

IDENTIFICATION AND CONTROL OF *EIMERIA* SPECIES-ASSOCIATED COCCIDIOSIS
IN NORTHERN BOBWHITES

by

RICHARD WILLIAM GERHOLD JR

(Under the Direction of Larry R. McDougald and Elizabeth Howerth)

ABSTRACT

Species specific polymerase chain reaction (PCR) primers were constructed against the three *Eimeria* species infecting Northern bobwhites (*Colinus virginianus*) including *E. lettyae*, *E. coloni*, and *E. dispersa*. The primers were used to determine the distribution of the three species in litter samples originating from thirty-one bobwhite captive facilities from multiple locations throughout the United States. Survey results demonstrated *E. lettyae* from 20 farms, *E. coloni* from 22 farms and *E. dispersa* from 29 farms.

Chemotherapeutic trials in bobwhites with 13 separate anticoccidial agents currently used in the poultry industry disclosed several useful effective compounds. Efficacy was good-excellent with narasin+nicarbazin (Maxiban[®]), sulfadimethoxine+ormetoprin (Rofenaid[®]), clopidol (Coyden[®]), decoquinate (Decox[®]), diclazuril (1 and 2 ppm) (Clinacox[®]), lasalocid (Avatec[®]), robenidine (Robenz[®]), and zoalene (150 ppm) (Zoamix[®]). Marginal protection was found with semduramicin (Aviax[®]), or semduramicin + roxarsone (3-Nitro[®]). Monensin (Coban[®]) and salinomycin (Bio-cox[®]) were marginal in some tests, moderately good in others. Amprolium (250 ppm) roxarsone, and zoalene (125 ppm) were ineffective.

To determine whether Northern bobwhites could be immunized against *E. lettyae* by a low dose inoculation of oocysts, we inoculated 30 birds each with either 100 or 1,000 oocysts at two days of age (given orally by pipette). Four weeks after immunization, the immunized birds and unimmunized controls were challenged with 1×10^6 *E. lettyae* oocysts. Effectiveness of the immunization was measured by analyzing weight gain, intestinal lesions, severity of diarrhea, feed conversion ratio, and oocysts production. After challenge, birds immunized with 100 or 1,000 oocysts gained an average of 33.3 gm and 28.9 gm, respectively; whereas unimmunized challenged birds gained an average of 11.5 gm. Immunized quail produced approximately 99.7% fewer oocysts, had minimal gross intestinal and cecal lesions, had minimal diarrhea, and had a 50% lower feed conversion ratio compared to unimmunized challenged controls. These findings indicate that vaccination is a viable option for controlling coccidiosis in quail

INDEX WORDS: Coccidia, Coccidiosis, Chemotherapy, *Colinus virginianus*, *Eimeria colini*, *Eimeria dispersa*, *Eimeria lettyae*, immunization, molecular detection, Northern bobwhite quail.

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DEDICATION

I would like to dedicate this dissertation to my loving wife, Kelly L. Lockerman, whose support and encouragement were essential to the completion of many of my degrees and essential to my success.

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CHAPTER 1

INTRODUCTION

Eimeria spp. are obligate intracellular apicomplexa protozoal parasites that infect and replicate within the intestinal epithelial cells of their respective hosts (Tyzzer, 1929). Coccidia within the family Eimeriidae have direct life cycles involving endogenous and exogenous development stages. Within the endogenous stages, schizogony is followed by merogony and gamogony, leading to oocyst formation. Oocysts are shed in the host's feces and once outside the host, undergo sporulation. Following ingestion by another host animal, sporulated oocysts rupture, releasing eight sporozoites which infect the host epithelial cells. Coccidiosis is the general term given to the disease caused by the lesions and clinical signs elicited by *Eimeria* spp. infections. Coccidiosis has been reported in numerous vertebrates and is a major economic problem in poultry and other livestock (Long, 1990). The disease most often occurs in immunologically naive animals or in animals that are stressed or crowded; both can result in overwhelming infections (McDougald, 2008).

Although, coccidiosis outbreaks have been reported from free-ranging wildlife (Farr, 1965; Yabsley et al., 2002; Yabsley 2008), the disease is most often observed in animals raised in confinement (Reid et al., 1984). This is for two reasons: 1) parasite replication is self-limiting given the fixed number of merogony cycles and 2) after infection, the host develops protective immunity. The lesions produced by the parasites are a function of the number of ingested oocysts, the immune status and age of the host, the site of infection, and other factors.

Coccidiosis has a major economic impact on the poultry industry due to the density of birds raised within facilities. The frequency of devastating coccidia outbreaks are a function of the bird density and reproductive potential of the organisms (Reid et al., 1984). The development of effective chemotherapy against coccidia was a major milestone in the evolution of the poultry industry; and without the use of these anticoccidial compounds, the broiler industry as we know it would not exist. Due to the development of drug resistance in coccidia (Jeffers and Bentley, 1980), live attenuated or non-attenuated vaccines are being used more frequently in domestic poultry (Shirley et al., 2005).

Free-ranging Northern bobwhite (*Colinus virginianus*) populations have significantly decreased in the southeastern United States due to habitat fragmentation and destruction, and increase of non-native and native predators (Droege and Sauer, 1990; Mueller et al., 1999). To compensate for this decrease, Northern bobwhites are often raised in confinement and subsequently released for population restocking. It is estimated that 30-40 million bobwhites are raised in captivity and then released. Some farms in the USA produce upwards of one million birds annually for this market (NAGA, pers. comm.).

Coccidiosis has been identified as a major disease of captive raised Northern bobwhites (Ruff, 1985; Ruff, 1986); however, little work has been conducted on coccidia of captive and free-ranging bobwhites (Yabsley, 2008). To date, *E. dispersa*, *E. colini*, and *E. lettyae* are the described species from Northern bobwhites (Tyzzer, 1929; Fisher and Kelly, 1977; Ruff, 1985). These species infect intestinal epithelial cells leading to decreased weight gain, morbidity, or mortality. We aim to identify the intestinal coccidial species from captive Northern bobwhites using traditional methods reinforced by molecular analysis. Classical biological characters used in identification of coccidia include oocyst morphology, gross and histological lesions, prepatent

period, and sporulation time (Tyzzer, 1943). Molecular methods include designing PCR (polymerase chain reaction) primers to detect specific coccidia species (Su et al., 2003; Schnitzler et al, 1998). Initial PCR primers were developed using genus wide primers to the ribosomal RNA (rRNA) internal transcribed spacer (ITS) region 1 to amplify the ITS-1 region of *Eimeria* spp. collected from Northern bobwhite fecal samples. The primers were then used to survey the collected bobwhite fecal samples to determine the prevalence and distribution of the various coccidia species.

Additionally we investigated potential methods of controlling coccidiosis in bobwhites. For short term control we determined the efficacy of selected currently available anticoccidial compounds used in the poultry industry (McDougald, 1993). For potential long term control we studied the development of protective immunity in bobwhites inoculated with low dose of oocysts. From this information we determined that the pursuit of a live coccidiosis vaccine for quail is feasible.

Specifically the project was designed to:

- 1) Identify the different *Eimeria* coccidia species in Northern bobwhite by molecular analysis of the ITS-1 region of the rRNA.
- 2) Develop species specific PCR primers for the different *Eimeria* species to be used for diagnostics and research.
- 3) Determine the efficacy of numerous anticoccidial compounds for control of coccidiosis in Northern bobwhites.
- 4) Determine if Northern bobwhites can be effectively immunized with non-attenuated vaccine strains against important *Eimeria* species.

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CHAPTER 2

LITERATURE REVIEW

History and taxonomy

Eimeria spp. are obligate intracellular coccidia protozoal parasites belonging to the phylum Apicomplexa Levine, 1970, class Sporozoasida Leukart, 1879, subclass Coccidiasina Leukart, 1879, order Eucoccidiorida Leger and Duboscq, 1910, suborder Eimeriorina Leger, 1911, and family Eimeriidae Minchin 1903 (Levine, 1953; Levine, 1982; Long, 1993). *Eimeria* are classified in the above taxa based on several factors including: Macrogametes and microgamonts develop independently; microgamont typically produces many microgametes; sporocysts enclose the sporozoites; development is in the host cell proper; they lack attachment organelles or a vaginal tube; merogony is within the same host; sporogony is outside of the host; and microgametes have 2-3 flagella (Levine, 1982).

Approximately, 1,500 named *Eimeria* species are known and although coccidia organisms have been found parasitizing invertebrates (Chinchilla et al., 1994), the majority of the coccidia species infect vertebrates (Long, 1990). Almost every vertebrate species harbors coccidia parasites, which are generally species-specific (Tyzzer, 1929). Avian coccidia were first documented in the in 1890's by Raillet and Lucet (1890) and Salmon (1899). Most coccidia species infecting vertebrates are homoxenous and most replicate within the intestinal epithelial cells (Long, 1990). Although exceptions exists such as *Eimeria stiedae* which infects the liver of

rabbits (Dobell, 1922) and *Eimeria truncata* and *Eimeria auritusi* which infect the kidneys of geese (*Branta* spp.) and double-crested cormorants (*Phalacrocorax auritus*), respectively (Reid et al., 1984; Yabsely et al., 2002). Disseminated multiple organ infection in cranes has been well documented in infections with *Eimeria gruis* and *Eimeria reichenowi* (Carpenter et al., 1980, Spalding et al., 2008). *Eimeria* are transmitted from one host to another by direct transmission through ingestion of environmentally resistant oocysts. Sporulated *Eimeria* spp. oocysts share a common morphological characteristic consisting of four internal sporocysts, each containing two sporozoites, for a total of eight sporozoites.

***Eimeria* genome and phylogenetic studies**

The majority of the research performed on genetic analyses in the genus *Eimeria* has focused on *E. tenella* from the chicken. The genome size of *E. tenella* is approximately 60 Mbp found within approximately 14 chromosomes that range from 1 to > 7 Mbp (Shirley, 1994). Research on other *Eimeria* spp. suggests that similar chromosome organization exists among the genus and estimates genome size to between 50-60 Mbp (Cornelissen et al., 1984; Shirley, 2010). Interestingly, the chromosome size and DNA content was found to be larger in wild type parent *E. tenella* strains than those of precocious vaccine strains (Shirley, 1994). In addition, extrachromosomal DNA has been observed in the mitochondria and plastid organelles. The plastid DNA analysis disclosed linear molecules ranging from 25 to 170 kbp; however, the location of the plastid within the *Eimeria* cell is unknown (Dunn et al., 1998). Circular 35 kbp extrachromosomal DNA has been found in other apicomplexans including *Toxoplasma gondii* and *Plasmodium falciparum* (Gardner et al., 1994; Kohler et al., 1997). The apicoplasts are believed to be of algal origin (Kohler et al., 1997) and are likely due to a secondary endosymbiotic event such that occurred with mitochondria and eukaryotes. In *T. gondii*, and *P.*

falciparum, the apicoplasts have lost the ability to photosynthesize, but are essential for cell multiplication and several metabolic pathways in the parasite (Ralph et al., 2004; Mazumdar et al., 2006), and thus are attractive antiparasitic drug targets. Double stranded RNA segments and virus like particles have also been described from *E. tenella* and other *Eimeria* spp. (Ellis and Revets, 1990; Lee et al., 1996). Phylogenetic analyses of *Eimeria* using whole genome sequencing have identified *E. tenella* to be monophyletic with *T. gondii*, but separate from *Cryptosporidium parvum* (Kuo et al., 2008).

Infection, replication, and oocyst survival

Endogenous development, including both asexual (merogony/ schizogony) and sexual (gamogony), occurs within the host cells. In the avian host, the grinding action of ventricular wall facilitates the breakage of the oocysts wall and thus freeing the sporocysts. Sporozoites undergo excystation through the stieda body of the sporocysts in response to several internal host factors including body temperature, carbon dioxide concentration, bile salts, and trypsin (Long, 1990). The eight motile zoites attach to host cells using secreted transmembrane proteins called micronemes (Labb de Venevelles et al., 2005). Attachment to host cells is additionally facilitated by lectin and mucin attachments and these interactions are suggested to define the site of infection for the various *Eimeria* sp. (Fuller, 1999). In *E. tenella*, the mucins produced in the duodenum suppressed the attachment of sporozoites in cell culture, while mucins produced from cecal sections enhanced attachment (Fuller, 1999).

Once attached the sporozoites actively infiltrate the host cells that lie in the extreme villus tips using organelles of the apical complex (Long, 1990; Dasak, 1999). Apicomplexa zoites move by gliding motility that occurs by filament interactions within the parasite (Long, 1990).

In *E. tenella*, zoites move into the lamina propria by gliding motility and are engulfed by macrophages (Levine, 1982). Following infection, the sporozoites transform into uninucleate meronts and asexual proliferation, characterized by concurrent mitotic divisions, occurs within the meront. Approximately 1,000 first generation 2-4 micron long merozoites are formed in the first stage meronts. The first generation meronts are generally found between the host cell nucleus and the brush border. This first generation of merogony is completed when the merozoites are released from the meronts and rupture the host cell. Depending on the species of *Eimeria*, three to five cycles of merogony may occur. The meronts in the later stage of merogony are found closer to the basement membrane of the host cell and as such the occurrence of the late stage meronts corresponds with the peak of clinical signs and lesions seen in coccidiosis (Long, 1990). Following the completion of the final merogony generation, the merozoites differentiate into either micro- or macrogametes, commencing the sexual (gamogony) stage. The cues governing the various cycles and the switching from merogony to gamogony are unknown. Fertilization occurs when microgametes exit the host cell and penetrate a host cell containing a macrogamete. Following fertilization, a zygote is formed and an oocyst develops around the developing zygote. Mature oocysts rupture from the host cell and are excreted in the feces of the host. In theory, each ingested *E. tenella* oocyst can produce 2.5 million last generation merozoites, each of which can develop into either a microgamont or macrogamete. However, the actual number of oocysts produced per ingested oocyst is considerably lower due merozoites being excreted in feces and the phenomena of crowding (Long, 1990). Crowding occurs when the number of merozoites produced in the asexual cycles exceeds the available intestinal epithelial cells for gamogony. The excess numbers of merozoites are excreted in the feces.

Once excreted, exogenous development begins (sporogony), leading to sporulated oocysts that are infectious to susceptible hosts. Sporogony is dependent on ambient factors including temperature, moisture, and oxygen tension (Marquardt et al., 1960). Sporogony commences with reduction division and loss of a polar body. The haploid sporont divides into four sporoblasts which leads to a sporocyst containing two sporozoites. Oocysts can remain viable in the environment for long periods of time and may even survive winters in northern regions including Canada (Skene et al., 1981). Temperatures greater than 65C and less than -15C are generally sufficient to inactivate sporulated oocysts (Long, 1993).

Pathology and pathogenicity

Because coccidia replication and thus coccidiosis is self limiting, often the number of ingested oocysts is the major factor in morbidity, severity, and/or mortality. This is in contrast to other protozoal diseases including trichomoniasis and histomoniasis which can cause morbidity and mortality after infection with a single parasite (Stabler and Kihara, 1954; Liebhart et al., 2008). Generally, in natural settings of wild birds and mammals, coccidia infections are transient and only a few oocysts are ingested due to natural animal dispersal and animal density. However, under confinement, a susceptible host can ingest a large number of oocysts in a short time period leading to increased morbidity or mortality. The severity of the lesions produced by the parasites are a function of numerous factors including the species of coccidia, number of ingested oocysts, the immune status of the host, the age of the host, and nutritional status and concurrent diseases of the host (Long, 1993). The intestinal epithelial cell damage results in interruption of feeding and digestive processes with subsequent decreases in nutrient absorption, growth, and a decline in the overall health of the host. Dehydration, anemia, and increased susceptibility to other disease agents are common sequelae of coccidiosis (McDougald and Hu,

2001; McDougald, 2008). In severe cases of coccidiosis, mortality can occur. Outbreaks causing 25-50% mortality in the flock are not uncommon (McDougald, 2008).

Several factors are associated with the pathogenicity of coccidia including dose of oocysts, viability of oocysts, site of development, age of bird, concurrent disease, bacteria flora, and nutritional status (Fernando, 1982). Gross lesions can vary from mild catarrhal enteritis to multifocal erythematous regions to severe hemorrhage and possible necrotic caseous material (cores) within the intestinal and cecal lumens. Depending on the severity and chronicity of the infection, microscopic lesions include mild to severe caseous and coagulative necrosis with variable numbers of mononuclear and granulocytic inflammatory infiltrates admixed with hemorrhage, fibrin, and/or fibrous connective tissue. Heterophils are found as early as 65 hr post infection and are found in the crypt lumen immediately following the first stage generation schizonts. Lymphocytes are often seen later in the infection, especially during the maturation of second to third generation schizonts. The lesions are generally most severe in about 5 days post infection that corresponds to the late stages of merogony (Fernando, 1982). The various coccidian life stages may be observed within the affected areas and to a lesser extent unaffected areas.

During coccidia infection of the host's intestines, changes in the physiological functions of the intestine contribute to the lesions produced. Several studies have demonstrated a decrease in intestinal pH during coccidia infection (Stephens et al., 1974; Ruff et al., 1975) and this pH decrease was unaffected by oocyst dose or severity of infection. The increase acidity of the intestines led to a decrease in absorption of vitamin A and xanthophyll (Ruff et al, 1974, Stephens et al., 1974). It was suggested that this loss of plasma xanthophylls in coccidia-infected chickens is associated with problems in transport mechanisms rather than difficulties in

absorption (Yvone and Mainguy, 1972). Along with decrease in transport, the decrease of sodium and chloride was concurrent with the appearance of mucoid diarrhea (Allen et al., 1973). Methemoglobin is increased in coccidia infection in birds (Mathis et al., 1984) and may contribute to the pathogenic effect of coccidia since elevated methemoglobin is associated with decreased ability of oxygen transport in blood. Death is considered to occur by shock that occurs due severe hypoproteinemia, the electrolyte disturbances, and the marked reduction in extracellular fluid (Hein, 1971).

In bobwhites, coccidiosis associated with *Eimeria lettyae* produced up to 89% and 25% mortality in 5-day-old bobwhites at doses of 1×10^6 and 5×10^5 oocysts, respectively (Ruff and Wilkins, 1987). In 2 ½ week-old bobwhites, the aforementioned dosages caused 56% and 0% mortality, respectively. Coccidia in game birds are often more prolific than those in chickens and turkeys and as high as 600,000 to 2,000,000 oocysts produced per oocyst given (Ruff, 1985). Oocyst production generally extends longer (Tyzzer, 1929; Ruff, 1985). *E. lettyae* oocysts production may continue to occur up to 60 days after inoculation (Ruff, 1985).

Diagnosis and distinction of *Eimeria* species

Different species of *Eimeria* were historically diagnosed based on classical techniques including sporulated oocysts morphology (size, shape, color, wall characteristics, and presence or absence of a micropyle, residual body, or polar granules), pre-patent periods, sporulation time, gross lesions and histological lesions produced by variable dose levels of the *Eimeria* sp., the time course, location, and progression of schizonts, meronts, microgametocyte, macrogametocyte, and zygote development within the intestines (Tyzzer, 1943). Additionally, cross transmission infection studies and the ability to immunize animals with putative novel

Eimeria species against challenges with other *Eimeria* spp. known to infect the particular host are useful techniques.

Recently, molecular techniques have been used to compare genetic sequences of different coccidia and create PCR primers for diagnostic testing (Su et al., 2003; Schnitzler et al, 1998). Previous investigations have demonstrated analysis of the internal transcribed spacer (ITS) regions useful in molecular comparison and distinguishing intraspecific variations in *Eimeria* and other protozoa including trichomonads (Felleisen, 1997), *Perkinsus marinus* (Brown et al., 2004), and *Entamoeba* spp. (Som et al., 2000). PCR testing has several advantages compared with historical diagnostics in coccidia identification, including reduction in research cost (housing and feeding of birds) and turnaround of results because PCR results can be obtained in hours compared with several weeks with traditional testing. Additionally PCR testing can allow for sequence and phylogenetic analysis of isolates aiding in determining the epidemiology and evolutionary relationships among isolates.

Although, coccidiosis outbreaks have been reported from free-ranging wildlife (Farr, 1965; Yabsley et al., 2002; Yabsley 2008), the disease generally is observed in domestic animals raised in confinement (Reid et al., 1984). This low risk of disease in wild animals is due to two reasons: 1) parasite replication is self-limiting given the fixed number of merogony cycles and 2) there is a low level of initial exposure and after infection, the host develops protective immunity. The lesions produced by the parasites are a function of the number of ingested oocysts, the immune status and age of the host, the site of infection, and other factors. Often coccidiosis outbreaks in free-ranging animals are associated with artificial situations including aeration systems at lakes harboring large number of birds (NWHC, unpublished data). Additionally wild animals gathering at artificial food sources can lead to ingestion of fecal material and thus

amplification of oocysts and leading to potential morbidity (Ruff et al., 1988; Gerhold and Ruder, unpublished data).

Coccidia in Northern bobwhites

Only a scarce amount of research has been performed on coccidia of captive and free ranging Northern bobwhite and often this work is incomplete or contradicting and frequently both. *Eimeria dispersa*, *E. lettyae*, and *E. colini* are the three relatively well described species from Northern bobwhite (Tyzzer, 1929; Levine, 1953; Fisher and Kelley, 1977; Ruff, 1985; Ruff and Wilkins, 1987). *Eimeria lettyae* is apparently the most pathogenic coccidia species in Northern bobwhites (Ruff, 1985). An unnamed *Eimeria* sp. capable of causing disease was reported from a quail farm in Georgia (Prostowo and Edgar, 1970) and a separate unidentified *Eimeria* was reported from quail in South Carolina (Waggoner, 1967). Venard (1933) reported finding oocysts morphologically consistent with *E. dispersa*, *E. tenella*, and *E. acervulina* from the intestinal contents of sixty-seven wild Northern bobwhites in Ohio; interestingly, the two later species are known to infect domestic chickens. Sporulated oocysts of the putative *E. tenella* and *E. acervulina* isolated from the bobwhites were administered to chickens and clinical disease, cecal lesions, and oocyst production was observed in chickens infected with putative *E. tenella*; however, chickens infected with the putative *E. acervulina* did not develop clinical signs, lesions, or oocysts, suggesting that the coccidia were mis-identified. In contrast, Vetterling (1976) reported that Northern bobwhites, Rock partridge (*Alectoris graeca*), Japanese quail (*Coturnix japonica*), Wild turkeys (*Meleagris gallopavo*), Ring-necked pheasants (*Phasianus colchicus*), Indian peafowl (*Pavo cristatus*), and Guinea fowl (*Numidia meleagris*) all failed to produce oocysts when infected with *E. tenella* isolated from domestic chickens. Patterson (1933) found similar negative results when he attempted to infect bobwhites with *E. tenella* and

E. acervulina. In the same study, Patterson was unable to infect domestic chickens with *E. dispersa* from bobwhites. It is doubtful that these two *Eimeria* spp. collected from bobwhites in Ohio were *E. tenella* and *E. acervulina*. A thorough investigation of the coccidia of bobwhites using molecular analysis is needed to elucidate the epidemiology and potential of cross infection of the different *Eimeria* spp.

Rare surveys of *Eimeria* spp. in wild Northern bobwhite disclosed varying prevalences. Duszynski and Gutierrez (1981) did not observe any oocysts from the intestinal contents of ten wild bobwhites from Roosevelt County, New Mexico. Williams et al. (2000) and Kocan et al. (1979) reported *Eimeria* spp. oocysts from 36% (N=9) of bobwhites from Eastern Kansas and 28% (N=30) of bobwhites from Oklahoma, respectively. The *Eimeria* spp. in these surveys were not identified, and no attempts were made to infect other hosts or to determine their pathogenicity by experimental infection. Coccidiosis has become a major issue in captive raised bobwhites. Historically, wild bobwhites have been an important game species and approximately 1 million hunters pursue bobwhites each year (USFWS, 2006). Due to the decrease in free-ranging Northern bobwhite populations in the southeastern United States due to habitat fragmentation and destruction, and increase of non-native and native predators (Droege and Sauer, 1990; Mueller et al., 1999), captive bobwhites are often raised to flight ready and then released for hunting. Some farms in the USA produce upwards of one million birds annually for this market (NAGA, pers. comm.).

Intestinal coccidiosis in the Chinese ring-necked pheasant (*Phasianus colchicus*) is caused by several species of *Eimeria* including *E. langeroni*, *E. duodenalis*, *E. pacifica*, *E. dispersa*, *E. phasiani* and *E. colchici* (Norton, 1976). *Eimeria phasiani* and *E. colchici* are considered the most pathogenic species in Ring-necked pheasants and gross lesions consisting of

caseous cecal cores and hemorrhagic typhlitis is often seen in severe infection (Norton, 1976). As pheasants are reared in large numbers for sale to sporting plantations (5-7 million/year), the farms where they are started and reared to 'flight ready' are also large (McQuiston, 1987). Some farms in the USA produce upwards of one million birds annually for this market. Coccidiosis is common during pheasant-rearing in the USA and United Kingdom (Swarbrick, 1985; McQuiston, 1987).

Levine (1939), discovered that *E. tenella* infections could be controlled with sulfanilamide and from this discovery, chemotherapy became a major focus for research. The development of chemotherapy against coccidia is a major milestone in the evolution of the poultry industry and without the use of anticoccidial compounds the broiler industry as we know it would not exist (McDougald, 1993). Anticoccidial compounds generally fall into one of two categories. The first are polyether ionophores which disrupt the proper intra and extracellular concentrations of the various cations and leads to cellular dysfunction. The second group includes compounds that cause an enzymatic reaction. The various classes of anticoccidial compounds and their mechanism of action are listed below.

Polyether ionophores fall into one of five categories including monovalent, monovalent glycosides, divalent, divalent glycosides, and divalent pyrrole ethers. Their mode of actions is to form lipophilic complexes with alkali metal cations and to transport these ions across biological membranes (Berger et al., 1951; Mehlhorn et al., 1983; McDougald, 1993). Anticoccidial protection from ionophores was unknown until testing and identification of monensin (Shumard and Callender, 1967). Mehlhorn et al (1983), found that only merozoites were susceptible to ionophores and that the drugs had no effect on intracellular stages or sporozoites. The end result is that the cell is unable to maintain the proper intra and extracellular concentrations of the

various cations which leads to cellular dysfunction. A main way this occurs is by the ionophores interfering with the K^+/Na^+ pump osmolarity. Ionophore drugs also allow some cycling of the coccidia in the bird which aids in producing immunity. Ionophores generally have lower rate of resistance development compared to the enzyme reaction drugs and often allow some low level cycling of the coccidia in the host leading to host immunity (Jeffers, 1978). Anticoccidial drugs belonging to the polyether ionophores that are used in poultry include lasalocid, salinomycin, maduramicin, monensin, narasin, lonomycin, and semduramicin.

Sulfonamides are folate antagonists and their mechanism of action (MOA) occurs by competitive inhibitors of PABA in the dihydropteroate synthetase reaction to make folate (Walter and Konigk, 1974). Normally, folate is reduced to tetrahydrofolate, using NADPH as a co-factor, and is used to create and methylate DNA. 2,4-diaminopyrimidines act in a similar fashion and block the reduction of folate to tetrahydrofolate. The combination of sulfonamides and 2,4-diaminopyrimidines is synergistic and is active on first and second generation schizonts and perhaps sexual stages as well (Wang et al., 1975). Amprolium is a thiamine antagonist and acts by competitively inhibiting the active transport of thiamine (Cuckler et al., 1961; James, 1980).

Several compounds have an MOA of inhibiting the mitochondrial energy metabolism at the cytochrome level including the 4-Hydroxyquinolones (buquinolate, methyl benzoquate, and decoquate) and clodolol (Wang, 1976). Although these compounds have similar MOAs, there is no cross resistance between the two classes, so they are likely effective at a different point in the mitochondrial energy metabolism (McDougald, 1993).

Many of the other compounds used in controlling coccidiosis have unknown MOAs. These compounds include nicarbazin, diclazuril, toltrazuril, butynorate, halofuginone, and nitrofurans (McDougald, 1982). In other compounds little is known regarding the MOA including robenidine which inhibited oxidative phosphorylation in rat livers (Wong et al., 1972). The biochemical action of nitromide, aklomide, and zoalene are not known, but are assumed to be nicotinamide antagonists (Joyner, 1960). Glycarbylamide and arprinocid are thought to be purine antagonists, however, they are no longer used due to rapid resistance development (Ball, 1966). Compounds belonging to organic arsenicals, including roxarsone, arsanilic acid, and arsenosobenzene, are suggested to possibly induce apoptosis due to cell signaling interference (Huang et al., 2002).

The efficacy of three anticoccidial compounds for the control of *E. dispersa* and *E. lettyae* in pen-raised quail was previously examined by Ruff et al., (1987). The investigators disclosed that 0.008% monensin or 0.0055% salinomycin prevented depression of weight gain when infected with 1×10^6 sporulated *Eimeria* oocysts. However, amprolium was ineffective. Similar results with amprolium were found in Japanese quail and Ring-necked pheasants (Ruff, 1986). Currently only monensin and salinomycin are approved for use in bobwhites (Feed additive compendium, 2007). Although numerous anticoccidial compounds are currently available and are effective in controlling coccidiosis in poultry (McDougald, 1993), experiments determining the efficacy of these compounds in bobwhites have not been performed. Although excellent protection was observed in pheasants treated with lasalocid and challenged (Fuller et al., 2008), extrapolating effective levels of compounds from previous research in other avian species has been shown to be unreliable, and it is imperative to test compounds in each target species to determine the most efficacious concentration (McDougald, 1982). It is necessary to obtain data

from several compounds, allowing rotation of the compounds to minimize breaks in drug resistance leading to coccidiosis outbreaks (Jeffers and Bentley, 1980; McDougald, 1982; and McDougald et al., 1986).

Eimeria are generally immunogenic and a single infection in an immunocompetent host will induce immunity to reinfection to some degree. However, exceptions to this generality have been noted (Todd and Hammond, 1968; Versenyi and Pellerdy, 1970). Immunity in coccidia occurs primarily as a result of the asexual replication, with the sexual stages contributing little additional protection (Rose and Hesketh, 1979). Immunity to a challenge inoculum is manifested as a reduction in clinical signs and reduced multiplication of the parasite (Rose, 1982). Circulating antibodies are effective at opsonization and enhancing the uptake of parasites by macrophages (Rose, 1982). The role of cell-mediated immunity has been shown to be the most important factor in host protection (Liburd et al., 1973). In this study, the researchers demonstrated that thoracic duct lymphocytes collected from infected donor rats were effective at protecting naïve recipient rats infected with *Eimeria nieschulzi*. Adoptive transfer of lymphocytes from *Eimeria* immunized chickens to naïve chickens leads to protection when challenged (Rose and Hesketh, 1979). Further research demonstrated that CD8⁺ cells were the most important for immunity especially for secondary infection in *E. acervulina* and *E. tenella* (Trout and Lillehoj, 1996). The authors also noted that anti-CD4⁺ treated chickens produced significantly greater number of oocysts during primary infection compared to the control. However, the oocyst production in secondary infection was not different in the anti-CD4⁺ treated chickens compared to the controls. An interesting finding in this research is that anti-CD8⁺ treated birds produce fewer oocysts after primary infection and the authors suggested this was due to fewer numbers of lymphocytes available for transporting sporozoites.

Live vaccines, consisting of infective oocysts of the important *Eimeria* species, are available for use in the poultry industry, providing an alternative to the use of anticoccidial drugs (McDougald, 2008). The development of vaccines for coccidia is possible due to the fact that replication is self-limiting and infected birds develop protective cell-mediated immunity (Shirley et al., 2005). Protective immunity develops rapidly after exposure, but depends on reinfection to reinforce the developing protection. There is no confirmed cross-protection between different species of *Eimeria* resulting in the requirement for multiple species of coccidia in vaccines. Vaccines are usually administered to day-old birds at the hatchery. Some vaccines are based on low doses of virulent organisms, while others contain strains attenuated by selection for the shortened life cycle. By selecting the earliest produced oocysts, strains were selected that genetically have mutated to skip the later stages of merogony (McDougald and Jeffers, 1976) and can be propagated to create strains of coccidia that immunize the host without causing clinical morbidity. Previous work disclosed that bobwhites immunized 25 times with either 100 or 1,000 *E. lettyae* oocysts did not stop oocyst production completely when challenged with a high dose of *E. lettyae* (Ruff et al., 1985). However, these trials did not determine whether the birds would be protected against clinical disease. The limited data from previous publications suggests that related game bird species (i.e. Ring-necked pheasants) develop protective immunity after initial infections with a low-dose of coccidia (Liou et al., 2001). No vaccines strains specific for Northern bobwhite *Eimeria* spp. or other gamebirds are currently available.

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CHAPTER 3

DETECTION AND SURVEILLANCE OF *EIMERIA* SPECIES IN NORTHERN BOBWHITES USING SPECIES SPECIFIC PRIMERS¹

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ABSTRACT: Coccidiosis is an important disease in captive gamebirds including Northern bobwhites (*Colinus virginianus*). *Eimeria lettyae*, *Eimeria dispersa*, and *Eimeria colini* are the three described species in bobwhites. However, distinguishing the various *Eimeria* spp. is often problematic due to similarity in oocyst morphology. Live bird infections are needed to distinguish the three species. To aid in parasite detection and distinction, 31 coccidia positive samples were collected from diagnostic cases or litter from captive bobwhite facilities originating from 11 states. From a subset of these samples, DNA amplification of the internal transcribed spacer region 1 (ITS-1) of the ribosomal RNA gene was performed using *Eimeria* genus-wide PCR primers. PCR products were cloned, sequenced, and aligned. Sequence alignment and phylogenetic analyses of the rRNA-ITS-1 region indicate that Northern bobwhites are hosts to three molecularly distinct *Eimeria* species. Species-specific ITS-1 sense and antisense PCR primers were constructed and matched to the appropriate previously described *Eimeria* spp. using classical techniques or reference strains. The primers were used to detect the three *Eimeria* spp. in the collected 31 samples. The PCR survey results disclosed *E. lettyae*, *E. dispersa*, and *E. colini* in 20 (64.5%), 22 (73.3%), and 29 (93.5%) of the samples, respectively. Thirteen (41.9%) samples had 3 *Eimeria* spp. detected, 14 (45.2%) samples had 2 spp. detected, and 4 (12.9%) samples had 1 sp. detected. Flock age or geographical location was not associated with the presence of particular *Eimeria* spp.

Introduction

Northern bobwhites (*Colinus virginianus*) have been an important game species and approximately 1 million hunters pursue bobwhites each year (USFWS, 2006). Free-ranging bobwhite populations have significantly decreased in the southeastern United States due to various reasons including habitat fragmentation (Droege and Sauer, 1990). To compensate for natural population declines and produce quail for recreational hunting, Northern bobwhites are often raised in confinement to 'flight ready' and subsequently released for hunting. It is estimated that 30-40 million bobwhites are raised in captivity and then released. Some farms in the USA produce upwards of one million birds annually for this market (NAGA, pers. comm.). Raising game birds in these densities facilitates the transmission of pathogenic organisms especially coccidia (Ruff, 1985; Ruff, 1986).

Coccidiosis is common in captive game birds for the same reasons it is important in the poultry industry, including bird density and high prolific rates of parasite replication (McDougald, 2008). Although coccidiosis is responsible for major economic losses in the game bird industry, the *Eimeria* species infecting captive-reared game birds are incompletely known. *Eimeria colini*, *Eimeria dispersa* and *Eimeria lettyae* are the three described species from Northern bobwhites (Tyzzer, 1929; Fisher and Kelly, 1977; Ruff, 1985). There are two reports of other unnamed *Eimeria* species (Waggoner, 1967; Prostowo and Edgar, 1970). All of the reported species above share similar morphological features including length to width size indices, high intraspecies size variability, lacking polar granules, and having an inconspicuous or no micropyle. Species identification often requires determining the pre-patent and sporulation periods, pathogenicity of graded doses in the host, and cross species transmission trials.

Alternative to classical techniques, molecular diagnostics involve detection of species using nucleotide primers to amplify DNA specific to the organism of interest. Given that PCR is becoming common place in veterinary diagnostic laboratories, molecular distinction and detection of *Eimeria* spp. is feasible if primers are available. The goal of our study was to construct PCR primers to specifically amplify and identify the various *Eimeria* spp. in Northern bobwhites and use these primers to survey the various *Eimeria* spp. from captive bobwhite facilities throughout the United States.

MATERIAL AND METHODS

Parasite collection and propagation. Fecal, intestinal, or litter samples were collected from 31 captive-reared Northern bobwhite facilities from various locations throughout the United States (Table 3.1). The samples were sieved through cheesecloth, placed in 2% potassium dichromate, and aerated to encourage sporulation for at least 48 hrs. The presence of *Eimeria* spp. in the aerated material was determined by microscopic examination. If no oocysts were observed, oral infections of sieved material were administered as a blind pass to the quail, raised in strict isolation to avoid coccidia contamination. Repeated propagation of the cultures was conducted and oocysts were morphologically examined and enumerated using a McMaster's chamber. Propagated samples were stored in 2% potassium dichromate at 10 C until further use.

DNA amplification and primer development. Following removal of the potassium dichromate by repeated washing and centrifugation, oocysts were concentrated using saturated NaCl solution. DNA amplification by PCR of the internal transcribed spacer-1 (ITS-1) region of the ribosomal RNA (rRNA) was performed using *Eimeria* genus wide primers BSEF and BSER (Schnitzler et al., 1998). PCR components included 1-2µl of oocysts in a 25-µl reaction

containing Ready-to-go PCR beads (GE Scientific, Piscataway, NJ) and 0.5 μ M of primers BSEF and BSER. Cycling parameters for the amplification were 95 C for 5 min followed by 40 cycles of 95 C for 15 sec, 45 C for 30 sec, and 72 C for 30 sec, and a final extension at 72 C for 15 min. A negative water control was included to detect contamination. PCR amplicons were separated by gel electrophoreses using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. An approximate 500 bp amplicon was excised and the DNA purified using a QIAquick® Gel Extraction kit (Qiagen Inc., Valencia, California) per the manufacturer's instructions. Extracted DNA was cloned using pCR®4-TOPO® plasmid (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Sequencing of the plasmid was performed using primers T3 and T7 at the Integrated Biotechnology Laboratories at the University of Georgia using an Applied Biosystems Inc., 3100 Genetic Analyzer.

Plasmid sequences were removed and the remaining sequences were subjected to BLAST analysis to ensure *Eimeria* genus sequences were amplified and cloned. The sequences were aligned using the multisequence alignment ClustalX program (Thompson et al., 1994). The DNA amplification procedure was replicated for 9 field isolates originating from multiple locations and four to six clones from each PCR amplicons were sequenced. To sort the various *Eimeria* sequences into like groups, phylogenetic analysis and bootstrapping distances were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 program (Kumar et al., 2007) utilizing the neighbor-joining and minimum evolution algorithms using the Kimura 2-parameter model. Bootstrap values of at least 95 were considered sufficient to separate clades into distinct species. Sense and antisense primers were constructed for each separate phylogenetic group by visually examining sequence alignments for nucleotide runs that were conserved among all sequences in a particular phylogenetic group but different from

sequences from other phylogenetic groups. Care was taken to minimize hairpins and dimers and to have similar melting temperatures in the antisense and sense primers whenever possible. The resultant primers were tested against a plasmid containing the DNA insert belonging to the phylogenetic group of the corresponding primers.

Matching primers and pure cultures. Pure cultures of *E. lettyae* were obtained by propagating few individually selected oocysts that were morphologically similar to those described by Ruff (1985). The propagated oocysts were confirmed to be *E. lettyae* by examining the pre-patent and sporulation periods, gross lesions and pathogenicity, performing cross-species infection trials, and by PCR and nucleotide sequencing as previously described (Gerhold et al., in press). Pure cultures of *E. dispersa* were obtained from previous propagated oocysts that were stored in liquid nitrogen in our laboratory. The correct primers for the putative *E. colini* were determined by process of elimination given there are three described species and we found three molecularly distinct sequence groups. Efforts to obtain reference strains of *E. colini* were unsuccessful.

Primer pairs were then matched to the corresponding species by using the propagated pure cultures (*E. lettyae* and *E. dispersa*) as template in a PCR reaction containing one of the species specific primer pairs. PCR components were the same as the aforementioned protocol as was the cycling parameters except the annealing temperatures of the individual primer pairs were set as shown in Table 3.2. The results of the PCR were used to determine which primer pair was specific to which *Eimeria* sp. and to ensure that cross-species detection did not occur with the primers. These species-specific primers were used to survey the collected and propagated quail coccidia samples to determine the distribution and frequency of the various *Eimeria* spp.

RESULTS

Thirty-one litter, fecal, or intestinal samples from captive quail facilities were collected from 11 states (Table 3.1). Oocysts were detected by microscopy in all samples at either time of submission or following one blind pass. Sequence alignment and phylogenetic analysis of rRNA-ITS 1 region indicate that Northern bobwhites contain three genetically distinct *Eimeria* species. Species-specific ITS-1 sense and antisense PCR primers were constructed to detect *E. lettyae*, *E. dispersa*, and *E. colini* (Table 3.2). Primer annealing temperatures ranged from 45-48 C and amplicon size ranged from 280-320 bp (Table 3.2).

Utilizing the species-specific primers, we detected *E. lettyae*, *E. dispersa*, and *E. colini* in 20 (64.5%), 22 (73.3%), and 29 (93.5%) of the 31 samples, respectively (Table 3.1). Thirteen (41.9%) samples had three *Eimeria* spp. detected, 14 (45.2%) samples had 2 species detected, and 4 (12.9%) samples had 1 species detected. Of the 14 samples containing two species, *E. dispersa* and *E. colini* were found in 7 (50%) samples, *E. lettyae* and *E. colini* were found in 6 (42.9%) samples, and *E. lettyae* and *E. dispersa* were detected in 1 (7.1%) sample. Detection of the various *Eimeria* spp. was not associated with flock age or geographical location.

DISCUSSION

The results of our study indicate the constructed PCR primers specifically detect and distinguish the three species of *Eimeria* in Northern bobwhites. The primer pairs of the three species have similar melting temperatures and thus likely would work well as a multiplex PCR reaction. However, the similarity in bp size would potentially make it difficult to distinguish the amplicon size by gel electrophoresis. Real time PCR machines are becoming more

commonplace in veterinary diagnostic laboratories which would allow for distinction of the PCR products. By being able to distinguish the various *Eimeria* spp. in quail, researchers can determine which spp. are most associated with morbidity and mortality and determine what vaccine strains should be produced.

Although there have been reports of 5 potential *Eimeria* spp. in bobwhites, only 3 have been well described. Our findings of 3 molecularly distinct *Eimeria* spp. suggest that the 2 unnamed *Eimeria* are not novel species. However, additional *Eimeria* spp. may be found if further molecular surveys of bobwhite coccidia are conducted. It is interesting that only 3 *Eimeria* spp. were found in bobwhites, given that chickens (*Gallus gallus*), turkeys (*Meleagris gallopavo*) and ring-necked pheasants (*Phasianus colchicus*) have 9, 7, and 7 species, respectively (Tyzzer, 1929, Norton, 1976; McDougald, 2008). In contrast Chukars (*Alectoris chukar*) have 2 reported species (Pellerdy, 1974). In our phylogenetic analyses we used a 95% bootstrap as a cutoff value to distinguish the various species. This value was chosen because phylogenetic analyses with Genbank-acquired ITS-1 rRNA sequences from seven chicken *Eimeria* spp. revealed that 95% was the lowest bootstrap value separating any two *Eimeria* species. The ITS-1 rRNA region was chosen for the molecular analysis for this study due previously documented use in distinguishing *Eimeria* spp. in chickens (Schnitzler et al., 1998) and the fact that the resultant sequences can be used for meaningful intraspecies phylogenetic analyses.

To our knowledge this is the first survey of coccidia in captive bobwhites. All of the samples contained coccidia organisms; however, oocysts were only detected from litter samples following examination of a blind pass. The majority of the samples (n=27; 87.1%) contained at least two species of coccidia. *Eimeria colini* was detected in 29 (93.5%) samples and was the

most frequently detected species in our study (Table 3.1). It is unknown if *E. colini* is pathogenic to quail. The least frequently detected (n=20; 64.5%), but most pathogenic species, was *E. lettyae* (Ruff and Wilkins, 1987). A survey of 109 pen raised wild turkeys from 12 locations disclosed that 74 (68%) of the birds were positive for coccidia (Ruff et al., 1988). Of the positive birds, *Eimeria meleagritidis* and *Eimeria gallopavonis*, which are two of the most pathogenic species, were the most frequently detected species. In contrast, McDougald et al. (1997) found *Eimeria acervulina* in 93% (n=40) and *Eimeria tenella* in 14% (n=6) of Argentina poultry farms. *Eimeria tenella* is the most pathogenic coccidia species in chickens, but was the least frequently detected species; whereas *E. acervulina* is mildly pathogenic, but was the most frequently detected species.

Rare surveys of *Eimeria* spp. in wild Northern bobwhite disclosed varying prevalences. Duszynski and Gutierrez (1981) did not observe any oocysts from the intestinal contents of ten wild bobwhites from Roosevelt County, New Mexico. Williams et al., (2000) and Kocan et al., (1979) reported *Eimeria* spp. oocysts from 36% (n=9) of bobwhites from Eastern Kansas and 28% (n=30) of bobwhites from Oklahoma, respectively. The *Eimeria* spp. in these surveys were not identified, and no attempts were made to infect other hosts or to determine their pathogenicity by experimental infection. It would be useful to use the species specific primers constructed in this study to determine the prevalence and distribution of the various *Eimeria* spp. in wild bobwhites from throughout their range. It is unknown if released quail can serve as a disease threat for wild quail; however, it would seem that coccidia would not be an issue in wild birds given the dispersal of wild animals and the self-limiting nature of coccidia infection.

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TABLE 3.1. List of *Eimeria* spp. isolates obtained from captive Northern bobwhites.

Isolate no.	State of origin	Bird age	<i>Eimeria</i> <i>lettyae</i>	<i>Eimeria</i> <i>dispersa</i>	<i>Eimeria</i> <i>colini</i>
1	GA	Adult	-	+	+
2	AL	Adult	-	+	+
3	AR	Undetermined	-	+	+
4	AL	Undetermined	-	+	+
5	AL	Undetermined	-	+	+
6	AL	Adult	+	-	+
7	MS	10 months	+	+	+
8	AL	12 months	+	-	+
9	MS	4 months	+	+	+
10	AL	9.5 months	+	-	+
11	LA	9 days	+	+	+
12	KS	1.5 months	+	+	+
13	KS	Adult	+	+	+
14	LA	7.5 months	+	-	+
15	MS	2 weeks	+	+	+
16	GA	2.5 months	+	+	+
17	WI	2 weeks	-	+	+
18	KY	Adult	+	+	+
19	PA	2 weeks	+	+	+
20	SC	2 months	+	+	+
21	MS	3.5 months	+	+	+
22	KY	3.5 weeks	+	+	+
23	PA	2 weeks	+	+	+
24	PA	1 month	-	-	+
25	PA	3 months	+	-	+
26	GA	4.5 months	+	-	+
27	MO	8 months	+	+	-
28	TX	1 month	-	+	-
29	IA	10 months	-	-	+
30	KS	2 months	-	+	+
31	MS	2.5 weeks	-	-	+

TABLE 3.2. Species specific PCR primers of *Eimeria* from Northern bobwhites.

Host	<i>Eimeria</i> spp.	Primer sequence	Annealing Temp (°C)	Amplicon size (bp)
Northern bobwhite	<i>Eimeria</i>	5'-ACATACTACTCCCCGTGC-3'	48	320
	<i>dispersa</i>	5'-TCCAGGACGCTCTTTCAG-3'		
Northern bobwhite	<i>Eimeria</i>	5'-ATTTCGCTGCGTCCTTCA-3'	45	307
	<i>colini</i>	5'-CCTGCATACTCCTTCAAA-3'		
Northern bobwhite	<i>Eimeria</i>	5'- GCGTATATAGTGACGCA-3'	45	280
	<i>lettyae</i>	5'-GCTTTGTTGGATTTTGTCG-3'		

CHAPTER 4

THE EFFICACY OF ANTICOCCIDIAL PRODUCTS AGAINST *EIMERIA* SPP. IN NORTHERN BOBWHITES¹

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Summary: To determine whether chemotherapeutic compounds available for use in domestic poultry are effective at controlling coccidiosis in Northern bobwhites (*Colinus virginianus*), we tested 13 chemotherapeutic anticoccidials including amprolium (250 ppm), clopidol (125 ppm), diclazuril (1 ppm and 2 ppm), decoquinate (30 ppm), lasalocid (120 ppm), monensin (90 ppm), narasin/nicarbazin (36/36 ppm), robenidine (33 ppm), roxarsone (50 ppm), sulfadimethoxine/ormetoprin (125/75 ppm), salinomycin (60 ppm), semduramicin (25 ppm), and zoalene (125 ppm and 150 ppm). Three tests were conducted using two replicates of 10 birds each: Infected, unmedicated controls (IUC) and medicated birds were challenged with 1×10^6 oocysts of a field isolate, consisting primarily of *Eimeria lettyae*. Subsequently, we tested clopidol, lasalocid, salinomycin, diclazuril (1 ppm), and monensin against mixed species field isolates containing *E. lettyae*, *E. dispersa*, and/or *E. colini*. Weight gain, gross intestinal lesions, severity of diarrhea, and feed conversion ratio (FCR) six days post infection were recorded. Lesion score, as previously reported, was unreliable as a measure of severity of infection, in comparison with weight gain, fecal scores, and FCR. Excellent to good efficacy was found with clopidol, decoquinate, diclazuril (1 and 2 ppm), lasalocid, narasin/nicarbazin, robenidine, sulfadimethoxine/ ormetoprin, and zoalene (150 ppm). Marginal protection was found using monensin, salinomycin, semduramicin, or a roxarsone /semduramicin combination. Amprolium, roxarsone, and zoalene (125 ppm) were ineffective at controlling coccidia. Two of the six isolates tested against diclazuril (1ppm) and clopidol demonstrated a high degree of resistance, but none of the six isolates was resistant to lasalocid. Four of the eight isolates showed mild to moderate and moderate to high resistance against monensin and salinomycin, respectively. These findings indicate that several available compounds are effective at controlling coccidiosis in bobwhites

Key words: Coccidia, Coccidiosis, Chemotherapy, *Colinus virginianus*, *Eimeria colini*, *Eimeria dispersa*, *Eimeria lettyae*, Northern bobwhite

Abbreviations: GLM= General linear model; *E. colini*= *Eimeria colini*; *E. dispersa*= *Eimeria dispersa*; *E. lettyae*=*Eimeria lettyae*; FCR= feed conversion ratio; NAGA=North American Gamebird Association; NC= not calculated, PI= days post infection; PPM= parts per million; PRC= Poultry Research Center. S.E.= Standard Error; UUC= uninfected, unmedicated control; IUC= infected, unmedicated control.

To compensate for Northern bobwhite (*Colinus virginianus*) natural population decreases (2, 10) and produce birds for recreational hunting, quail are often raised in confinement to ‘flight ready’ and subsequently released for hunting or population restocking. It is estimated that 30-40 million bobwhites are produced annually for release. Some farms in the USA produce upwards of one million birds annually (NAGA, pers. comm.). Raising quail in these densities facilitates the transmission of pathogens such as ulcerative enteritis, viral diseases, and coccidiosis (13, 15).

Coccidiosis is important in the poultry industry due to bird density and high prolific rates of parasite replication (9). Coccidiosis is also common for the same reasons during bobwhite and other captive game bird rearing (11, 13, 14, 15). The three described coccidia species from the Northern bobwhite are *Eimeria colini*, *Eimeria dispersa* and *Eimeria lettyae* (3, 4, 14, 18). There are reports of other unnamed *Eimeria* species, but these have not been confirmed (12, 19). Among these species, *E. lettyae* is the most pathogenic and has been associated with mortality and morbidity (16, 20). Currently, coccidiosis is controlled by prophylactic use of anticoccidial compounds, as is practiced in other poultry (15, 17). In previous studies, monensin and salinomycin were effective at controlling coccidiosis, and these are the only products currently approved for use in quail in the USA (1). Coccidia are known to develop drug resistance without proper drug rotation or shuttle programs (6, 7, 8, 9). Efficacy trials to determine which chemotherapeutic compounds are adequate at controlling coccidiosis in bobwhites are needed to provide veterinarians and game bird producers with effective control programs. This report describes the efficacy of 13 anticoccidial compounds in Northern bobwhites challenged with *Eimeria* spp.

MATERIALS AND METHODS

Experimental animals. Northern bobwhite eggs were purchased from a commercial quail producer (Wadley Quail Hatchery, Wadley, GA) and hatched at the University of Georgia's Poultry Research Center (PRC). Newly hatched birds were housed in colony cages in a room used only for brooding and given unmedicated gamebird starter feed and water *ad libitum* until experimental procedures commenced.

Experimental rations and medicaments. Quail starter feed was given unmedicated to both the uninfected unmedicated (UUC) and infected unmedicated (IUC) controls or was blended with medicated premix at the manufacturer's recommendation or at the desired concentrations. Medications used in this study include lasalocid (Avatec[®], Alpharma, Bridgewater, New Jersey), monensin (Coban[®], Elanco Animal Health, Indianapolis, Indiana), diclazuril (Clinacox[®], Huvepharma Inc., Peachtree City, Georgia), decoquinate (Deccox[®], Alpharma), roxarsone (3-Nitro 20[®], Alpharma), amprolium (Amprol[®], Merial Select, Gainesville, Georgia), clopidol (Coyden[®], Merial Ltd., St. Louis, Missouri), narasin + nicarbazin (Maxiban[®], Elanco Animal Health), sulfadimethoxine + ormetoprin (Rofenaid[®], Alpharma), robenidine (Robenz[®], Alpharma), salinomycin (Bio-cox[®], Alpharma), semduramicin (Aviax[®], Phibro Animal Health, Ridgefield Park, New Jersey), and zoalene (Zoamix[®], Alpharma).

Collection and propagation of *Eimeria* spp. Litter, intestinal, or fecal samples originating from Northern bobwhite farms from multiple locations throughout the United States were submitted to our laboratory in 500 ml plastic containers containing 2% aqueous potassium dichromate. To propagate the isolates, the fecal or intestinal material was sieved through cheesecloth and aerated for at least 48 hrs to encourage sporulation. The presence of *Eimeria*

spp. was determined by microscopic examination. If no oocysts were observed, we orally infected bobwhites in a blind pass and examined feces from the infected birds. Repeated propagation of the cultures was conducted and oocysts were morphologically examined and enumerated using a McMaster counting chamber.

Coccidia for the first three experiments originated from a farm in Missouri experiencing morbidity and mortality associated with coccidiosis. The majority of oocysts were morphologically similar to the original description of *E. lettyae* (14). In subsequent trials, we used field samples containing a mixture of *E. lettyae*, *E. dispersa*, and/or *E. colini*. The infection dosage was chosen after an assay, wherein groups of young bobwhites were inoculated with graded doses of oocysts. The chosen inoculum was 1×10^6 oocysts, which could be expected to produce significant effects on weight gain while causing little or no mortality (16).

Experimental procedures. A total of 5 tests were conducted. Birds to be infected were removed from the brooding room and randomly distributed to cages in Petersime broiler finishing batteries (Petersime Inc., Gettysburg, Ohio). Batteries consisted of 15 cages, arranged in 5 tiers of 3 cages each, with stainless steel feeding and watering troughs and droppings trays. The cages were modified with 0.5 inch wire mesh on the floor and sides to keep quail from escaping. In three initial experiments, the efficacy of amprolium (250 ppm), lasalocid (120 ppm), semduramicin (25 ppm), monensin (90 ppm), diclazuril (1 or 2 ppm), decoquinate (33 ppm), roxarsone (50 ppm), narasin + nicarbazin (Maxiban[®], 36/36 ppm), sulfadimethoxine + ormetoprin (Rofenaid[®], 125/75 ppm), robenidine (33 ppm), and zoalene (125 or 150 ppm) were evaluated. In these experiments, each treatment was replicated in 2 cages of 10 birds each. In the subsequent two experiments, the efficacy of selected anticoccidial compounds (lasalocid, salinomycin, diclazuril, monensin, or clopidol) was assessed against mixed field isolates. Each

treatment consisted of one unreplicated cage of 10 birds each. Treatments were randomized to cages in a complete block design. Drug treated and IUC birds were weighed, tagged with numbered wing bands, and orally inoculated with 1×10^6 sporulated oocysts from the respective isolates suspended in 0.5 ml of water. The UUC birds were weighed and given a sham inoculation of tap water. Feed was weighed as issued, and any remaining feed weighed at termination.

Six days after challenge, all birds were euthanized by cervical dislocation, individually weighed, and the intestines examined for gross lesions consistent with coccidia infection. The upper (i.e. duodenal and jejunum), lower (ileum), and cecal sections of the intestines were scored separately, based on a 0-3 scale constructed by the authors from prior oocyst titration assays with the isolates in birds of similar age to those used in this investigation. Diarrhea was estimated with a subjective score on a scale of 0-3, where 0=normal and 3 = most severe diarrhea, as previously described (5). Feed and water were given *ad-libitum* throughout.

Statistical analysis. Data were analyzed in SAS version 9.1 using a general linear model test at $\alpha = 0.05$. For initial trials statistical analysis was performed on all categories and for the subsequent trials only the mean weight gain were analyzed statistically. Means were separated after ANOVA by the Duncan's multiple range test.

Results

In some of the tests, lesion scores were difficult to assess and differed little between IUC and medicated treatments even when other parameters were greatly improved. The most reliable parameters were weight gain, FCR, and possibly fecal scores. In Test 1, the potentiated sulfa

product (Rofenaid[®]) was highly effective, with weight gains as good as the UUC, lesion scores greatly reduced, and fecal scores of 0 (Table 4.1). In comparison, diclazuril and robenidine were moderately effective, with significant improvement in all parameters over the IUC. Amprolium gave no improvement in comparison with the IUC.

In Test 2, diclazuril (2 ppm) improved weight gain better than the UUC, and significantly ($P<0.05$) reduced lesion and fecal scores (Table 4.2). Decoquinate, lasalocid, and zoalene (150 ppm), were moderately effective, based on the same parameters. Roxarsone and semduramicin gave some improvement in weight gain, but little improvement in lesion scores (not significantly different).

Rofenaid was the most effective product in Test 3, with weight gains near normal and lesion and fecal scores significantly reduced in comparison with IUC and UUC (Table 4.3). The combination product (narasin/nicarbazin) also gave significant improvement in weight gain and reduction of lesion and fecal scores, in comparison with IUC and UUC. Zoalene (125 ppm), roxarsone, and monensin gave little improvement in any parameter.

A comparison of monensin and salinomycin against eight field isolates (Test 4) showed variable results. In most instances, monensin and salinomycin significantly or numerically improved weight gains relative to the IUC (Table 4.4). However, the efficacy of each product varied significantly between isolates. Weight gain for monensin treatments averaged 12.6 g/bird, compared with 12.5 g/bird for salinomycin, 3.3 g/bird for the IUC and 17.8 g/bird for the UUC, respectively. The two products were approximately equal in efficacy against isolates 8, 10, 16, and 20. Monensin gave better results with isolates 4 and 5 while salinomycin was more effective against isolate 22 and 24. Four of the eight isolates showed mild-moderate or moderate-high

resistance against monensin or salinomycin, respectively. Lesion scores did not appear to be a reliable measure of infection, as they did not correlate well with other parameters. Fecal droppings scores and feed conversion agreed better with mean weight gain.

In the final experiment (Test 5), the efficacy of lasalocid, diclazuril (1 ppm), and clopidol were examined against six isolates. The weight gains of the IUC treatment for the 6 isolates ranged from 10.1 to 15.8 gm, compared with 19.0 g/bird for the UUC (Table 4.5). In isolates 4 and 5, weight depression of the IUC were significantly different from the UUC ($P < 0.05$). Lasalocid improved weight gain with most isolates (mean 18.3 g/bird), but this only was significantly different from the respective IUC values in isolates 5 and 22. For diclazuril (1 ppm), the gain averaged 13.1 to 21.1 g/bird (mean 16.3 g/bird). The gain of clopidol treated birds ranged from 11.8 to 20 g/bird (mean of all isolates, 16.1 g/bird). Both clopidol and diclazuril were significantly different from the IUC value in isolate 5. All six isolates were sensitive to lasalocid, while two were tolerant to diclazuril (1ppm) and clopidol. As in previous tests, lesion scores did not correspond well with weight gain or fecal scores. Fecal scores and feed conversion ratios corresponded better with weight gains.

DISCUSSION

Several anticoccidial compounds mixed in game bird starter provided significant protection against coccidia infection in Northern bobwhites as shown by protection of weight gain after infection, and reduction in diarrhea (droppings scores). Efficacy was good-excellent with narasin+nicarbazin, sulfadimethoxine+ormetoprin, clopidol, decoquinate, diclazuril (1 and 2 ppm), lasalocid, robenidine, and zoalene (150 ppm). Marginal protection was found with

semduramicin, or semduramicin + roxarsone. Monensin and salinomycin were marginal in some tests, moderately good in others. Amprolium (250 ppm), roxarsone, and zoalene (125 ppm) were ineffective. Amprolium has historically been one of the most widely used anticoccidial compounds in game birds, probably accounting for apparent drug resistance in the tested coccidia. Ruff also found amprolium at 250 ppm to be ineffective in controlling coccidiosis in quail (17). The tested level of amprolium in the present study (250 ppm) is actually higher than the approved level (175 ppm).

The inoculum for the first three tests consisted of *E. lettyae* (5, 14). Other inocula were not pure cultures, and contained a mixture of this and other species. *E. lettyae* appears to be the most important species in quail in commercial production (14, 20). Our findings of morbidity, depressed weight gain, and minimal to moderate mortality using 1×10^6 oocysts of *E. lettyae* agrees with previous studies (16). The farm this isolate was acquired from reportedly did not have any history of anticoccidial drug use. Thus, the response to anticoccidial products might be expected to be unaffected by drug resistance. Other isolates were from farms where various anticoccidials had been used for some time, although records on this usage were incomplete or not available.

Our results indicate lesions scores corresponded poorly with weight gains. Similar findings were reported by others with *E. lettyae* in bobwhites (17). In other experiments, we have seen better correlation of lesion scores with other parameters (5). It is possible that lesion appearance could vary greatly with the exact time of necropsy relative to infection, and other factors. Because of the unreliability of lesion scores, more dependence was given to weight gains and fecal scores. We would not recommend the use of lesion scores for coccidiosis in this host for future studies.

Monensin (90 ppm) and salinomycin (55 ppm) were tested more extensively than other products because they are approved for use in bobwhites (1). Overall, both of these compounds provided good to moderate protection against some isolates as evidenced by the improvement in weight gain in comparison with infected controls. These results are consistent with previous reports on 80 ppm of monensin and 44 ppm of salinomycin in bobwhites (17). Four of the eight isolates had mild-moderate or moderate-high resistance against monensin or salinomycin, respectively, based on weight gains of medicated birds in comparison to the IUC. Similar conclusions were made with diclazuril (1ppm) and clopidol, with two of the six isolates demonstrating a high degree of resistance. None of the six isolates was resistant to lasalocid suggesting that lasalocid has not been used extensively in quail and may represent a possible future replacement for the other ionophores. The ionophores have more complicated modes of action than other drugs, with the result that resistance develops slower to these compounds than synthetic compounds (8). Lasalocid is approved for use in Chukars (*Alectoris chukar*) (1). In other poultry, the ionophores allow for some reproduction of coccidia in the host resulting in birds developing immune protection (8). Similar results could be expected in quail. Some of the synthetic chemical products were effective and could be used in quail feed if veterinary prescription can be obtained for specific applications. Fortunately, no adverse side effects were seen at the use levels employed in our tests. The effective use levels for anticoccidials in bobwhite quail were similar to those used in other poultry, except for amprolium, where a higher dosage in feed is required (175 ppm approved). To minimize resistance development, non-ionophores should be used sparingly and for short periods before switching to other products.

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TABLE 4.1. Efficacy of anticoccidial drug investigation with Northern bobwhites (Test 1).[†]

Treatment (dosage) ^A	Mean weight gain (gm) ^B	Percent wt gain ^C	Upper GI score ^D	Lower GI score ^D	Cecal lesion score ^D	Fecal score ^E	Feed Conversion
UUC	27.8 (± 1.4) ^{aF}	100	0 ^a	0 ^a	0 ^a	0 ^a	4.3 ^a
IUC	7.8 (± 1.3) ^c	28.1	2 ^d	0.2 ^a	2.7 ^b	3 ^c	11.8 ^b
Sulfadimethoxine/ormetoprin (125/75 ppm)	30.6 (± 1.4) ^a	110.1	0.4 ^{a b}	0.1 ^a	0.7 ^c	0 ^a	4.3 ^a
Diclazuril (1 ppm)	23.3 (± 1.4) ^b	83.8	0.6 ^b	0.1 ^a	0.7 ^c	1 ^b	4.9 ^a
Robenidine (33 ppm)	21 (± 1.4) ^b	75.5	0.9 ^c	0.1 ^a	1.4 ^d	1 ^b	5.3 ^a
Amprolium (250 ppm)	4.9 (± 1.4) ^c	17.6	1.6 ^d	0.2 ^a	2.7 ^b	3 ^c	27.9 ^c

[†] Two week old birds were placed on respective medicated feed two days prior to oral inoculation of 1×10^6 sporulated oocysts of *Eimeria lettyae*. Experiments were terminated six days post inoculation.

^A Indicates the final dosage of the compound mixed in game bird starter mash diet. UUC=Uninfected, unmedicated controls; IUC=Infected, unmedicated, controls.

^B Mean \pm S. E. of replicates for each treatment.

^C Gain as a percent of the UUC.

^D Mean gross lesion score (0=no lesion; 1=mild lesion; 2=moderate lesion; 3=severe lesions).

^E Mean fecal score (0=no diarrhea; 1=mild diarrhea; 2=moderate diarrhea; 3=severe diarrhea).

^F Values with different superscript letters are significantly different at $\alpha=0.05$.

TABLE 4.2. Anticoccidial drug investigation with Northern bobwhites (Test 2). [†]

Treatment (dosage) ^A	Mean weight gain (gm) ^B	Percent weight gain ^C	Upper GI score ^D	Lower GI score ^D	Cecal lesion score ^D	Fecal score ^E	Feed to gain conversion
UUC	18.4 (± 1.7) ^{ab F}	100	0 ^a	0 ^a	0 ^a	0 ^a	8.3 ^a
IUC	0.6 (± 1.6) ^e	3	1.6 ^b	0.8 ^b	2.5 ^b	3 ^c	41 ^d
Decoquinate (30 ppm)	13.3 (± 1.7) ^{bc}	72.3	0.9 ^b	0.4 ^{ab}	1.2 ^c	1 ^b	9.6 ^{ab}
Diclazuril (2 ppm)	19.8 (± 1.6) ^a	107.6	0.9 ^b	0.5 ^{ab}	1.4 ^c	1 ^b	7.1 ^{ab}
Lasalocid (120 ppm)	13 (± 1.6) ^c	70.7	1.2 ^b	0.6 ^{ab}	1.6 ^c	2.25 ^c	9.7 ^{ab}
Roxarsone (50 ppm)	4.8 (± 1.7) ^d	26.1	1.6 ^b	1.1 ^b	2.2 ^{bc}	2.75 ^c	25 ^c
Semduramicin (25 ppm)	9.3 (± 1.7) ^{cd}	50.5	1.3 ^b	0.6 ^{ab}	2 ^c	3 ^c	13.7 ^{bc}
Zoalene (150 ppm)	13.7 (± 1.7) ^{bc}	74.5	1.5 ^b	0.9 ^b	2.1 ^{bc}	2.5 ^c	8.6 ^{ab}

[†] Two week old birds were placed on respective medicated feed two days prior to oral inoculation of 1×10^6 sporulated oocysts of *Eimeria lettyae*. Experiments were terminated six days post inoculation.

^A Indicates the final dosage of the compound mixed in game bird starter mash diet. UUC=Unmedicated, uninfected controls; IUC=Unmedicated, infected controls.

^B Mean \pm S. E. of replicates for each treatment.

^C Gain as a percent of the UUC.

^D Mean gross lesion score of treatment group (0=no lesion; 1=mild lesion; 2=moderate lesion; 3=severe lesions).

^E Mean score fecal severity group (0=no diarrhea; 1=mild diarrhea; 2=moderate diarrhea; 3=severe diarrhea).

^F Values with different letter superscripts are significantly different at $\alpha=0.05$.

TABLE 4.3. Anticoccidial drug investigation with Northern bobwhites (Test 3). [†]

Treatment (dosage) ^A	Mean weight gain (gm) ^B	Percent weight gain ^C	Upper GI score ^D	Lower GI score ^D	Cecal lesion score ^D	Fecal score ^E	Feed to gain conversion
UUC	20.4 (\pm 2.2) ^{aF}	100	0 ^a	0 ^a	0 ^a	0 ^a	5.5 ^a
IUC	-2.3 (\pm 3.0) ^c	-11	1.5 ^{cd}	1.2 ^{bc}	2.3 ^c	3 ^d	NC ^G
Monensin (90 ppm)	6.8 (\pm 2.2) ^b	33.3	1.5 ^{cd}	1.3 ^{bc}	1.7 ^b	2 ^c	24 ^a
Zoalene (125 ppm)	0.95 (\pm 2.3) ^{b c}	4.7	1.6 ^d	1.5 ^c	1.8 ^b	2.5 ^c	155 ^b
narasin/nicarbazin (90 ppm)	17.21 (\pm 2.2) ^a	84.4	0.9 ^{bc}	0.6 ^{a b}	1.1 ^b	1.5 ^{bc}	8.6 ^a
Roxarsone (50ppm)/semduramicin (25ppm)	4.7 (\pm 2.3) ^{b c}	23	1.7 ^d	1.4 ^c	2 ^c	2 ^c	28.4 ^a
Sulfadimethoxine/ormetoprin (200 ppm)	19.6 (\pm 2.2) ^a	96.1	0.6 ^{ab}	0.3 ^a	0.4 ^a	0.5 ^{ab}	7.6 ^a

[†] Two week old birds were given feed treatments two days prior to oral inoculation of 1X10⁶ sporulated oocysts. Experiments were terminated 6 days post-inoculation.

^A Indicates the final dosage of the compound mixed in game bird starter mash diet. UUC= Uninfected, unmedicated controls. IUC=Infected, unmedicated, infected controls.

^B Mean \pm S. E. of all replicates for the treatment groups.

^C Gain as a percent of the uninfected control group

^D Mean gross lesion score (0=no lesion; 1=mild lesion; 2=moderate lesion; 3=severe lesions).

^E Mean score fecal (0=no diarrhea; 1=mild diarrhea; 2=moderate diarrhea; 3=severe diarrhea).

^F Values with different letter superscripts are significantly different at $\alpha=0.05$.

^G Feed conversion not calculated due to negative weight gain.

TABLE 4.4. A comparison of monensin and salinomycin for efficacy in Northern bobwhites (Test 4). [†]

Isolate	Treatment (dosage) ^A	Mean weight gain (gm) ^B	% Gain ^C	Upper GI score ^D	Lower GI score ^D	Cecal lesion score ^D	Fecal score ^E	Feed to gain conversion
-	UUC	17.8 (\pm 1.6)	100	0	0	0	0	8.3
	Monensin (90 ppm)	14.2 (\pm 2.9) ^{b F}	79.8	1.6	1.6	2.4	1	11.2
10	Salinomycin (60ppm)	11 (\pm 2.9)	61.8	2.1	2.1	2.4	2	12.5
	IUC	1.7 (\pm 2.7) ^a	9.6	2	2.1	2.4	3	82.5
	Monensin (90 ppm)	14.6 (\pm 2.6)	82	2.1	1.8	2.8	2	6.5
16	Salinomycin (60ppm)	17.3 (\pm 2.2)	97.2	1.8	2	2.1	1	11.8
	IUC	9.9 (\pm 2.4) ^a	55.6	2.2	2.5	2.7	3	55.8
	Monensin (90 ppm)	13.1 (\pm 2.3) ^{ab}	73.6	2	2.2	2.6	3	9.7
20	Salinomycin (60ppm)	9.9 (\pm 2.5) ^b	44.4	2.4	3	2.7	3	8.4
	IUC	-9 (\pm 2.3) ^a	-50	1.5	1.5	3	3	NC ^G

	Monensin (90 ppm)	7.7(<u>±</u> 2.7) ^{ab}	43.3	2	2.1	2	2	18.5
22	Salinomycin (60ppm)	13.1 (<u>±</u> 2.7) ^b	73.6	2.6	2.8	3	2	8.6
	IUC	-0.3 (<u>±</u> 3.0) ^a	-1.7	1.5	1.7	2.4	3	NC ^G
	Monensin (90 ppm)	18.9 (<u>±</u> 2.4)	106	1.5	1.8	1.8	1	7.1
24	Salinomycin (60ppm)	24 (<u>±</u> 3.7)	135	1.6	1.2	1.6	2	7.4
	IUC	5.4 (<u>±</u> 2.3)	30.3	1.6	1.8	2.4	2	13.9
	Monensin (90 ppm)	10.8 (<u>±</u> 2.4) ^b	60.7	1.6	1.8	2.6	2	6.5
4	Salinomycin (60ppm)	5.4 (<u>±</u> 3.8)	30.3	1.6	2	2.6	1	11.8
	IUC	2.5 (<u>±</u> 2.7) ^a	14	3	2.5	2.6	1	55.8
	Monensin (90 ppm)	11.7 (<u>±</u> 3.4)	65.7	2	2.2	3	3	7.4
5	Salinomycin (60ppm)	7.5 (<u>±</u> 2.4) ^a	42.1	2.1	2	2.2	2	18.7
	IUC	10.2 (<u>±</u> 2.4)	57.3	2.3	2.3	2.6	1	11.1

8	Monensin (90 ppm)	12.3 (± 2.4) ^b	69.1	1.9	1.9	2.3	2	8.9
	Salinomycin (60ppm)	11.9 (± 2.8) ^b	66.9	1.9	1.9	2.4	2	11.3
	IUC	6 (± 3.3) ^a	33.7	1.2	1.4	1.8	3	23.6

[†]Two week old birds were given feed treatments two days prior to oral inoculation of 1×10^6 sporulated oocysts. Experiments were terminated 6 days post-inoculation.

^A Indicates the final dosage of the compound mixed in game bird starter mash diet. UUC= Uninfected, unmedicated controls; IUC=Infected, unmedicated controls.

^B Mean \pm S. E. of replicates for the treatment groups.

^C Gain as a percent of the UUC group.

^D Average gross lesion score (0=no lesion; 1=mild lesion; 2=moderate lesion; 3=severe lesions).

^E Average score fecal (0=no diarrhea; 1=mild diarrhea; 2=moderate diarrhea; 3=severe diarrhea).

^F Values with “a” superscript are significantly different from UUC and values with “b” superscript are significantly different from the IUC value for the respective isolate at $\alpha=0.05$.

^G NC= not calculated because of negative weight gain.

TABLE 4.5. Anticoccidial drug investigation with numerous Northern bobwhite coccidia isolates (Test 5).[†]

Isolate	Treatment (dosage) ^A	Mean weight gain (gm) ^B	% gain ^C	Upper GI score ^D	Lower GI score ^D	Cecal lesion score ^D	Fecal score ^E	Feed to gain conversion
-	UUC	19.0 (\pm 2.5)	100	0	0	0	0	8.9
	Lasalocid (120 ppm)	16.3 (\pm 2.3)	85.8	1.3	0.9	1.3	1	8.5
4	Diclazuril (1 ppm)	13.1 (\pm 3.3)	68.9	0.8	0.5	1.6	2	16.1
	Clopidol (125)	11.8 (\pm 2.8)	62	1.4	1.3	1.6	2	15.8
	IUC	10.1 (\pm 2.3) ^{aF}	53.2	1.3	0.4	1.4	1	12.3
	Lasalocid (120 ppm)	17.5 (\pm 2.5) ^b	92	1.1	0.6	1.1	1	7.6
5	Diclazuril (1 ppm)	18.5 (\pm 2.4) ^b	97.4	1.3	1.5	1.7	1	6.7
	Clopidol (125)	20 (\pm 2.4) ^b	105.3	0.8	0.6	1.4	1	6.8
	IUC	11.7 (\pm 2.5) ^a	61.6	1.3	1.2	1.6	1	12.7
	Lasalocid (120 ppm)	15.9 (\pm 2.4)	83.7	1.3	1	1.5	1	8.5

10	Diclazuril (1 ppm)	17.4 (± 3.3)	91.6	1.2	1	1.3	1	7.8
	Clopidol (125)	12 (± 2.2)	63.2	1.7	1.5	1.8	2	9.5
	IUC	12.6 (± 2.3)	66.3	1.5	1.2	1.6	1	8.4
	Lasalocid (120 ppm)	15.6 (± 2.7)	82.1	1.1	1.1	1.1	1	9.5
16	Diclazuril (1 ppm)	18 (± 2.8)	94.7	1.9	1.6	1.9	1	10.3
	Clopidol (125)	19.1 (± 2.4)	101	1.5	1.3	1.6	2	8.5
	IUC	15.8 (± 2.8)	83.2	0.7	0.6	0.9	3	11.8
	Lasalocid (120 ppm)	18 (± 2.5)	94.7	1.4	0.8	1.7	2	12.9
20	Diclazuril (1 ppm)	21.1 (± 2.4)	111	1	0.8	1.2	0	9.6
	Clopidol (125)	14.6 (± 2.2)	76.8	1.3	1.2	1.2	1	11.7
	IUC	10.5 (± 2.4)	55.3	1.4	1.1	1.8	2	11.2
	Lasalocid (120 ppm)	26.2 (± 2.5) ^b	137.9	1.1	0.6	1.2	2	8.5

22	Diclazuril (1 ppm)	15 (\pm 2.3)	78.9	1	1	1.3	2	12.1
	Clopidol (125)	19.1 (\pm 2.2)	101	1.5	1	1.6	1	8.4
	IUC	14.2 (\pm 2.2)	74.7	1.3	1.3	1.4	2	13

[†] Two week old birds were placed on respective medicated feed two days prior to oral inoculation of 1×10^6 sporulated oocysts. Experiments were terminated six days post inoculation.

^A Indicates the final dosage of the compound mixed in game bird starter mash diet. UUC=Uninfected, unmedicated controls; IUC=Infected, unmedicated controls.

^B Mean \pm S.E. of all replicates for the treatment groups.

^C Gain as a percent of the UUC.

^D Average gross lesion score (0=no lesion; 1=mild lesion; 2=moderate lesion; 3=severe lesions).

^E Average fecal score (0=no diarrhea; 1=mild diarrhea; 2=moderate diarrhea; 3=severe diarrhea).

^F Values with “a” superscript are significantly different from UUC and values with “b” superscript are significantly different from the IUC value for the respective isolate at $\alpha=0.05$.

CHAPTER 5

LOW DOSE IMMUNIZATION OF NORTHERN BOBWHITES WITH *EIMERIA LETTYAE* PROVIDES PROTECTION AGAINST A HIGH DOSE CHALLENGE¹

¹R.W. Gerhold, A. L. Fuller , R. B. Beckstead , and L. R. McDougald

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Summary: To determine whether Northern bobwhite quail (*Colinus virginianus*) could be immunized against *Eimeria lettyae* by a low dose inoculation of oocysts, we inoculated 30 birds each with either 100 or 1,000 oocysts at two days of age (given orally by pipette). Four weeks after immunization, the immunized birds and unimmunized controls were challenged with 1×10^6 *E. lettyae* oocysts. Eight days after challenge, birds were killed, weighed, and intestines examined for gross lesions. Effectiveness of the immunization was measured by analyzing weight gain, intestinal lesions, severity of diarrhea, feed conversion ratio, and oocysts production. After challenge, birds immunized with 100 or 1,000 oocysts gained an average of 33.3 gm and 28.9 gm, respectively; whereas unimmunized challenged birds gained an average of 11.5 gm. Immunized quail produced approximately 99.7% fewer oocysts, had minimal gross intestinal and cecal lesions, had minimal diarrhea, and had a 50% lower feed conversion ratio compared to unimmunized challenged controls. These findings indicate that vaccination is a viable option for controlling coccidiosis in quail and that further research into vaccination is warranted.

Key words: Coccidia, *Colinus virginianus*, *Eimeria lettyae*, immunization, Northern bobwhite

Abbreviations: GLM= General linear model; *E. colini*= *Eimeria colini*; *E. dispersa*= *Eimeria dispersa*; *E. lettyae*=*Eimeria lettyae*; NAGA=North American Gamebird Association; PI= days post infection; PRC= Poultry Research Center.

Free-ranging Northern bobwhite (*Colinus virginianus*) populations have significantly decreased in the southeastern United States due to various reasons including habitat fragmentation and increases of non-native predators, especially fire-ants (1, 7). To compensate for this decrease and to produce quail for recreational hunting, Northern bobwhites are often raised in confinement to ‘flight ready’ and subsequently released for hunting or population restocking. It is estimated that 30-40 million bobwhites are raised in captivity and then released. Some farms in the USA produce upwards of one million birds annually for this market (NAGA, pers. comm.).

Coccidiosis is important in the chicken and turkey industries because of bird density and high reproductive rates of the parasites (6). Coccidiosis is also common for the same reasons during bobwhite-rearing in the USA (10, 11, 13). However, little work has been conducted on coccidia of captive bobwhites. To date, *E. colini*, *E. dispersa* and *E. lettyae* are the three described species from Northern bobwhites (2, 3, 10, 18). There are two reports of other unnamed *Eimeria* species (9, 19). Among these species, *E. lettyae* is the most pathogenic and has been associated with mortality and morbidity (12). Currently, coccidiosis in Northern bobwhites is controlled by chemotherapeutic use of anticoccidial compounds, particularly monensin and amprolium (13). However, coccidia are known to develop drug resistance without proper drug rotation or shuttle programs (4, 5, 6).

Live vaccines consisting of infective oocysts of the important *Eimeria* species are available for use in the poultry industry, providing an alternative to the use of anticoccidial drugs (6). Vaccination for control of coccidiosis depends on the self-limiting nature of the organism and the development of cell-mediated immunity (17). Because no cross-immunity exists between different species of *Eimeria* multiple species of coccidia are necessary in vaccines.

Previous work disclosed that bobwhites immunized 25 times with either 100 or 1,000 *E. lettyae* oocysts did not stop oocyst production completely when challenged with a high dose of *E. lettyae* (11). However, trials to determine whether such inoculations provided ample immune protection under practical conditions were not conducted. The present studies were conducted to study the protection afforded by low dose inoculation of bobwhite quail with oocysts of *E. lettyae*, to assess its potential for practical immunization of commercial flocks.

MATERIALS AND METHODS

Experimental animals. Northern bobwhite eggs were purchased from a commercial quail producer (Wadley Quail Hatchery, Wadley, GA) and hatched at the University of Georgia's Poultry Research Center (PRC). For propagation of parasites for use in these studies, newly hatched birds were placed into brooding chambers in a parasite free room and strict biosecurity protocols were followed to ensure no parasite contamination. Birds to be infected were removed from the brooding room and placed in sterilized infection chambers in a separate room. For purposes of immunization trials, newly hatched birds were randomly separated into two treatment groups including immunized and unimmunized. Immunized birds were placed into sterilized infection chambers equipped with brooding lamps and unimmunized birds were placed into a brooding chamber in the parasite free room until the challenge portion of the experiment. All birds were given nonmedicated quail starter feed and water *ad libitum*.

Identification and propagation of *Eimeria lettyae*. An intestinal sample originating from a Northern bobwhite quail farm in Missouri that experienced morbidity and mortality associated with coccidiosis was submitted to our laboratory. Examination of sporulated oocysts in the sample revealed that the majority of oocysts were morphologically similar to previous

descriptions of *E. lettyae* (10). To acquire pure cultures of *E. lettyae*, individual sporulated oocysts were selected by performing serial dilutions and microscopic examination. Three oocysts containing the morphological properties of *E. lettyae* were orally inoculated into a Northern bobwhite raised in strict isolation to avoid coccidia contamination. Seven days post infection, the inoculated bird was euthanized and the intestines dissected with sterile scissors and placed into a sterilized one-liter plastic container containing 2% potassium dichromate. During repeated propagation of the pure culture, various biological characters were observed, including prepatent and sporulation periods, gross pathology in the host, and general oocyst morphology.

Cross transmission studies were also performed by infecting five Wild turkeys (*Meleagris gallopavo*) and three Ring-necked pheasants (*Phasianus colchicus*), raised in strict isolation, with 10,000 oocysts each. Feces produced from days 5-9 PI were collected, sieved through cheesecloth, and the sediment examined for the presence of oocysts by direct microscopic exam and by examining a sampled diluted 1:10 in a saturated salt solution using a McMaster chamber at 100x magnification.

Additionally, an aliquot of the oocysts was used for DNA amplification by PCR to confirm that the sample was pure culture. Amplification of the internal transcribed spacer-1 (ITS-1) region of the ribosomal RNA (rRNA) was performed using *Eimeria* genus specific primers BSEF and BSER that anneal to the 3' end of the 18S short subunit (SSU) and 5' end of the 5.8S of the rRNA, respectively (16). PCR components included 2µl of oocysts in a 25-µl reaction containing 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 0.01% Triton X-100, 1.5 mM MgCl₂, 0.2 mM of each dNTP (Promega, Madison, Wisconsin), 0.75 U *Taq* DNA polymerase (Promega), and 0.5 µM of primers BSEF and BSER. Cycling parameters for the amplification were 95 C for 5 min followed by 40 cycles of 95 C for 30 sec, 45 C for 30 sec, and 72 C for 30

sec, and a final extension at 72 C for 15 min. A negative water control was included to detect contamination. PCR amplicons were separated by gel electrophoreses using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. An approximate 500 bp amplicon was excised and the DNA purified using a QIAquick® Gel Extraction kit (Qiagen Inc.) per the manufacturer's instructions. Extracted DNA was sequenced at the Integrated Biotechnology Laboratories, The University of Georgia, Athens, Georgia using amplification primers BSEF and BSER.

Immunization trials of bobwhite quail. At two days of age, 40 chicks each were inoculated with 100 or 1,000 sporulated oocysts in 0.25 ml water. Chicks were then placed into steam-sterilized infection chambers, given nonmedicated quail starter feed and water *ad libitum* and examined daily. At 28 days PI, inoculated and non-inoculated chicks were placed into modified Petersime broiler finishing batteries (Petersime Inc., Gettysburg, OH) consisting of 15 cages each, arranged in 5 tiers of 3 cages each, with stainless steel feeding and watering troughs. The cages were modified with 0.5 inch wire mesh on the floor and sides to keep quail from escaping. The four treatments consisted of 1) unimmunized, unchallenged (UI) and 2) unimmunized, challenged (UU), 3) immunized with 100 oocysts and challenged, and 4) immunized with 1,000 oocysts and challenged. Each was replicated in 3 cages of 10 birds each, for a total of 12 cages of birds. Challenged and infected control birds were weighed, wing banded with a unique identification number, orally inoculated with 1×10^6 sporulated *E. lettyae* oocysts suspended in one ml of water, and given nonmedicated quail starter feed and water *ad libitum*. Uninfected controls were weighed and given a sham inoculation of tap water. Feed was weighed as issued, and any remaining feed weighed at termination. Treatments were assigned to the various cages using a randomized complete block design.

Eight days after challenge, all birds were euthanized by cervical dislocation, individually weighed, and the intestines examined for gross lesions consistent with coccidia infection. The upper (i.e. duodenal and jejunum), lower (ileum), and cecal sections of the intestines were scored based on a 0-3 scale constructed by the authors from prior oocyst titration assays with *E. lettyae* in birds of similar age to those used in this investigation. The scoring system for gross lesions was as follows: 0=no lesions; 1=mild to moderate catarrhal enteritis but no attenuation of mucosal; 2= mild to moderate mucosal attenuation; 3= marked to severe mucosal attenuation. Similarly, the severity of diarrhea was scored on a 0-3 scale by subjectively examining the fecal droppings collected between days 5-9 post challenge. The fecal scoring system was as follows: 0=>90% of solid fecal matter; 1= \leq 90%, but >75 % percent solid fecal matter; 2= \leq 75%, but >50% solid fecal matter; and 3= <50% solid fecal matter. Lesion and fecal scoring were performed blindly by the same individual to allow consistency in scores. Unconsumed feed for all pens was weighed back, to determine feed consumption. Fecal materials were collected by cage on days 5-9 PI for oocyst counts. Feces were blended in known volumes of water. Oocysts were counted in aliquots of the blended fecal material using a McMaster chamber, and extrapolated to total oocysts/bird for the 5-9 day period.

Statistical analysis. Data were analyzed in SAS version 9.1 using a general linear model test with an alpha = 0.05 (15). Means were separated after ANOVA by the Duncan's (1955) multiple range test.

RESULTS

Parasite identification. The mean length and width (standard deviation) of 100 *E. lettyae* oocysts was 20.0 (\pm 2.5) x 16.9 (\pm 1.7) μ m. The prepatent and sporulation periods were 90-99

hrs and 21-25 hrs, respectively. Cross transmission studies did not yield any oocyst production in either Ring-necked pheasants or the Wild turkeys. The resultant sequence did not contain any nucleotide polymorphisms and indicated that only one *Eimeria* sp. was amplified by the *Eimeria* genus specific primers. The sequence will be published in a separate manuscript describing the phylogenetic relationship of the various bobwhite *Eimeria* spp.

Immunization trials. The infected controls (IUC) gained an average of 11.5 gm/bird during the eight day challenge period, compared to 27.9 g/bird for uninfected controls (UUC). Birds immunized with 100 or 1,000 oocysts and challenged gained an average of 33.3 gm or 28.9 gm (significantly improved in comparison with the IUC at $P < 0.0001$) (Table 5.1, Fig.5.1). The 100 oocysts immunization treatment groups had a significantly improved weight gain compared to 1,000 oocysts immunization treatment group ($P < 0.05$). Both immunized groups were not significantly different from the UUC group, although on average the 100-immunized group gained more weight than the UUC group. The immunizing dose did not appear to have a weight effect in immunized birds illustrated by no significant difference in pre-challenged weight between the UUC and the birds immunized with 100 or 1,000 oocysts.

Eimeria lettyae is known to infect the duodenum, jejunum, ileum, and ceca so examination of the entire intestinal tract was required (10). Gross lesion scores for the upper (i.e. duodenum and jejunum), lower (i.e. ileum), and cecal sections of the infected controls were significantly ($P < 0.0001$) reduced in the immunized treatment groups compared with IUC (Table 5.2). The upper intestinal and cecal lesions were significantly increased in the 1,000 oocysts immunization group compared to the 100 oocyst immunization group; however, the severity of the lower lesions were not significantly different between the two immunization groups. The fecal scores were significantly lower than those of the IUC ($P < 0.05$) (Table 5.3). Similarly,

oocyst production in the immunized groups was significantly reduced compared to IUC ($P<0.0001$). Feed to gain conversion was significantly improved in the immunized challenged birds compared to the IUC ($P<0.001$); however, neither immunized challenged group was significantly different than the UUC. Although the average fecal score, feed conversion, and oocysts production was lower for the birds immunized with 100 oocysts compared to those immunized with 1,000 oocysts, none of these parameters were significantly different from each other (Tables 5.2 and 5.3).

Discussion

In the present study, immunization of Northern bobwhites by low dose inoculations of *E. lettyae* provided significant protection against *E. lettyae* as shown by the suppression of decreased weight gain and the significant suppression of gross intestinal and cecal lesion scores, and oocyst production. Of particular interest was the dramatic decrease (99.7%) in oocyst production in the immunized groups compared with the IUC. This suggests that protective immunity develops after exposure to only a few live oocysts in the same manner as reported for chickens and turkeys.

The species *E. lettyae* was studied because it is apparently the most pathogenic coccidia species in Northern bobwhites (12, 20). Pure cultures of *E. lettyae* established using birds hatched at our facility and housed in strict isolation ensured no cross-contamination of other *Eimeria* spp. The oocyst size and morphology, pre-patent period and sporulation period was consistent with that reported previously by (10). Differentiation from *E. dispersa* was shown by cross- transmission studies in Wild turkeys and Ring-necked pheasants, both of which are known to be susceptible to infection with *E. dispersa* (8, 14). These studies did not yield any patent

infections. Similar to previous reports for *E. lettyae* (12), our titration assays disclosed that inoculation of birds with 1×10^6 caused severe morbidity with minimal to moderate mortality, justifying the choice of this dosage as the challenge dose. Sequence analysis of the ITS-1 region of the rRNA indicated that the propagated oocysts consisted of a pure culture.

Immunization doses of 100 and 1,000 oocysts were chosen as starting doses to determine the minimum needed dose to afford protection. Immunization against coccidia occurs via cell mediated immunity since this form of protective immunity has been previously demonstrated for other gallinaceous birds (17). As shown by studies in poultry, an initial dose of live oocysts stimulates a T-cell response and immune protection. By the use of low doses of live oocysts, protective immunity can be stimulated without causing weight loss or secondary diseases (6). The dose of 100 oocysts provided excellent protection; on average these challenged birds had marginally greater weight gain compared to the uninfected controls. Further experimentation should be conducted to determine whether a lower immunization dose (i.e. 10-50 oocysts) would afford adequate protection. Other studies are needed on practical means of dosing (feed treatment, spray cabinet, etc.). Other studies are needed on the effects of vaccination of birds on litter vs. wire floored cages. Another important consideration is whether the other species (*E. colini* and *E. dispersa*) should be included in proposed vaccines, and whether attenuation of the coccidia would be required for successful use in commercial production.

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TABLE 5.1. Average weight gain, percent weight gain, and feed conversion of Northern bobwhites.

Treatment group	Mean weight gain (gm) [*]	Percent weight gain (%) [†]	Feed to gain conversion ^{**}
Unimmunized uninfected	29.7 (± 5.5) ^{ab‡}	100	4.1 ^a
Unimmunized infected	11.5 (± 8.7) ^c	38.7	8.7 ^b
Immunized with 100 oocysts and challenged	33.3 (± 4.5) ^a	112.1	4.4 ^a
Immunized with 1,000 oocysts and challenged	28.9 (± 7.6) ^b	97.3	4.3 ^a

^{*}Represents mean \pm the standard deviation of all replicates for the treatment group.

[†](Mean weight gain of treatment group/ mean weight gain of unimmunized uninfected treatment)*100.

^{**} Grams of feed ingested per gram of weight gain.

[‡]Values with different letter superscripts are significantly different at alpha=0.05.

TABLE 5.2. Lesion scores of upper and lower small intestines and ceca of Northern bobwhites.

Treatment group	Upper small intestine lesion score*	Lower small intestine lesion score*	Cecal lesion score*
Unimmunized uninfected	0 ^{a†}	0 ^a	0 ^a
Unimmunized infected	2.4 ^b	2.2 ^b	2.2 ^b
Immunized with 100 oocysts and challenged	0.8 ^c	0.6 ^c	0.7 ^c
Immunized with 1,000 oocysts and challenged	1.3 ^d	1.0 ^c	1.5 ^d

* Mean gross lesion score of treatment group based on subjective scoring scale (0=no lesion; 1=mild lesion; 2=moderate lesion; 3=severe lesions).

† Values with different letter superscripts are significantly different at alpha=0.05.

TABLE 5.3. Fecal score and oocyst production in Northern bobwhites

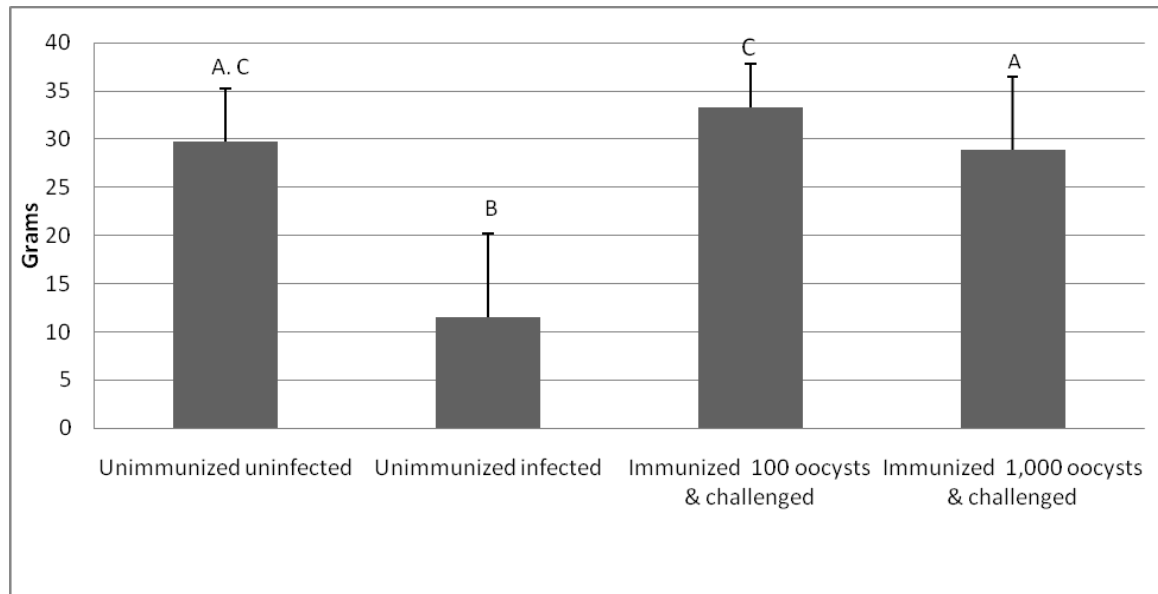
Treatment group	Mean fecal score*	Mean oocyst production**
Unimmunized uninfected	0 ^{a†}	0 ^a
Unimmunized infected	2.5 ^b	39.9 ^b
Immunized with 100 oocysts and challenged	1.0 ^a	0.10 ^a
Immunized with 1,000 oocysts and challenged	1.0 ^a	0.13 ^a

*Mean fecal consistency score of treatment group based on subjective scoring scale (0=>90% of solid fecal matter; 1= ≤ 90%, but >75 % percent solid fecal matter; 2= ≤ 75%, but >50% solid fecal matter; and 3= <50% solid fecal matter).

**Millions of oocysts produced per bird per day for days 5-9 post challenge

†Values with different letter superscripts are significantly different at alpha=0.05.

FIGURE 5.1. Weight gain of immunized and unimmunized Northern bobwhites. Average weight gains (gm) and standard deviations of immunized birds along with uninfected and infected controls are shown. Means with different superscript letters above error bars numbers are significantly different at $\alpha = 0.05$



Chapter 6

OOCYST PRODUCTION OF *EIMERIA LETTYAE* IN NORTHERN BOBWHITES FOLLOWING LOW DOSE INOCULATIONS¹

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ABSTRACT: Inoculation of bobwhite quail with low doses of *E. lettyae* oocysts stimulates a protective immune response, suggesting immunization may be an option for controlling coccidiosis. However, the oocyst production of inoculated birds could be considerable, leading to subsequent outbreaks. To determine the oocyst production following inoculation with *E. lettyae*, we orally infected 12-week old bobwhites with 100, 1,000, or 10,000 sporulated oocysts. Fecal materials were collected on days 5-9 post inoculation (PI) and total oocyst production counted in McMaster chambers. Oocyst production/bird was 49.75, 89.5, or 436×10^6 for 100, 1000, or 10,000 oocysts administered, respectively. Estimated oocysts produced/oocyst administered was 49.75, 8.95, or 4.36×10^4 for 100, 1000 or 10,000 oocysts administered, respectively. These findings not only illustrate the crowding effect of larger oocyst inocula, but also illustrate the fecundity of *E. lettyae* at low doses. This suggests that successful immunization of bobwhites against coccidiosis with live vaccines might require attenuated strains with reduced reproductive potential.

Captive propagation and rearing of game birds for subsequent released for hunting is common in North America and Europe. Some farms in the United States raise over 1 million Northern bobwhites quail (*Colinus virginianus*) per year (NAGA, pers. comm.). Coccidiosis is common in quail propagated in captivity (Ruff, 1986). Given the bird density and high reproductive rates of *Eimeria* spp., mortality events can often be dramatic, producing up to 50% morbidity and mortality in a flock. Northern bobwhites are hosts to three described *Eimeria* spp., *E. colini*, *E. dispersa* and *E. lettyae* (Tyzzer, 1929; Fisher and Kelley, 1977; Duszynski and Guthierrez, 1981; Ruff, 1985). There are two reports of other unnamed *Eimeria* spp. (Waggoner, 1967; Prostowo and Edgar, 1970). Among these species, *E. lettyae* is the most pathogenic and is the most often associated with mortality and morbidity (Ruff, 1986; Ruff and Wilkins, 1987; Yabsley, 2008).

Crowding as a factor in reproductive potential of *Eimeria* is well known in chickens (Brackett and Bliznick, 1952; Williams, 2001). Crowding occurs when the number of merozoites produced in the asexual cycles exceeds the available intestinal epithelial cells for parasitism (Tyzzer et al., 1932). The excess numbers of merozoites are excreted in the feces. Crowding generally increases as the number of oocysts administered is increased (Williams, 1973). The host immune response likely also contributes to crowding (Brackett and Bliznick, 1952).

We have previously demonstrated that inoculation of bobwhites with 100 oocysts of *E. lettyae* stimulates protective immunity without causing clinical signs, suggesting the practical use of live vaccination as is practiced in other poultry (Gerhold et al., in press). However, the reproductive potential of *E. lettyae* and effects of crowding has not been determined. The

purpose of this study was to determine the oocyst production of *E. lettyae* given three levels of inoculation and to further understand the biology of *E. lettyae*.

Northern bobwhite eggs were purchased from a commercial quail producer (Wadley Quail Hatchery, Wadley, GA) and hatched at the University of Georgia's Poultry Research Center (PRC). For propagation of parasites for use in these studies, newly hatched birds were placed into brooding chambers in a parasite-free room. Strict biosecurity protocols were followed to ensure no parasite contamination. Birds to be infected were removed from the brooding room and placed in sterilized infection chambers in a separate room. All birds were given nonmedicated quail starter feed and water *ad libitum*.

Pure cultures of *E. lettyae* were acquired and identified as previously described (Gerhold et al., In press). Briefly, individual sporulated oocysts were isolated by serial dilutions and oral inoculation of young bobwhites. Birds were kept in isolation for 7 days PI, then euthanized. The intestines were removed and aerated in a solution of 2% potassium dichromate for sporulation. During repeated propagation of the pure culture, prepatent and sporulation periods, gross pathology in the host, and general oocyst morphology were determined to aid in identification. An aliquot of oocysts was used for DNA amplification of the internal transcribed spacer-1 (ITS-1) region of the ribosomal RNA by PCR. The resultant amplicon was sequenced and examined for nucleotide polymorphisms to confirm that the sample was a pure culture. A culture identified as *E. lettyae* was used for further work.

A broiler finishing battery (Petersime Inc., Gettysburg, OH) consisting of 15 cages arranged in 5 tiers of 3 cages each, with stainless steel feeding and watering troughs, was cleaned and steam-sterilized. The cages were modified with 0.5 inch wire mesh on the floor and sides to

keep quail from escaping. Twenty-eight, 12-week old bobwhites were randomly assigned to four pens. One pen was kept uninfected as a control (UI), and others were inoculated with 100, 1000 or 10,000 oocysts, respectively. Oocysts were administered to birds in 1 ml of water. Birds were given nonmedicated quail starter feed and water *ad libitum*. Fecal materials were collected by cage on days 5-9 PI for oocyst counts. Feces were blended in known volumes of water. Oocysts were counted in aliquots of the blended fecal material using a McMaster chamber for calculation of total oocysts/bird for the 5-9 day period and oocysts per individual inoculated oocyst.

The mean length and width of the measured oocysts and the prepatent and sporulation periods were consistent with previous reports of *E. lettyae* (Ruff, 1985; Gerhold et al., in press). The resultant sequence did not contain any nucleotide polymorphisms and indicated that only one *Eimeria* sp. was amplified by the *Eimeria* genus specific primers (Gerhold et al. In press).

Oocyst production per bird for days 5-9 PI totaled 49.7, 89.5 or 436×10^6 for birds given 100, 1000 or 10,000 oocysts, respectively (Table 6.1). Oocyst production per oocyst inoculated averaged 49.75, 8.95, 4.36×10^4 oocysts for the 100, 1000, or 10,000 oocyst groups, respectively. No oocysts were observed in the feces of UI birds.

Our results suggest that crowding occurred with doses above 100 *E. lettyae* oocysts, resulting in a reduction in reproductive index (oocysts produced/oocysts administered). Crowding was seen in *Eimeria acervulina* in chickens with greater than 1,000 oocysts; whereas <100 oocysts caused crowding in *Eimeria tenella* (Williams, 2001). The reason for the differences in crowding thresholds for the two species is because the site of infection for *E. acervulina* is larger than that of *E. tenella* (Tyzzer, 1929). *Eimeria lettyae* infects the duodenum,

jejunum, ileum, cecae and there are five separate merogony cycles (Ruff, 1985). The greater number of merogony cycles in *E. lettyae* compared to *E. acervulina* likely explains why the former has a lower crowding threshold.

The actual production of oocysts increased with higher doses of inoculum, although not proportionately. In the formulation of a live vaccine for coccidiosis it is important to choose a level of inoculation which ensures the establishment of an infection without causing clinical effects. Equally, it is important for the vaccine strain to be propagated sufficiently to cause reinfection and reinforcement of the developing immunity. This was clearly accomplished with the 100 oocyst dose. However, it is also important to consider that the large number of oocysts produced by vaccinated birds might unduly expose the flock to a heavy challenge during the second and third cycles of infection. Additional studies are needed to determine whether bobwhites on litter floors could develop practical protection rapidly enough to ward off the successive challenges. While the bulk of oocysts production would be found at 5-9 days, the production is known to continue for much longer. Ruff (1985) found that *E. lettyae* oocyst production could last up to 67 days PI, with multiple recurring peaks, suggesting that the birds did not become completely immune to reinfection. Ruff (1985), performed infections with graded doses of *E. lettyae* in 2.5 week old birds and found that 100 and 1,000 inoculated birds produced 5.9×10^7 and 1.74×10^8 oocysts during a 7 day period, respectively which was much higher production compared to our results. Birds used in the earlier experiments were 2.5 weeks old birds, compared with 12 weeks in the present study. Older birds have larger intestinal tracts with greater number of epithelial cells resulting in less crowding compared to younger birds, so there is no clear explanation for the difference in our results.

If a live vaccine is to be used in bobwhites for control of coccidiosis, it would seem that more attention should be given to the strain of *E. lettyae*. In chickens, coccidia have been attenuated by selection for shortened life cycle (Jeffers, 1975). Such modified strains have been used successfully in live vaccines (Williams et al., 1999). Precocious strains lack one or more schizogony cycles and thus produce fewer oocysts to contaminate the litter (McDougald and Jeffers, 1976). Given the natural reproductive capacity of *E. lettyae*, it may be necessary to vaccinate with attenuated strains to avoid overwhelming exposure and clinical signs of disease in bobwhite quail flocks. Furthermore, precocious vaccine strains in poultry are less associated with secondary bacterial diseases (*i.e.* clostridial enteritis). Ulcerative enteritis, caused by *Clostridium colinum*, has been reported as a major disease in captive quail (Raid, 2004). In similar diseases of chickens, it is believed that exposure to coccidia at the same time as *Clostridium* triggers for toxin production by the bacteria. Precocious strains appear less likely to interact in this way because of reduced tissue damage during vaccination and reinfection.

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TABLE 6.1. Oocyst production of *Eimeria lettyae* in 12-week-old Northern bobwhite quail on days 5-9 post- inoculation.

Inoculation dose per bird	Total oocyst production (x 10 ⁶)	Daily oocysts production (x 10 ⁶)	Daily oocyst production per inoculated oocyst (x 10 ⁴)	Oocyst production per inoculated oocyst (x10 ⁴)
100	49.7	9.95	99.5	497.5
1,000	89.5	17.9	17.9	89.5
10,000	436.0	87.5	8.75	43.6

CHAPTER 7

AN UNUSUAL CASE OF COCCIDIOSIS IN LABORATORY REARED PHEASANTS RESULTING FROM A BREACH IN BIOSECURITY

R.W. Gerhold, S. M. Williams A. L. Fuller , and L. R. McDougald. 2010. Avian Diseases 53:
1112-1114. Reprinted here with permission of editor.

Summary: An outbreak of coccidiosis in laboratory reared Chinese ring-necked pheasants (*Phasianus colchicus*) resulted in high morbidity and moderate mortality. The outbreak was associated with a breach in biosecurity caused by the cleaning of a sewer line with a mechanical device, resulting in extensive splattering of fecal material throughout the ‘clean room’ where birds were held prior to use in coccidiosis experiments. Mortality and morbidity in the affected birds was seen exactly 5 days after the incident, after birds had been moved to another room for experimental use, corresponding closely with the known prepatent or preclinical period of *Eimeria phasiani* and *Eimeria colchici*. Gross lesions in the affected birds varied from dehydration to intestinal and ventricular hemorrhage. Microscopic examination confirmed a diagnosis of severe intestinal coccidiosis. This report underscores the ease of contamination of experimental birds leading to coccidiosis outbreaks during breaches of management and biosecurity.

Keywords: Biosecurity, coccidiosis, contamination, drains, *Eimeria colchici*, *Eimeria phasiani*, *Phasianus colchicus*, Chinese Ring-necked Pheasants

Abbreviations: *E. colchici* = *Eimeria colchici*, *E. phasiani* = *Eimeria phasiani*, and *E. duodenalis* = *Eimeria duodenalis*, NAGA = North American Gamebird Association, PDRC= Poultry Diagnostic and Research Center, USA= United States of America

Intestinal coccidiosis in the Chinese ring-necked pheasant (*Phasianus colchicus*) is caused by several species of *Eimeria* (7). As pheasants are reared in large numbers for sale to sporting plantations (5-7 million/year), the farms where they are started and reared to 'flight ready' are also large (6, 8). Some farms in the USA produce upwards of one million birds annually for this market. Coccidiosis is common during pheasant-rearing in the USA and United Kingdom (3, 5, 8), for the same reasons (i.e. density of birds and high parasite reproduction) coccidiosis is important in the chicken and turkey industries (4).

Case Report

In a study sponsored by the North American Gamebird Association (NAGA), we were conducting tests to survey the pathogenic species of coccidia in pheasants and to test the efficacy of anticoccidial drugs available for use in other gallinaceous birds, when we experienced an outbreak of disease in pheasant chicks recently transferred from a 'parasite-free' holding area.

Within two days after moving the chicks from the brooding area to battery cages in the treatment room for a drug study, approximately 150 of the 425 birds (33%) became depressed, had ruffled feathers, labored breathing, and were inappetent and within 3 days, 19% of the birds were dead (Table 7.1). The dead birds appeared dehydrated and had various lesions in the small intestine including hemorrhage and catarrhal enteritis. Ventricular erosion and hemorrhage was found in several birds as well. Fifteen dead or moribund birds were submitted for necropsy at the Poultry Diagnostic and Research Center (PDRC), The University of Georgia, Athens, Georgia (USA).

Suspected causes based on the gross lesions and previous management or disease issues encountered at our research facility, included biogenic amine contamination of feed, poor water

supply, bacterial contamination of feed, pesticide or rodenticide toxicosis, and fungal toxins. However, birds from the same hatch, given the same food and water from the same source, but held in isolation in another animal room, remained healthy. Given the concern of feed contamination and potential bacterial infection as the cause of the morbidity/mortality event, the remaining birds in the affected group were placed on commercially obtained game bird starter (Purina®, St. Louis MO), given water supplemented with 400 mg/ gal of bacitracin methylene disalicylate (BMD®, Alpharma Inc., Fort Lee, NJ) and commercial vitamin and electrolyte solution (Durvet Inc., Blue Springs, MO) at manufacturers' recommended doses. These treatments seemed to have little effect on the condition of the birds.

Upon microscopic examination of multiple sections of intestines, the duodenum contained multiple foci of coagulative necrosis. Within and surrounding the necrotic areas, large numbers of 1-7 μ m diameter basophilic developing schizonts were apparent in the superficial aspect of the villi, between the brush border and host cell nucleus (Fig. 7.1). Several 6-12 μ m macrogametes and developing oocysts were scattered within the infected intestines. No other significant microscopic findings were apparent. Microscopic examination of fecal droppings revealed oocysts consistent in size and shape with *Eimeria phasiani*, *Eimeria colchici*, and other unidentified species, resulting in a diagnosis of severe coccidiosis.

Discussion

Several days following the postmortem diagnosis of coccidiosis, events became apparent at the holding facility that revealed the source of the infection. The brooding area is adjacent to the treatment rooms; however, unlike the infection rooms, it is equipped with colony cages and electric heat lamps for brooding. The testing/brooding facility consists of a long building with

eleven rooms in a row, with a separate outside entrance to each room (Fig.7.2). As a matter of practice, personnel are advised strictly to plan their work so that there is no movement from a contaminated room to the clean holding room. Despite the proximity of animal rooms housing uninfected and infected birds, this system had been used successfully for 35 years with chicken and turkey coccidia research projects. Immediately prior to the outbreak, the adjacent infection room had been used to house birds for propagation of coccidia field isolates in two-week old pheasant chicks. About a dozen isolates containing *E. colchici*, *E. phasiani*, and *E. duodenalis* were propagated in individual cages of 5-10 birds. During the cleaning of the coccidia propagation room, custodial workers washed a considerable amount of fecal matter down the drain, which subsequently became clogged.

The drains for the entire building flow into a common pipe running beneath all the rooms, including the parasite-free holding room (Fig. 7.2). When the university physical plant plumbers were called to open the drain they entered the 'clean' room and ran a rotary power tool through the drain into the common pipe to the source of the clog under the next room. This resulted in oocyst-laden fecal material from the next room being splattered extensively over the walls, ceilings, floors and holding cages (containing brooding pheasants), in the clean room. The subsequent ingestion of the oocysts by the pheasants resulted in the infection and clinical signs of coccidiosis 5 days later, the normal pre-clinical period for *E. phasiani* and *E. colchici*.

The investigators were not told of these events until considerable effort had been expended to determine the cause of the morbidity and mortality event as well as the source of the contamination. In the past, the simple measures taken to limit movement of personnel from contaminated to uncontaminated areas were sufficient to prevent this type of breach of biosecurity. In the present example, the breaching of biosecurity protocols in place and the lack

of communication allowed massive contamination to go unreported until clinical disease was rampant. Morbidity and mortality were first noted five days post contamination and was most prominent six days post contamination, which corresponds with the formation of second to third generation schizonts, the most pathogenic stages of the coccidian life cycle. This was supported by the microscopic findings of large number of schizonts infecting the duodenum villi epithelial cells of the affected birds. The birds surviving the infection were considered compromised for further use in coccidiosis studies and were euthanized.

Contamination of poultry and other food animals with microbial pathogens have previously been associated with contaminated drains and sewer lines (1, 2). Although these previous examples all involve bacterial contamination, our findings indicate that parasites of food animals can also lead to contamination. The current disinfection protocol for the clean room on our research farm includes removing all organic matter followed by cleaning equipment and cages with detergents and then power washing the entire room, including the drains, with 150 C steam. Similar drain cleaning protocols should be utilized in other research facilities and processing plants.

These findings underscore the importance of having strict protocols for researchers, caretaking, and custodial staff regarding biosecurity and movement between various infection and brooding rooms. In addition, it is necessary that personnel hired from outside agencies for mechanical or plumbing repair be advised of biosecurity protocols and be escorted by researchers to ensure that biosecurity protocols are followed. Furthermore, this case emphasizes the importance of thorough postmortem diagnostics in cases of unknown morbidity and mortality events in research animals to ensure the results of the research are not influenced by unintended infection. Given the environmental resistance of oocysts, the proliferative reproduction of

coccidia, and the susceptibility of game bird to coccidiosis, it is imperative that game bird propagators and researchers adhere to strict protocols to minimize accidental contamination.

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TABLE 7.1. Progression of morbidity and mortality in brooding pheasant chicks due to coccidiosis contamination.

Days post - contamination	Morbidity (no. and %) ^a	Mortality (no. and %) ^b
5	150 (33%)	21(5%)
6	250 (60%)	60 (14%)
7	100 (29%)	0
Total	325 (75%)	81 (19%)

^a Estimated number and percent total morbid birds per day

^b Percent of dead birds each day calculated based on number of total live birds for that day

FIGURE 7.1. Duodenum of pheasant infected with coccidia. Sections of duodenum contain large numbers of developing 2-7 μm schizonts (arrows) and 6-12 μm macrogametocytes and microgametocytes (arrowheads). H&E. Bar=50 μm .

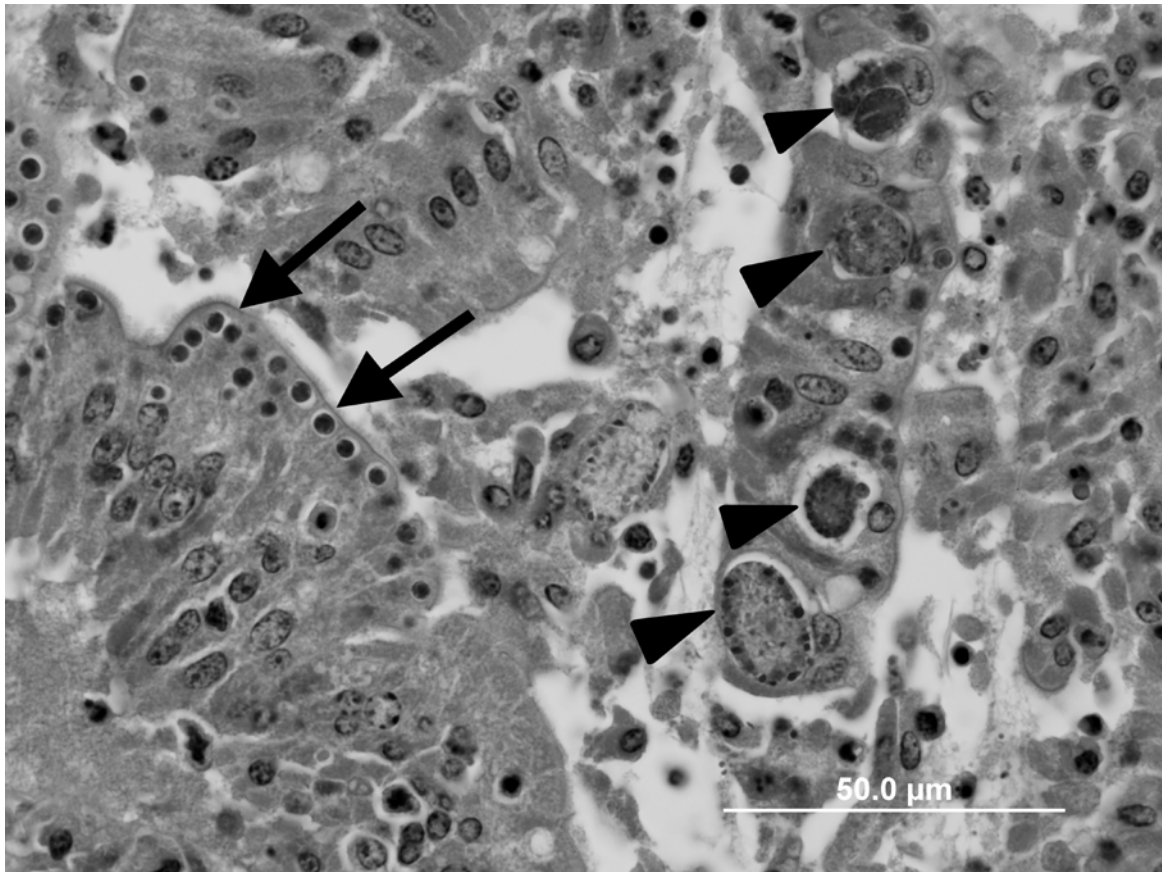
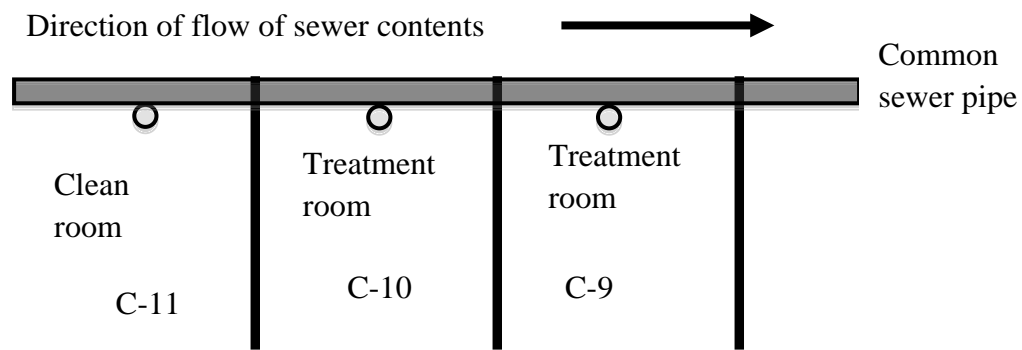


FIGURE7.2. Placement of treatment and clean rooms in poultry research facility. Note the individual drains (round open holes) for each room are connected by a common sewer pipe.



CHAPTER 8

CONCLUSIONS

The major objective of this research was to determine the major coccidia species causing disease in Northern bobwhites, perform a survey of the *Eimeria* spp., and determine potential control measures against coccidiosis. The disease has been diagnosed in a large number of captive quail and it is considered to cause major economic losses for quail producers.

Study 1 (Chapter 3)

The goal of this project was to create PCR primers to molecularly distinguish and detect the various *Eimeria* spp. causing disease in Northern bobwhites. Coccidia samples were collected from 31 captive facilities from 11 states. PCR primers were constructed in a two step fashion: 1) *Eimeria* genus wide primers were used to amplify the ITS-1 region of the rRNA gene and the PCR products were cloned and sequenced; 2) sequences were aligned and placed into like groups followed by construction of sense and antisense PCR primers for the various species. The primers were used to perform a molecular survey of the *Eimeria* spp. from the collected samples. The results demonstrated the following: 1) Three molecularly distinct *Eimeria* spp. were found in quail, corresponding to the three described species (Tyzzer, 1929; Fisher and Kelly, 1977; Ruff, 1985); 2) *E. lettyae*, *E. dispersa*, and *E. colini* were found in 20 (64.5%), 22

(73.3%), and 29 (93.5%) of the 31 samples, respectively; 3) Thirteen (41.9%) samples had 3 *Eimeria* spp. detected, 14 (45.2%) samples had 2 spp. detected, and 4 (12.9%) samples had 1 sp. Detected; 4) Flock age or geographical location was not associated with the presence of particular *Eimeria* spp.

Study 2 (Chapter 4)

Thirteen anticoccidial compounds used in poultry were evaluated against coccidia in Northern bobwhites. Five tests were done in total. *Eimeria lettyae* was used in the first three trials and combinations of *E. lettyae*, *E. dispersa*, and/or *E. colini* were used in the last two studies. Weight gain, severity of diarrhea, and feed conversion ratio (FCR) six days post infection were recorded. Lesion score, as previously reported (Ruff, 1986), was unreliable as a measure of severity of infection, in comparison with weight gain, fecal scores, and FCR. Excellent to good efficacy was found with clodolol, decoquinate, diclazuril (1 and 2 ppm), lasalocid, narasin/nicarbazin, robenidine, sulfadimethoxine/ ormetoprin, and zoalene (150 ppm). Marginal protection was found using monensin, salinomycin, semduramicin, or a roxarsone /semduramicin combination. Amprolium, roxarsone, and zoalene (125 ppm) were ineffective at controlling coccidia. Two of the six isolates tested against diclazuril 1ppm and clodolol demonstrated a high degree of resistance, but none of the six isolates was resistant to lasalocid. Four of the eight isolates showed mild to moderate and moderate to high resistance against monensin and salinomycin, respectively. These findings indicate that several available compounds are effective at controlling coccidiosis in bobwhite quail. Similar to Ruff et al

(1987), we found that amprolium, which is one of the most used anticoccidials, is ineffective at controlling coccidiosis in bobwhites.

Study 3 (Chapter 5)

This study investigated the ability to immunize young Northern bobwhites with a low dose of *E. lettyae*, because it is the most pathogenic species in quail (Ruff and Wilkins, 1987). Immunize birds and non-immunized controls were challenged with a high dose of *E. lettyae*. Birds immunized with 100 or 1,000 oocysts gained an average of 33.3 gm and 28.9 gm, respectively; whereas unimmunized challenged birds gained an average of 11.5 gm. Immunized quail produced approximately 99.7% fewer oocysts, had minimal gross intestinal and cecal lesions, had minimal diarrhea, and had a 50% lower feed conversion ratio compared to unimmunized challenged controls. These findings indicate that vaccination is a viable option for controlling coccidiosis in quail and that further research into vaccination is warranted.

Study 4 (Chapter 6)

We examined the oocyst production in *E. lettyae* following inoculation with low doses. This was performed by orally infecting 12-week old bobwhites with 100, 1,000, or 10,000 sporulated oocysts. Fecal materials were collected on days 5-9 post inoculation (PI) and total oocyst production counted in McMaster chambers. Oocyst production/bird was 49.75, 89.5, or 436×10^3 for 100, 1000, or 10,000 oocysts administered, respectively. Estimated oocysts produced/oocyst administered was 49.75, 8.95, or 4.36×10^3 for 100, 1000 or 10,000 oocysts administered, respectively. These findings not only illustrate the crowding effect (Williams, 2001) of larger oocyst inocula, but also illustrate the fecundity of *E. lettyae* at low doses. This

suggests that successful immunization of bobwhites against coccidiosis with live vaccines might require attenuated strains with reduced reproductive potential.

Study 5 (Chapter 7)

This study describes an outbreak of coccidiosis in laboratory reared Chinese ring-necked pheasants (*Phasianus colchicus*). The outbreak was associated with a breach in biosecurity caused by the cleaning of a sewer line with a mechanical device, resulting in extensive splattering of fecal material throughout the ‘clean room’ where birds were held prior to use in coccidiosis experiments. Mortality and morbidity in the affected birds was seen exactly 5 days after the incident, after birds had been moved to another room for experimental use, corresponding closely with the known prepatent or preclinical period of *Eimeria phasiani* and *Eimeria colchici* (Norton, 1976). Gross lesions in the affected birds varied from dehydration to intestinal and ventricular hemorrhage. Microscopic examination confirmed a diagnosis of severe intestinal coccidiosis. This report underscores the ease of contamination of experimental birds leading to coccidiosis outbreaks during breaches of management and biosecurity.

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