.

- The thoroughly mixed inoculum was loaded into a repeater pipette whose output volume is set to 1ml.
- The first dose from the pipette was discarded in order to avoid wrong dosage.
- The birds were restrained in hand by holding legs and wings with the help of an assistant and its head was elevated to extend the neck.
- Bird's mouth was opened by applying gentle pressure at the temporo-mandibular junction.
- The pipette was inserted into the mouth directing it to the esophagus to inject the dose.

The birds were kept in hand for some time to allow them to swallow the inoculum before placing back into the cage/pen.

Appendix 8: Procedure for counting oocysts from the litter.

- Litter materials were collected from multiple spots in each pen to get a representative sample and was transferred to labelled plastic Ziploc bags.
- 200 g of each sample were soaked overnight, in water in separate plastic beakers and were covered with aluminum foils.
- It was then mixed in an electric paddle stirrer for 10 minutes.
- One ml of the thoroughly mixed samples were pipetted out into labelled glass test tubes.
- Contents in the test tubes were diluted to tenfold with saturated salt solution, for better counting of oocysts. Sometimes the dilution can be 100 fold.
- Then it was mixed well and allowed to stand for 5 minutes.
- The samples were then loaded into 2 sections of the counting chambers with the help of a pipette.
- After 2 minutes the oocysts are counted under the microscope from the entire two sections of the counting chamber separately to give a mean count.
- Oocysts number can be calculated by;

Oocysts per gram (OPG) of litter =

Mean oocysts count x dilution x volume of the sample

0.15 x weight of the sample



MEMORANDUM

TO: Dr. H.D. Chapman

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee

DATE: July 7, 2014

SUBJECT: IACUC APPROVAL
Expiration date: July 7, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED protocol 14057: "Development of immunity in young chicken egg replacement stock to Eimeria spp.". You may begin work immediately

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond July 7, 2017 you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian